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## Correlation between segmental flexibility and effector function of antibodies

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*Mouse monoclonal anti-dansyl antibodies with the same antigen-binding sites but different heavy chain constant regions were generated. The extent of segmental flexibility in times of nanoseconds and the capacity to fix complement were greatest for IgG2b, intermediate for IgG2a, and least for IgG1 and IgE. Hence, the effector functions of immunoglobulin isotypes may be controlled in part by the freedom of movement of their Fab arms.*

IMMUNOGLOBULIN G is a flexible Y-shaped molecule. The two antigen-binding Fab units of IgG are joined to an Fc unit at a hinge that allows the angle between the Fab parts to vary over a broad angular range, as shown by hydrodynamic<sup>1</sup>, electron microscopic<sup>2,3</sup>, and X-ray crystallographic studies<sup>4-6</sup>. Nanosecond fluorescence polarization measurements have demonstrated that immunoglobulin molecules exhibit segmental flexibility in the nanosecond time range<sup>7-9</sup>. Segmental flexibility is likely to be important in enabling immunoglobulins to bind optimally to multivalent antigens and to carry out certain effector functions. Studies of polyclonal antibody populations have suggested that immunoglobulin classes differ in their degree of segmental flexibility<sup>10-11</sup>. We have explored the relationship between hinge motion and effector function in specially constructed families of homogeneous immunoglobulins. Mouse monoclonal anti-dansyl antibodies with the same antigen-combining sites but different heavy chain constant regions (Fig. 1) were generated by selecting somatic variants in hybridoma cell lines. The extent of segmental flexibility in times of nanoseconds (measured by fluorescence spectroscopy) and the capacity to fix complement were greatest for IgG2b, intermediate for IgG2a, and least for IgG1 and IgE. Hence, the effector functions of immunoglobulin isotypes may be controlled in part by the freedom of movement of their Fab arms.

In most antibody-producing hybridoma cell lines, variants arise which produce antibody containing a different heavy chain<sup>12</sup>. For example, cells producing IgG2b sometimes arise from a cell line producing IgG1. The frequency of these heavy chain switch variants is ordinarily low ( $10^{-5}$ - $10^{-6}$  per generation). These rare cells can be separated by fluorescence-activated cell sorting on the basis of the appearance of a different heavy chain on their cell surface<sup>13,14</sup>. In these switch variants the light chain remains the same, whereas  $V_H$  becomes joined to a different  $C_H$ . Consequently, the antigen-combining site is the same as in the parent, despite the change in the heavy chain constant region. Many of these switch variants are stable and can be cloned.

We chose to generate a family of homogeneous mouse monoclonal (DNS) antibodies for two reasons. First, the fluorescence emission spectrum of the bound dansyl chromophore is responsive to the polarity of its environment<sup>15</sup>, making it a convenient and sensitive indicator of whether the antigen-combining site of the switch variant antibody is in fact the same as that of the parent line. Second, the bound dansyl chromophore has an excited state lifetime suitable for determining the segmental flexibility of immunoglobulin isotypes by nanosecond fluorescence polarization spectroscopy<sup>16</sup>. Variant hybridoma cell lines producing different immunoglobulin isotypes

Fig. 1 Schematic diagram of immunoglobulin G. In each family of isotypes, the V<sub>L</sub>, C<sub>L</sub> and V<sub>H</sub> regions are the same. The hinge is located between the C<sub>H1</sub> and C<sub>H2</sub> domains.

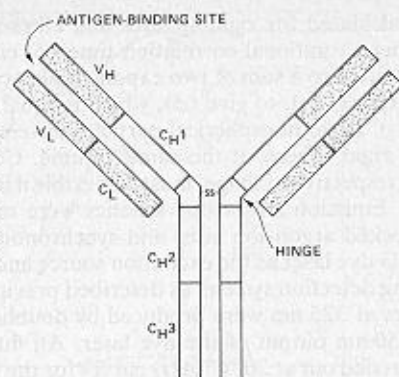


Table 1 Mean rotational correlation times of immunoglobulin isotypes and their Fab fragments

	$\langle\phi\rangle$ (ns)	
	No DTT	With DTT
<b>DNS1 family</b>		
Intact immunoglobulin		
IgE	124	88
IgG1	81	58
IgG2a	60	51
IgG2b	47	42
Fab fragment		
IgG1	28	28
IgG2a	28	28
IgG2b	29	29
<b>DNS3 family</b>		
Intact immunoglobulin		
IgG1	93	55
IgG2b	46	45

ected by using affinity-purified goat and rabbit anti-mouse heavy-chain sera and a panel of hybridoma anti-mouse immunoglobulin allotype antibodies<sup>14</sup>. IgG2b, IgG2a and IgE anti-DNS antibody-producing cell lines were isolated starting with the mouse IgG1 anti-DNS hybridoma cell line DNS1 (Igh<sup>a</sup> allotype, also designated as 27-44). Our previous nanosecond fluorescence study<sup>16</sup> showed that IgG1 from this mouse cell line is more rigid than is polyclonal rabbit anti-dansyl antibody. The cell lineage of the DNS1 family is: IgG1 (27-44) → IgG2b (27-13) → IgG2a (27-13) → IgE (27-74). A cell line containing a different IgG1 anti-dansyl combining site, DNS3 (Igh<sup>b</sup> allotype, also designated as 44-10), served as the source of another anti-dansyl IgG2b (44-32).

Two-dimensional gel electrophoresis of antibodies<sup>17,18</sup> produced by the DNS1 family shows that the heavy chain synthe-

sized by each member has the electrophoretic mobility expected for  $\gamma 1$ ,  $\gamma 2b$ ,  $\gamma 2a$  and  $\epsilon$  chains (Fig. 2). Also, these gels show that the light chains produced by this family are identical. Corresponding results were obtained for the DNS3 family. Heavy chains synthesized by these variant cells in the presence of tunicamycin, a known inhibitor of asparagine-linked glycosylation<sup>19-21</sup>, have a greater electrophoretic mobility than do those synthesized in its absence. This finding suggests that each variant isotype is normally glycosylated in the absence of tunicamycin. The fluorescence emission spectra of  $\epsilon$ -dansyl-L-lysine bound to switch variants of the DNS1 cell line are identical to that of the parent cell antibody (Fig. 3), strongly suggesting that the DNS1 family has the same antigen-combining site. Likewise, both DNS3 isotypes have identical dansyl fluorescence emission spectra. Also, the dissociation constant of the antibody-hapten complex is the same within the DNS family ( $K_d = 17 \pm 1$  nM) and the DNS3 family ( $K_d = 3 \pm 1$  nM).

### Segmental flexibility

The segmental flexibility of these anti-dansyl immunoglobulins was determined by measuring their fluorescence polarization kinetics in the nanosecond time range<sup>7,16,22</sup>. A solution of immunoglobulin containing bound  $\epsilon$ -dansyl-L-lysine is excited by a picosecond pulse of vertically-polarized light. Photoselection produces an ensemble of excited chromophores preferentially aligned in the vertical direction. The orientations of these excited molecules then become randomized by rotational brownian motion. The rate of randomization depends on the size and shape of the immunoglobulin, and also on its modes of flexibility. These motions can be monitored by measuring the intensities of the vertically-polarized [ $y(t)$ ] and horizontally-polarized [ $x(t)$ ] components of the fluorescence emission as a function of time following the excitation pulse. The most informative parameter is the emission anisotropy  $A(t)$ , which is defined as

$$A(t) = \frac{y(t) - x(t)}{y(t) + 2x(t)}$$

The emission anisotropy kinetics of a chromophore rotating in common with a rigid sphere is given by  $A(t) = A_0 \exp(-t/\phi)$ , where  $A_0$  is the initial value of the emission anisotropy and  $\phi$  is the rotational correlation time. For a nonspherical particle,  $A(t)$  is more complex, consisting of up to five exponential terms. For a rigid ellipsoid,  $A(t)$  is a sum of three exponential decays that depend on the volume and axial ratio of the ellipsoid and on the directions of the transition moment of the emitting chromophore. A rigorous theory for the emission anisotropy kinetics of rigid and segmentally flexible Y-shaped molecules has not yet been developed. Emission anisotropy data for immunoglobulins have instead been interpreted phenomenologically by comparing observed  $A(t)$  curves with those

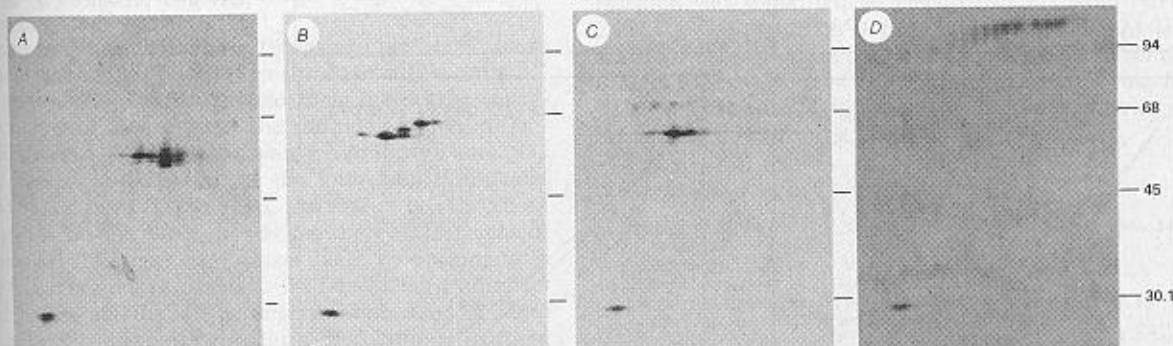
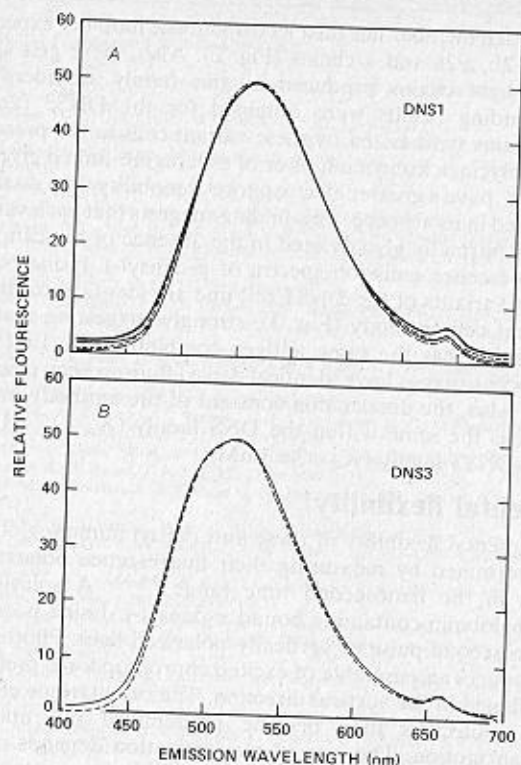
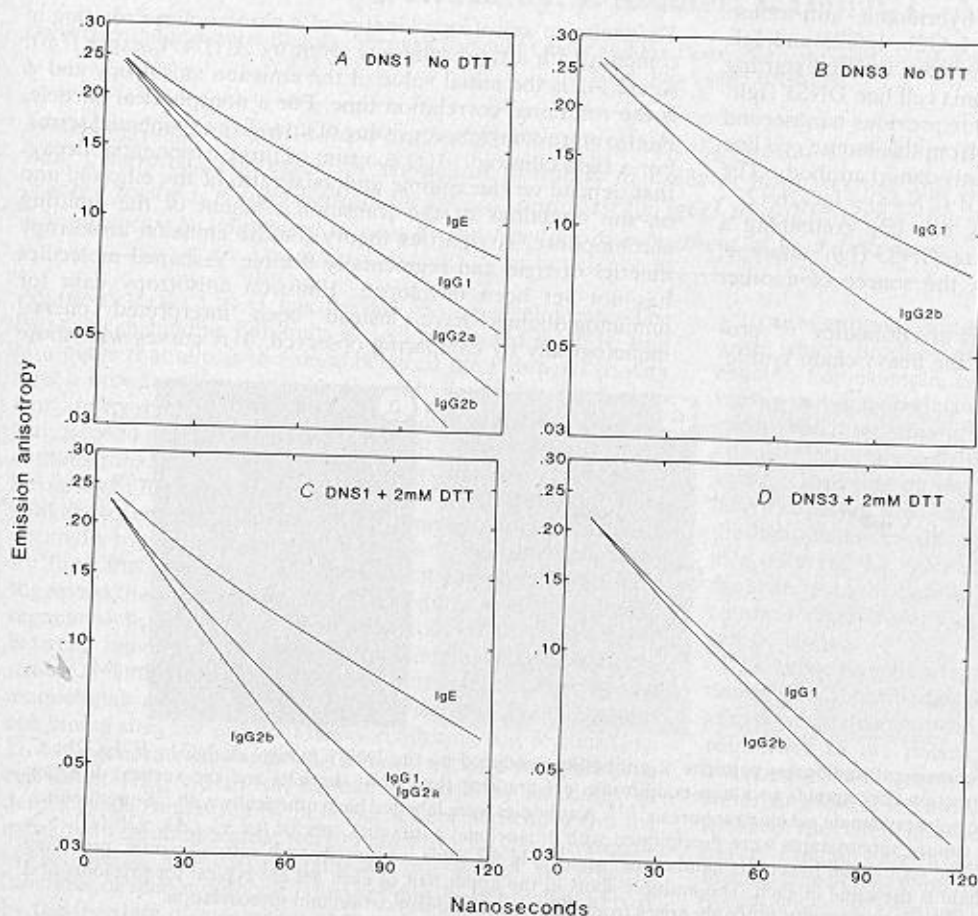


Fig. 2 Two-dimensional gel electrophoresis autoradiogram patterns of antibodies produced by the DNS1 family. A, IgG1; B, IgG2b; C, IgG2a; and D, IgE. The horizontal direction corresponds to a non-equilibrium pH gradient (low pI at the left) and the vertical direction corresponds to sodium dodecyl sulphate polyacrylamide gel electrophoresis<sup>17,18</sup>. Antibodies were labelled biosynthetically with <sup>35</sup>S-methionine. Secreted anti-dansyl antibodies in the culture supernatants were precipitated with monoclonal antibodies specific for  $\gamma 1$  (A),  $\gamma 2b$  (B),  $\gamma 2a$  (C) and a rabbit anti-mouse IgE (D). The spot in the lower left-hand corner appears in the same position in each autoradiogram, providing strong confirmation that the  $\kappa$  light chain is the same in each. The multiple spots in the upper half of each gel are typical for myeloma and hybridoma heavy chains. The heterogeneity probably arises from differences in deamidation and glycosylation.





**Fig. 3** Fluorescence emission spectra of  $\epsilon$ -dansyl-L-lysine bound to isotypes of the DNS1 (A) and DNS3 (B) families. The spectra of isotypes within a family are virtually superposable. The excitation wavelength was 340 nm.



**Fig. 4** Nanosecond emission anisotropy kinetics of  $\epsilon$ -dansyl-L-lysine bound to immunoglobulins of the DNS1 family in the absence (A) and presence (B) of 2 mM DTT; and the DNS3 family in the absence (C) and presence (D) of 2 mM DTT. The temperature was 20 °C.

calculated for rigid spheres and ellipsoids. In this regard, the mean rotational correlation time  $\langle\phi\rangle$  can be informative. It is fitted to a sum of two exponential decays,  $a_1 \exp(-t/\phi_1) + a_2 \exp(-t/\phi_2)$ , to give  $\langle\phi\rangle$ , which is equal to  $(a_1\phi_1 + a_2\phi_2)/(a_1 + a_2)$ . Rigid nonspherical particles have a  $\langle\phi\rangle$  greater than that of a rigid sphere of the same volume. Consequently, a particle, irrespective of shape, must be flexible if its  $\langle\phi\rangle$  is less than that of a rigid sphere of the same volume.

Emission anisotropy kinetics were measured using a pulsed argon-ion laser and synchronously pumped rhodamine 6G dye laser as the excitation source and a single-photon counting detection system, as described previously<sup>16</sup>. Picosecond pulses at 325 nm were produced by doubling the frequency of the 650 nm output of the dye laser. All fluorescence studies were carried out at 20 °C.  $A(t)$  curves for the DNS1 family are shown in Fig. 4A. The striking finding is that the IgG1, IgG2a, and IgG2b isotypes of this family display markedly different  $A(t)$  curves. Their  $\langle\phi\rangle$  values are 81, 60 and 47 ns, respectively. These immunoglobulins have virtually identical molecular weights and sedimentation coefficients (6.8, 6.67, and 6.70, respectively; Schumaker and M. Phillips, personal communication), which means that their molecular volumes and time-averaged shapes are nearly the same. Furthermore, the Fab fragments obtained from these isotypes have nearly the same  $\langle\phi\rangle$  values (Table 1). The orientation of the bound dansyl chromophore with respect to the long axis of the Fab unit is identical or nearly so in all isotypes because they possess identical antigen-combining sites. Thus, the large difference in the  $A(t)$  curves (Fig. 4A) is almost certainly due to different extents of segmental flexibility in the intact immunoglobulins. IgG1 is the most rigid, IgG2a is intermediate, and IgG2b is the most flexible of the three isotypes.

IgE is like IgG1 in being more rigid than IgG2a and IgG2b (Fig. 4A). The  $\langle\phi\rangle$  value for IgE can be compared with that of the three IgG isotypes by scaling its  $\langle\phi\rangle$  to take into account its 190,000 molecular weight<sup>23</sup> compared with 150,000 for

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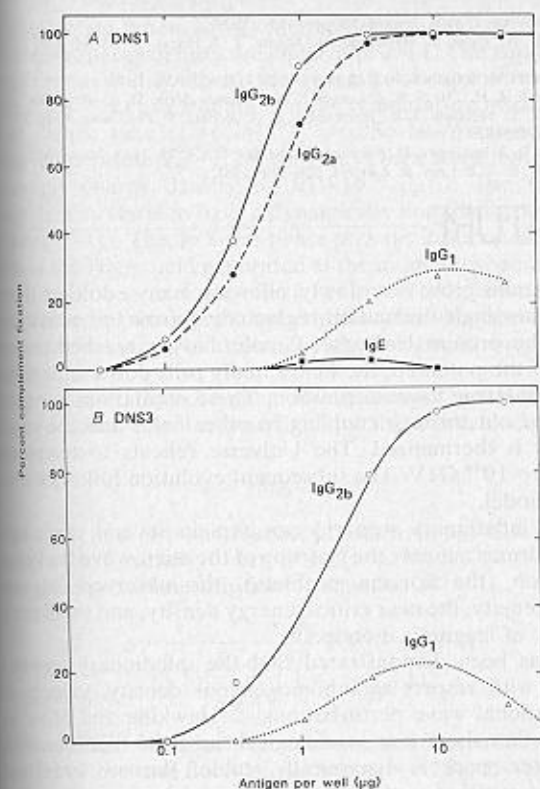


Fig. 5 Complement-fixation by the DNS1 (A) and DNS3 (B) family. A microcomplement consumption assay using  $^{51}\text{Cr}$  release from haemolysin-treated sheep erythrocytes similar to that described previously<sup>25</sup> was used to measure complement fixation by the DNS1 and DNS3 family. Immunoglobulin (10  $\mu\text{g}$  per 25  $\mu\text{l}$ ), DNS-bovine serum albumin dilutions (in 25  $\mu\text{l}$ ) and complement (2 CH-50 units in 25  $\mu\text{l}$ ) were combined in wells of a 96 well microtitre plate and incubated for 45 min at 37 °C. Next, 50  $\mu\text{l}$  of a 2% solution of activated,  $^{51}\text{Cr}$ -loaded erythrocytes was added to each well and after a further 45 min incubation at 37 °C the non-lysed cells were pelleted by centrifugation of the plates. 50- $\mu\text{l}$  aliquots of supernatant were collected from each well and counted on a gamma counter set for  $^{51}\text{Cr}$ .

The scaled  $\langle\phi\rangle$  value for IgE is  $124 \times (150/190) = 98$  ns, which is even larger than the 81 ns value for IgG1. The sedimentation coefficient of 8.35 S for this IgE (V. Schumaker and M. Phillips, personal communication) corresponds to 6.59 S when scaled for the molecular weight difference between IgE and IgG. This is consistent with the time-averaged shape (as expressed by the translational diffusion coefficient) of IgE being very similar to that of the three IgG isotypes.

Reduction of the disulphide bonds between the heavy chains in the vicinity of the hinge between the Fab and Fc units<sup>16,24</sup> provided additional information concerning the degree of segmental flexibility of the isotypes. The addition of 2 mM dithiothreitol (DTT) leads to a large decrease in  $\langle\phi\rangle$  for IgE and IgG1 (Fig. 4B, Table 1). Reduction of the interchain disulphides lowers  $\langle\phi\rangle$  of IgE from 124 to 88 ns, and that of IgG1 from 81 to 58 ns. DTT has no effect on  $\langle\phi\rangle$  for the Fab fragment of IgG1. Thus, DTT lowers  $\langle\phi\rangle$  of intact IgG1 by opening up a restricted hinge region. In contrast, DTT has almost no effect on  $\langle\phi\rangle$  of IgG2a and IgG2b because the Fab units of these antibody molecules have a high degree of rotational freedom even before reduction of the interchain disulphides.

A large difference in segmental flexibility was also observed within the DNS3 family (Fig. 4C and Table 1). IgG2b is again much more flexible than IgG1, as evidenced by their  $A(t)$  curves and  $\langle\phi\rangle$  values. Also, DTT markedly decreases  $\langle\phi\rangle$  of IgG1 and has little effect on  $\langle\phi\rangle$  of the more flexible IgG2b (Fig. 4D and Table 1).

## Complement-fixation effectiveness

The ability of these antibody molecules to fix complement was measured using a micro-complement consumption assay<sup>25</sup>. Dansylated bovine serum albumin served as the antigen. In the DNS1 family, IgG2b is most effective in fixing complement, and IgG2a is also highly effective (Fig. 5A). In contrast, IgG1 fixes less complement and required ten-fold more antigen to trigger complement fixation. IgE exhibits negligible complement-fixation activity. Likewise, in the DNS3 family, IgG2b fixes more complement than does IgG1 and does so at a lower concentration of antigen (Fig. 5B). The isotypes that fix complement most effectively, IgG2a and IgG2b, also exhibit the highest degree of segmental flexibility.

Two families of immunoglobulin isotypes with identical antigen-binding sites were constructed using somatic cell genetics techniques. Nanosecond fluorescence polarization studies of these monoclonal anti-dansyl isotypes have revealed striking differences in their extents of segmental flexibility. IgG2b is the most flexible, IgG2a is less flexible, and IgG1 and IgE are relatively rigid. The degree of molecular motion at the hinge in times of nanoseconds is correlated with the ability of these molecules to fix complement. This correlation may be more than fortuitous. Segmental flexibility may promote complement fixation by enhancing multivalent binding, facilitating the transmission of conformational changes from the Fab units to the effector domain on the Fc unit, or enabling the Fc unit to assume a conformation favourable for complement fixation. It will be very interesting to further explore the relationship between flexibility and effector function by engineering a series of immunoglobulins in which the hinge is deleted, elongated, or modified to alter the rotational mobility of the Fab arms. For example, it would be instructive to study a chimaeric immunoglobulin containing an IgG1 hinge and IgG2b  $\text{C}_{H2}$  and  $\text{C}_{H3}$  domains. We predict that such an immunoglobulin would fix complement less effectively than a normal IgG2b molecule. Segmental flexibility is likely to be one factor correlated with complement fixation. The construction of immunoglobulin genes with novel combinations of domains or with site-specific mutations and their subsequent insertion into lymphoid cell lines should lead to deeper insights into how immunoglobulins mediate their distinctive functions. Methods for expressing designed immunoglobulins have recently been developed<sup>26,27</sup>.

Monoclonal antibodies directed against tumour-specific antigens are now being used to treat certain malignancies<sup>28</sup>. It will be important to learn whether the therapeutic effectiveness of a tumour-specific antibody depends on its isotype as well as on its antigen-binding properties. The capacity of such antibodies to interact optimally with tumour-specific antigens on cell surfaces may be influenced by their segmental flexibility.

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## LETTERS TO NATURE

### Large anisotropy in the Universe does not prevent inflation

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The inflationary model in which the Universe passes through an exponentially expanding phase can explain a few cosmological conundrums of the standard big-bang model. It is therefore interesting to study the stability of this model. It has been demonstrated that the inflationary scenario is stable with respect to density, velocity and gravitational wave perturbations<sup>1-3</sup>. Here I show that the new inflationary model is compatible with large initial anisotropy. The initial anisotropic energy density could be many orders of magnitude larger than the initial radiation energy density.

The standard hot big-bang model of the Universe is remarkably simple and provides an amazingly accurate description of its evolution. There are, however, several observational facts which remain unexplained in this model, for example, the present high degree of isotropy of the microwave background radiation, the large scale homogeneity, and near critical energy density of the Universe.

The inflationary model of the early evolution of the Universe<sup>4,6</sup> explains these facts without altering the basic predictions of the big bang model. Guth<sup>4</sup> noticed that the first-order phase transition predicted by grand unified theories (GUTs), which occurs when temperature  $T$  is of the order of  $10^{14}$  GeV, could have profound cosmological consequences. The inflationary scenario requires Higgs field effective potential  $V(\phi)$  to have a global minimum at zero temperature. This global minimum is called the true vacuum. The zero temperature effective potential should also have a second metastable extremum at  $\phi=0$  (the false vacuum). Furthermore, one assumes that there is a critical temperature  $T_c \approx 10^{14}$  GeV above which the finite temperature effective potential has a lower value near the false vacuum than it has near the true vacuum. For any small temperature  $T > 0$  the false vacuum is stabilized by a barrier in the finite temperature effective potential. The height of this barrier is of the order of  $T^4$  and its width is of the order of  $T$ .

If at the very early stages of evolution the Universe contained a hot region ( $T > 10^{14}$  GeV), which was isotropically expanding fast enough to cool to  $T_c$  before gravitational effects could cause it to collapse, then this region will cool to  $T_c$  and supercool. The constant false vacuum energy density determines then the rate of expansion and this region expands exponentially. This expansion can be described by the de Sitter metric with the false vacuum energy density having the role of a cosmological constant.

The Higgs field fluctuates around the false vacuum. Some fluctuations begin to grow and at some point become large enough to be described by a classical evolution equation. At the moment when evolution of fluctuations could be described by the classical equation the fluctuations should be small. If the

fluctuations grow very slowly, allowing many  $e$  folding time to pass, this single fluctuation region could grow to become larger than the present Universe. Finally, having reached the top of the potential, the field rapidly rolls down and oscillates about the true vacuum position. These oscillations are exponentially damped out through coupling to other fields and the released energy is thermalized. The Universe reheats to temperature  $T = T_c \approx 10^{14}$  GeV. The subsequent evolution follows the standard model.

The inflationary scenario can explain several cosmological conundrums, namely the isotropy of the microwave background radiation (the horizon problem), the observed large scale homogeneity, the near critical energy density, and small number density of magnetic monopoles<sup>7</sup>.

It has been demonstrated that the inflationary scenario is stable with respect to inhomogeneous density, velocity, and gravitational wave perturbations<sup>1-3</sup>. Hawking and Moss<sup>8</sup> suggested that there is a cosmological 'no hair' theorem and de Sitter space is dynamically stable. Barrow and Turner<sup>9</sup> pointed out that in the inflationary model proposed by Guth, large anisotropy prevents the de Sitter phase from occurring. I now show that the new inflationary scenario can tolerate large homogeneous initial anisotropy.

Let us consider an anisotropic homogeneous Bianchi type I universe filled with radiation and constant false vacuum energy density  $\rho_v$ . The evolution of this model is described by

$$\dot{R}^2 = \frac{8\pi G}{3} (\rho_r + \rho_v) R^2 + \frac{8\pi G}{3} \frac{a^2}{R^4}$$

and

$$\rho_r R^4 = \text{constant}$$

where  $R$  is related to the volume expansion parameter  $V/V = 3\dot{R}/R$ ,  $\rho_r$  is the radiation energy density, and  $a$  is a constant related to the initial anisotropy. Equation (1) can be rewritten in the form

$$\frac{dz}{d\tau} = 2 \left( z^2 + 1 + \frac{\beta}{z} \right)^{1/2}$$

where

$$\tau = \chi t = \left( \frac{8\pi G}{3} \rho_v \right)^{1/2} t$$

$$z = \left( \frac{R}{R_0} \right)^2 \left( \frac{\rho_v}{\rho_r(0)} \right)^{1/2}$$

and

$$\beta = \frac{a^2}{R_0^6 \rho_r(0)} \left( \frac{\rho_v}{\rho_r(0)} \right)^{1/2} = \frac{\rho_{an}(0)}{\rho_r(0)} \left( \frac{\rho_v}{\rho_r(0)} \right)^{1/2}$$

where  $R_0$  is a constant, and  $\rho_r(0)$  and  $\rho_{an}(0)$  are the initial energy densities of radiation and anisotropy. In this equation  $z^2$  is related to the false vacuum energy density,  $1$  to the radiation energy density, and  $\beta/z$  to the anisotropic energy density.

At the very early stage of expansion, when  $z$  is very large, the anisotropic energy density determines the expansion rate of the Universe, so that  $z = \beta^{1/3} (3\tau)^{2/3}$ . We are interested in the situation when the anisotropic energy density determines the expansion rate of the Universe at an epoch when the Uni-

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