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Kinetics of protein–protein association explained by Brownian dynamics computer simulation

(diffusion controlled reactions/antibody–antigen complexation/self-assembly/lengthy collisions)

SCOTT H. NORTHRUP*† AND H. P. ERICKSON‡

*Department of Chemistry, Tennessee Technological University, Cookeville, TN 38505; and ‡Department of Cell Biology, Duke University Medical Center, Durham, NC 27710

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ABSTRACT **Protein–protein bond formations, such as antibody–antigen complexation or aggregation of protein monomers into dimers and larger aggregates, occur with bimolecular rate constants on the order of 10^6 $M^{-1}\cdot s^{-1}$, which is only 3 orders of magnitude slower than the diffusion-limited Smoluchowski rate. However, since the protein–protein bond requires rotational alignment to within a few angstroms of tolerance, purely geometric estimates would suggest that the observed rates might be 6 orders of magnitude below the Smoluchowski rate. Previous theoretical treatments have not been solved for the highly specific docking criteria of protein–protein association—the entire subunit interface must be aligned within 2 Å of the correct position. Several studies have suggested that diffusion alone could not produce the rapid association kinetics and have postulated "lengthy collisions" and/or the operation of electrostatic or hydrophobic steering forces to accelerate the association. In the present study, the Brownian dynamics simulation method is used to compute the rate of association of neutral spherical model proteins with the stated docking criteria. The Brownian simulation predicts a rate of 2×10^6 $M^{-1}\cdot s^{-1}$ for this generic protein–protein association, a rate that is 2000 times faster than that predicted by the simplest geometric calculation and is essentially equal to the rates observed for protein–protein association in aqueous solution. This high rate is obtained by simple diffusive processes and does not require any attractive or steering forces beyond those achieved for a partially formed bond. The rate enhancement is attributed to a diffusive entrapment effect, in which a protein pair surrounded and trapped by water undergoes multiple collisions with rotational reorientation during each encounter.**

The association of protein molecules to form dimers or larger complexes is characterized by second-order rate constants that are typically in the range $0.5–5 \times 10^6$ $M^{-1}\cdot s^{-1}$. The polymerization of ATP actin onto the barbed end of an actin filament occurs with $k_2 = 2–8 \times 10^6$ $M^{-1}\cdot s^{-1}$ (1). The association of hemoglobin dimers to form tetramers has $k_2 = 0.4–0.6 \times 10^6$ $M^{-1}\cdot s^{-1}$ (2, 3). A very general example of protein–protein association is antibody association to protein antigens. Values of $k_2 = 0.6–1.0 \times 10^6$ have been reported for polyclonal antibodies binding to hemoglobin and cytochrome *c* (4). The binding of Fab fragments and recombinant domains of antibody D1.3 to its antigen occurs with $k_2 = 2–4 \times 10^6$ $M^{-1}\cdot s^{-1}$ (5). Many protein associations occur at slower rates, no doubt reflecting a variety of energy barriers. Faster bimolecular rates have been reported (6) for insulin dimerization ($k_2 = 10^8$ $M^{-1}\cdot s^{-1}$) and for interaction of cytochrome *c* with cytochrome *c* peroxidase (7) and cytochrome b_5 (8) (k_2 varies from 10^7 to 10^9, with the faster rates at low ionic strength). These very fast reactions are the results of strong attractive coulombic forces that highly favor formation of the productive reaction complexes (8–11). Since rates of $k_2 = 0.5–5 \times 10^6$ $M^{-1}\cdot s^{-1}$ are achieved by many protein associations, including the very general reaction of antibodies with protein antigen, this range appears to represent the typical rate for proteins associating and docking at the precise orientation for bond formation, without any special steering forces.

When one considers the steric specificity of the bond connecting protein subunits, this rate seems incredibly fast. If the proteins were spheres of 18 Å radius (typical of a small protein), and if the spheres associated with every contact, without regard to orientation, the diffusion-limited association rate constant would be given by the Smoluchowski (12) rate constant, $k_2 = 7 \times 10^9$ $M^{-1}\cdot s^{-1}$. That the observed rates are substantially slower than the diffusion-limited encounter of spheres is easily explained as being due to steric specificity—the proteins associate only by docking of very specific patches on their surfaces. However, that the observed rates are only 1000-fold slower than the Smoluchowski rate is actually surprising given the extremely high steric specificity that we now understand for the protein–protein bond.

The protein–protein bond, as described by Chothia and Janin (13), consists of multiple noncovalent interactions across an extensive interface. The interface is typically ≈20 Å, accounting for ≈10% of the surface area of the protein. Most important for our analysis, the surfaces of the two subunits are highly complementary over the entire interface, fitting snugly together with multiple van der Waals contacts, as well as some ionic and hydrogen bonds formed across the interface. Displacement of >2 Å from the maximally bonded docking position would significantly compromise the chemical contacts and the overall strength of the protein–protein bond. An intuitive but incorrect approach to account for this steric specificity would be to calculate the probability that a random encounter would occur with the precise fit required for bonding and to multiply this by the Smoluchowski rate. As we show in the next section, this would predict a rate of only 7×10^2 $M^{-1}\cdot s^{-1}$, 10^4 less than the observed values.

In a kinetic study that is closely related to the question of protein–protein association, Sommer *et al.* (14) exploited a chemical method for creating reactive patches on the surface of proteins and measured the kinetics of interaction of these patches. The reactive patches were disulfide anion radicals, presumably one radical per protein molecule. Decay of the radicals required a "contact" of radicals on two protein molecules. Thus, the kinetics of radical decay indicated the rate of these protein molecules interacting with their anion radicals in contact. Rates of decay for a variety of proteins gave second-order association rates in the range 10^8 and 10^9 $M^{-1}\cdot s^{-1}$ in low and high salt buffers, respectively. These values were considered so high, and so close to the Smolu-

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Abbreviation: BD, Brownian dynamics.

†To whom reprint requests should be addressed.

chowski limit, that some mechanism for markedly accelerating the association was sought.

Sommer *et al.* (14) proposed a mechanism that they called "lengthy collisions between proteins" to accelerate the interaction of the anion radicals. The hypothesis is that protein molecules in solution form weakly bonded and rotationally nonspecific complexes, in which the molecules are held closely together for an extended period but are free to rotate. Rotational diffusion during this lengthy collision would eventually bring the reactive regions into contact. Sommer *et al.* (14) started with the Smoluchowski diffusion equation and used the theory of Solc and Stockmayer (15) to estimate the probability of a correct encounter. From this, they calculated the lifetime of the lengthy collision that would be needed to produce the accelerated reaction kinetics. Berg (16) extended and corrected this analysis, concluding that the lengthy collision complex would have a lifetime of a few microseconds and a K_d of $\approx 10^{-4}$ M.

The hypothesis that proteins associate to form nonspecific complexes with $K_d = 10^{-4}$ M is, however, not supported by experimental studies of several proteins at high concentration. Chymotrypsinogen (17), bovine serum albumin (18), and hemoglobin (19) showed no evidence of association at the highest concentrations examined (40, 100, and 300 mg/ml), corresponding to protein concentrations of 1.5–6 mM. Since even a 10–20% association would have been detected in these studies, nonspecific complexes of these proteins would have to have K_d larger than $\approx 10^{-2}$ M and a lifetime of <10 ns. These observed lifetimes are at least 100 times shorter than the lengthy collisions required for the hypothesis of Sommer *et al.* (14). Thus, these lengthy collisions are not a generic property of proteins in solution.

If the lengthy collisions do not exist, how can we explain the rapid kinetics for the anion decay reaction and for the formation of protein–protein bonds? The idea that these rates require a special mechanism was based first on the simple geometric probability calculation (see below). It has long been recognized that reactions in solution, which are governed by diffusion, should be faster than the geometric rate constants (15, 20, 21). Perhaps the most comprehensive treatment is the paper of Shoup *et al.* (21), which provides a general mathematical framework that incorporates and extends the previous treatments (15, 20). However, these analytical approaches have never been developed to provide a rate for protein–protein association. In particular, the mathematical formulations include steric limitations about two axes only (needed to define a "reactive patch"), whereas the protein–protein interface is now known to require precise orientation about three axes of each subunit. Since no previous treatment has provided a satisfactory and quantitative explanation of the observed kinetics of protein–protein association, we decided to undertake an alternative approach of computer simulation through a Brownian dynamics model.

Simulation Method

Brownian Dynamics (BD) Approach. The BD simulation approach has been developed as an alternative to analytical diffusion theories to study the diffusive dynamics and interaction between macromolecules (22). We use BD here in conjunction with simple spherical models of proteins to calculate the rates of protein–protein association. In BD, the Brownian trajectory of the two interacting molecules in a solvent is simulated as a series of small stochastic displacements chosen from a distribution that is equivalent to the short time solution of the diffusion equation with forces. Trajectory statistics are then related to bimolecular rate constants. The first application of BD to macromolecules was made successfully to the reaction of a small structureless substrate with an enzyme—namely, the reaction of superoxide anion with superoxide dismutase (23, 24). Later, the method was extended to treat the diffusion and interaction of two whole proteins and was applied to association of cytochromes (8–11, 25). In these systems, both simple spherical models and more robust models based on complex surface topography and charge distribution have been used. The former have been successful in yielding important qualitative understanding of the interplay between diffusion dynamics, intermolecular forces, and steric considerations, while the latter treatment has been found to be essential for more quantitative predictions for specific systems and for predicting effects of site mutations. In the present study, we return to a simple and general model of two interacting protein subunits and incorporate steric restraints appropriate for the highly specific docking at the protein–protein interface.

Idealized Model System. The simplified and idealized model system we used to study bimolecular rates of protein–protein association is depicted in Fig. 1. Each protein is treated as a diffusing hard sphere of $R = 18$ Å, typical of a small globular protein, with translational and rotational diffusion coefficients determined by the Stokes–Einstein equation for spheres in H_2O at 25°C. To emulate the steric complementarity of the protein–protein bond in a simple and computationally convenient fashion, we mounted on a particular "face" of each spherical protein a set of four contact points in a 17 × 17 Å square arrangement on a plane tangential to the sphere. The docking face of the model thus approximates the actual size of the interface between assembled semispherical globular proteins. We assume that the proteins are correctly docked when three of the four contact points are correctly matched and are within a tolerance of 2 Å.

Simulation of Rate Constants. The Ermak and McCammon (26) BD algorithm for displacements $\Delta\mathbf{r}$ in the relative separation vector $\mathbf{r}$ of reactant centers of mass in time step Δt is

$$\Delta\mathbf{r} = D\mathbf{F}(k_BT)^{-1}\Delta t + \mathbf{S}. \quad [1]$$

Here D is the translational diffusion coefficient for relative motion and is assumed to be spatially isotropic. The vector $\mathbf{F}$ is the systematic interparticle force, k_BT is the Boltzmann constant times absolute temperature, and $\mathbf{S}$ is the stochastic component of the displacement arising from random collisions of particles with solvent molecules and is generated by taking normally distributed random numbers obeying the relationship

$$\langle S^2 \rangle = 2D\Delta t. \quad [2]$$

Identical equations govern the independent rotational Brownian motion of each particle, with force replaced by torque and D replaced by an isotropic rotational diffusion coefficient D_r for each particle. Diffusion coefficients D and D_r are given by the Stokes–Einstein relations $D = k_BT/6\pi\eta R$ and $D_r = k_BT/8\pi\eta R^3$, which yield numerical values of 1.36×10^{-6} cm^2·s^{-1} and 3.16×10^7 s^{-1}, respectively, for 18-Å spheres in water at 25°C.

The important connection of BD trajectory fate statistics with bimolecular rate constants is made as follows (22). Trajectories of diffusing species are begun at random orien-

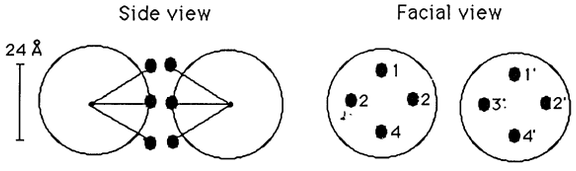

FIG. 1. Schematic of the idealized model system constructed to mimic the association of two proteins requiring strict docking complementarity. All four contacts lie in a tangential plane and must align to within 2 Å to fully specify complementary fit.

tations from a separation $r = b = 42$ Å and are calculated continuously until they reach a separation $r = c = 200$ Å, at which point the trajectory is terminated (the particles are then sufficiently far apart that another collision is very improbable). A large number of trajectories are monitored to obtain the probability β of association of pairs in favorable geometries for reaction before ultimate separation to distance $r = c$. The diffusion-controlled bimolecular reaction rate constant k may then be extracted from these probabilities by applying the formula

$$k = k_D(b)\beta/[1 - (1 - \beta)k_D(b)/k_D(c)]. \quad [3]$$

Here the diffusive rate constant for first arrival at arbitrary surface s is

$$k_D(s) = 4\pi sD, \quad [4]$$

where s is a starting or truncation surface radius (i.e., b or c, respectively). The denominator of Eq. **3** corrects for trajectories terminated at $r = c$ that may return to $r = b$ with some small but finite probability.

Brownian simulation of 500,000 trajectories was performed both in the absence of any forces other than hard sphere exclusion forces and in a parallel case in which a locking potential is turned on when the $N = 2$ intermediate state is achieved. This additional potential is described below.

Pathway and Rates for Forming the Protein–Protein Bond. The formation of a fully orientation-specific complex of two protein subunits can be viewed as a stepwise process, in which the proteins initially encounter in an orientationally nonspecific fashion, followed by rotational motions that lead to formation of an increasing number of contacts ($N = 1$, 2, and 3). The intermediate states are described below, and the estimated rate constants of achieving these states are given in Table 1.

Collisions, encounters, and the state N = *0*. The first step is formation of a nonspecific collision complex, in which the surfaces of the two proteins are within 2–4 Å, but the orientations are such that none of the four contact points is within 2 Å of its mate. It is important to differentiate here between the terms collision and encounter. A collision is defined as taking place when a protein pair attains a specified small distance from one another, here taken to be 2 Å; a collision is terminated when the proteins move farther than 4 Å apart. (The 4-Å specification for termination of a collision was chosen to avoid counting the very large number of diffusive recrossings of the 2-Å surface as separate collisions.) When the collision is terminated at this 4-Å separation, the BD calculation shows that there is actually a high probability of recolliding. Each time the proteins approach within 2 Å is counted as another collision. Eventually, in most cases, the two proteins will separate far enough that the probability of another collision is negligible. The complete set of interactions from the initial collision to this eventual separation constitutes an encounter. In our simulations we found that there were an average of nine collisions per encounter.

Table 1. Bimolecular rate constants for formation of successively more constrained protein–protein complexed states N

Complexation state N	BD-simulated rate constant, $M^{-1}\cdot s^{-1}$	Geometric rate constant, $M^{-1}\cdot s^{-1}$
1	3.8×10^8	2.2×10^6
2	4×10^6	1×10^4
3 (purely diffusive)	1×10^5	1×10^3
3 ($N = 2$ locking potential)	2×10^6	—

Comparison is made of the BD-simulated diffusion-controlled rate constant with the hypothetical geometric rate constant.

The situation for protein molecules in water may be contrasted with the behavior of gas molecules. In a gas, there is only one collision per encounter, and it is of very short duration; if the collision occurs outside the correct orientation, the two particles separate with negligible probability of recolliding. The geometric rate constant in Table 1 is based on the probability that diffusing spheres collide for the first time in the correct geometric alignment. Specifically, this rate is calculated as $k_D p_N$, where k_D is the Smoluchowski rate constant, and p_N is the geometric probability of correct alignment of N contact points. We determined p_N by generating random collisions and counting the number in correct alignment. Values obtained were $p_1 = 3.0 \times 10^{-4}$, $p_2 = 1.3 \times 10^{-6}$, and $p_3 = 2 \times 10^{-7}$. These small values are consistent with the high degree of specificity (2 Å tolerance) that constitutes correct docking. The geometric rate constant for the $N = 3$ complex is thus more than 3 orders of magnitude below the rates observed for protein–protein association.

In water, however, as shown by the BD simulation, each encounter comprises multiple collisions over an extended time duration, during which the proteins can reorient by rotational diffusion. The multiple collisions within an encounter greatly enhance the probability of correct interaction. This point is best understood with numbers derived from the simulation. First, for comparison, we note that the rotational correlation time for an 18-Å sphere is 5.3 ns. In the BD simulation, the duration of the average collision is only 0.38 ns, so there will be very little rotational reorientation within a single collision. The duration of the average encounter, however, is 6.3 ns, essentially the same as rotational correlation time. Thus, the interacting proteins can explore a substantial fraction of rotational orientations during each encounter.

State N = *1*. This state is defined as a collision complex in which one of the four pairs of contact points is matched within 2 Å, without any further orientational restriction (see Fig. 2). The geometric rate constant for forming such an alignment in a single, rapid collision is only $2.2 \times 10^6\ M^{-1}\cdot s^{-1}$. However, the BD simulation shows that the probability of achieving the alignment during the extended time of the encounter is increased almost 200-fold, giving a rate constant of $3.8 \times 10^8\ M^{-1}\cdot s^{-1}$. This is approximately the same as the rate of anion radical decay observed by Sommer *et al.* (14), which requires essentially the $N = 1$ state (see *Discussion*).

State N = *2*. In the present analysis, we do not postulate any stabilization of the $N = 1$ state. When the complex is terminated, however, the proteins do not become rotationally uncoupled until a time equivalent to the rotational relaxation time. There is therefore an enhanced probability that in the next collision the original contact points will still be close. But in the time between collisions there is also the possibility for rotation to bring another pair of contact points close to each other. One additional factor will enhance the probability of achieving the $N = 2$ state. Since the four contact points are all on the same face of the protein molecule, once an $N = 1$ state is achieved the other contact points will necessarily be closer than for random orientations. Although most $N = 1$

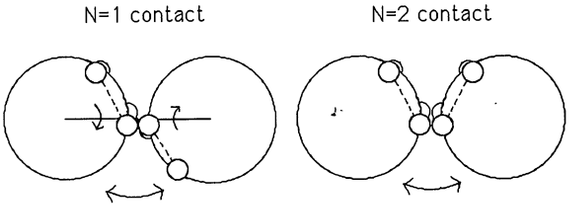

FIG. 2. Schematic showing loss of orientational freedom between $N = 1$ and $N = 2$ contacts.

complexes eventually separate, the BD simulation shows that ≈1% of them actually lead to the $N = 2$ state. The BD-simulated rate is 400 times greater than the geometric rate constant, the enhancement being attributed to the multiple collisions and rotational reorientation during the encounter. Most important, the BD-simulated rate for $N = 2$ formation, 4×10^6 $M^{-1}\cdot s^{-1}$, is very close to the rate observed for typical protein–protein bonds.

The achievement of the $N = 2$ state can be argued as the key point in the pathway, leading with high probability to completion of the final complex. This is because the area of contact in this state is an extended zone of contact along the entire line connecting the two points and constitutes a substantial fraction of the complete interface. Since this zone is aligned in the same fashion as in the final complex, van der Waals bonds, complementary ionic and hydrogen bonds, and hydrophobic interactions will be virtually complete over this entire zone. If the contact points are 17 Å apart and the zone of correct fit is ≈4 Å wide, this $N = 2$ zone will comprise 23% of the area of the complete interface. We can expect, therefore, that the $N = 2$ complex will be stabilized by a bond energy ≈1/4 of the total for the completely correct interface. This stabilization significantly prolongs the lifetime of the $N = 2$ collision complex beyond that obtained when no stabilizing forces are involved.

To include this important effect in our model, we performed a simulation in which a locking potential was added to stabilize specifically this $N = 2$ intermediate state. This potential energy was estimated to be −4.2 kcal/mol (1 cal = 4.184 J), 1/4 of the total typical intrinsic protein–protein bond energy.[§] No special forces are invoked until this state is attained, after which the locking potential is turned on. Any Brownian displacements taken in the $N = 2$ state that cause the loss of either of the two contacts are accepted only with a probability of $\exp(-4.2/RT)$, with rejected steps being repeated. In these simulations we found a lifetime of 2.3 ns for the $N = 2$ state. This is only a 6-fold increase over the duration of the average nonspecific collision (0.38 ns) but is sufficient to achieve a substantial rate enhancement for achievement of $N = 3$ (Table 1).

State N = *3*. The stabilization of the $N = 2$ complex not only prolongs the time available for reaching $N = 3$, but it also tacitly steers the complex in the correct direction, with the main freedom of movement in the $N = 2$ complex being a rocking along the line of contact. Any other movement (i.e., away from the $N = 2$ complex) would require breaking the specific bonds and hydrophobic interactions in the zone of contact. Moreover, rocking away from the correct position will similarly involve breaking bonds, while rocking toward the correct position will lead to formation of additional favorable bonds as the area of the bonded interface is increased. Therefore, once the $N = 2$ complex is formed, the rocking toward the fully docked interface is ensured with a high probability. As we show in Table 1, the rate constant for the formation of $N = 3$, which represents the fully formed protein–protein bond, is 1×10^5 $M^{-1}\cdot s^{-1}$ without the locking potential, and 2×10^6 $M^{-1}\cdot s^{-1}$ with the locking potential. Even without the locking potential, the $N = 3$ state is formed at a rate 2 orders of magnitude faster than would be expected based purely on geometric probability arguments. With the locking potential invoked, 50% of the $N = 2$ complexes proceed to the fully docked $N = 3$ state, and the calculated rate constant is equal to that observed in typical protein–protein associations.

[§]Assume for the completely docked complex $K_d = 10^{-7}$ M, the overall free energy for complex formation, $RT \ln K_d$, is −9.7 kcal/mol, but we need the intrinsic bond energy (see ref. 27 for this calculation). Assuming an entropic free energy of +7 kcal/mol, the intrinsic bond energy for the full complex is −16.7 kcal/mol. We set the intrinsic bond energy of the $N = 2$ state to 1/4 of this (−4.2 kcal/mol).

Discussion

The BD analysis has correctly predicted the rate constants observed for typical protein–protein interactions as arising directly from the diffusive interactions of the protein molecules in solvent. The 400-fold difference between the geometric rate constant and the BD-simulated rate for achieving the $N = 2$ intermediate complex, the key step along the pathway, may be attributed to the diffusion entrapment effect. Protein–protein association in solution must proceed by diffusive transport, which is very different from the interaction of molecules in the gas phase. In diffusive transport, the BD simulation shows that each encounter consists of a large number of diffusive steps. During a single encounter, the two molecules have time to undergo substantial rotational reorientation while remaining trapped in the vicinity of each other and undergoing multiple collisions.

Besides explaining the rate of protein–protein bond formation, the BD simulation also explains the very high rates of anion decay observed by Sommer *et al.* (14). The decay reaction they studied required contact of a single point on each of two protein molecules, without any additional orientational restriction. This is essentially the formation of the $N = 1$ complex in our scheme,[¶] for which the BD simulation gives a rate of 3.8×10^8 $M^{-1}\cdot s^{-1}$. This is very close to the rates of 10^8–10^9 $M^{-1}\cdot s^{-1}$ that Sommer *et al.* (14) observed for reactions on a wide variety of proteins and for which they invoked a nonspecific attractive force to achieve a lengthy collision. One might well describe the encounter as a lengthy collision, but we now see that this is a natural result of the diffusive entrapment effect and does not require any nonspecific attractive forces.

In the present analysis, we have not invoked any attractive or steering forces, apart from stabilization of the $N = 2$ complex by the partially formed bond. The rate of formation of this complex is already equal to that of a typical protein–protein association. Previous applications of BD dynamics have demonstrated further enhancements in rates by including the net charge of the molecules and dipole moments. Clearly, these can be invoked as necessary to explain the examples of particularly rapid protein–protein association. In particular, the extremely large rate constants observed for electron transfer reactions between oppositely charged cytochromes (10^7–10^9 $M^{-1}\cdot s^{-1}$ for reactions of cytochrome *c* with cytochrome b_5 or cytochrome *c* peroxidase) are accurately reproduced in BD simulations (8–11) that rigorously consider the detailed electrostatic forces and torques operating between these molecules.

[¶]There are two compensating numerical differences between our $N = 1$ complex and the anion-decay reaction. Our rate was calculated for overlap of any one of the four possible pairs of contacts, so the rate for a single contact pair should be reduced by a factor of 4. On the other hand, our contact was specified with a tolerance of 2 Å, while the anion decay was proposed to require only a 5-Å contact. Calculating the volumes of the contacts, we should increase our rate by a factor of $(5/2)^3 = 16$. Overall, the anion decay might be expected to occur ≈4 times faster than our BD-simulated rate for $N = 1$—namely, 1.5×10^9.

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