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Evaluation of the Distribution of Distances Between Energy Donors and Acceptors by Fluorescence Decay

(energy transfer/fluorescence/decay/conformation/polymers)

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ABSTRACT **When transfer of electronic excitation energy occurs between a donor–acceptor pair by the Förster mechanism, the decay of fluorescence of the donor follows first-order kinetics, with a rate constant that depends on the distance from donor to acceptor. In a system that contains donor–acceptor pairs of different separations, the fluorescence decay of the donors will not be exponential, but will depend on the distribution function of donor–acceptor distances, $f(r)$. Various approaches are outlined for the extraction of information about $f(r)$ from the decay curve of donor fluorescence. Specifically, if a plausible expression with adjustable parameters is assumed for $f(r)$, numerical methods can be used to evaluate the parameters that yield the closest fit between the observed decay curve and that calculated from the assumed $f(r)$. The technique of fluorescence decay may prove to be useful for determination of distribution functions of end-to-end distances of polymers to the edges of which suitable donor–acceptor chromophore pairs have been attached.**

Transfer of electronic excitation energy by the mechanism of dipole–dipole interaction may occur over appreciable distances—of the order of 50 Å—between an electronically-excited energy donor, A, and an acceptor, B. The various factors that contribute to the probability of energy transfer by this mechanism have been evaluated by Förster (1). The efficiency of energy transfer, E, is given by

$$E = \frac{R_0^6}{R_0^6 + r^6} \qquad [1]$$

where r is the distance between the donor and the acceptor chromophores, and R_0 is a quantity of dimension length given by

$$R_0^6 = \frac{9000\,(\ln 10)\,\chi^2\eta_0}{128\pi^5 n^4 N} \int_0^\infty f_A(\bar{\nu})\epsilon_B(\bar{\nu})\,\frac{d\bar{\nu}}{\bar{\nu}^4} \qquad [2]$$

In Eq. (2) η_0 is the fluorescence quantum yield of A in the absence of acceptor molecules, n is the index of refraction of the medium, N is Avogadro's number, $f_A(\bar{\nu})$ is the normalized fluorescence intensity of A at the wavenumber $\bar{\nu}$, $\epsilon_B(\bar{\nu})$ is the extinction coefficient of B at $\bar{\nu}$, and χ^2 is a dimensionless geometric factor determined by the orientation in space of the transition dipole moments of A and B. For donors and acceptors that rotate fast enough to randomize their orientations during the donor lifetime, χ^2 assumes the value $^2/_3$. Arguments have been presented for the use of this value for χ^2 in a few cases in which energy transfer between chromophores attached to polymer chains has been considered (2–4).

For the present discussion, the strong dependence of the probability of energy transfer on the separation r between donor and acceptor is noteworthy. This characteristic of long-range energy transfer has led Stryer and Haugland to propose the use of nonradiative energy transfer as a spectroscopic ruler for the determination of the distance between pairs of chromophores properly chosen and separated (2). The study and application of long-range energy transfer of the above type has been quite extensive, especially in the field of polypeptides and proteins (5).

Many cases of interest arise in which the donor–acceptor pairs are not uniformly separated; that is, their relative distance follows a certain distribution function. As an example one may quote energy transfer between donor and acceptor choromophores that are situated on polymer chains that do not assume a unique conformation. A few interesting cases of this type have been documented (5). Under such circumstances, the efficiency of energy transfer is given by (4)

$$E = \int_0^\infty f(r)\,\frac{R_0^6}{R_0^6 + r^6}\,dr \qquad [3]$$

where $f(r)$ represents the normalized distribution of distances between donor–acceptor pairs. The relative donor–acceptor distance is assumed not to vary during the donor lifetime. Obviously, under such circumstances the efficiency of energy transfer does not correspond to any trivial average of the distribution of distances between donor–acceptor pairs. Cantor and Pechukas have recently proposed that the entire distribution function, $f(r)$, be obtained by performance of a series of energy transfer experiments in which the R_0 value is varied. An iterative procedure was developed for the evaluation of $f(r)$ from such measurements (4).

We will show that it is not actually necessary to use various R_0 values for the evaluation of $f(r)$ from energy transfer studies, provided one studies the decay of fluorescence with time of the donor or acceptor instead of measuring transfer efficiency. Difficulties are thus obviated in obtaining systems that have different R_0 values, but are otherwise identical. It will be demonstrated that the decay curve is rather sensitive to the distribution function $f(r)$. Procedures will be presented to extract information from the fluorescence decay about the distribution of distances between donor–acceptor pairs. This approach is believed to be promising in the study of conformation of short polymer chains to the ends of which suitable chromophores have been attached, and the average end-to-end

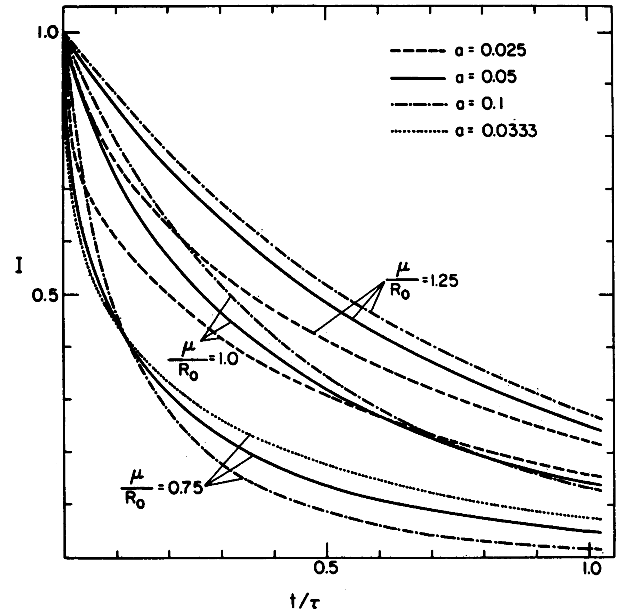

FIG. 1. Computer-simulated donor fluorescence decay curves, $I(t)$, for ensembles of donor–acceptor pairs the distance between which is described by parabolic distribution functions. $I(t)$ was obtained by use of Eq. 5, and the distribution functions shown in Fig. 2.

distance of which is of the order of R_0 of the attached pair of chromophores [30 ± 20 Å for typical cases (5)].

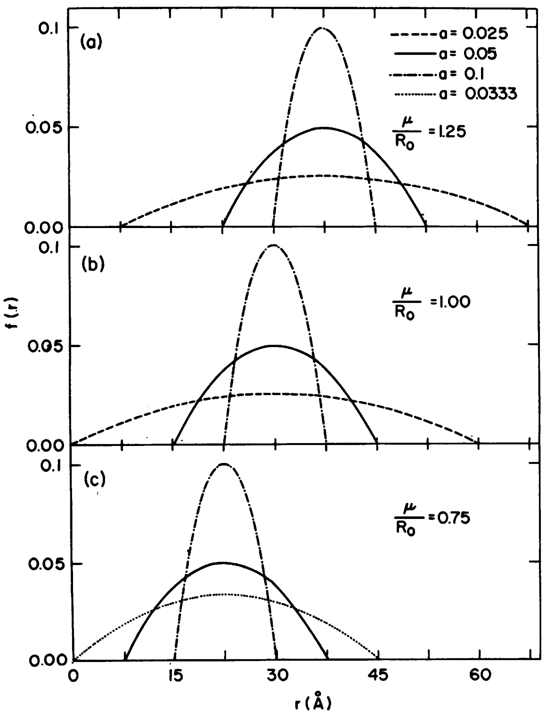

FIG. 2. Hypothetical distributions of distances between donors and acceptors in an ensemble of donor–acceptor pairs. The distribution functions, $f(r)$, are normalized parabolas of the form $a - {}^{16}/_{9}\, a^3 (r - \mu)^2$, where a and μ are the height and the mean of the parabola, respectively, and r is the donor–acceptor distance in a pair. $R_0 = 30$ Å.

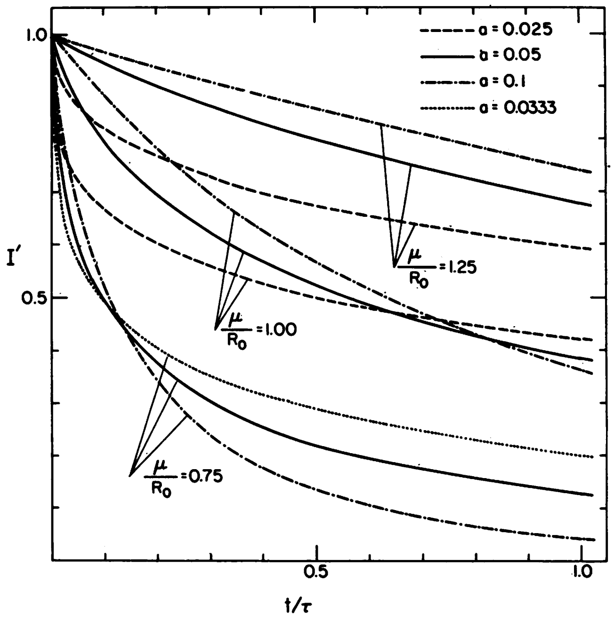

FIG. 3. Computer-simulated curves of $I'(t)$ for various distribution functions $f(r)$. $I'(t)$ was calculated according to Eq. 6 for the distribution functions $f(r)$ shown in Fig. 2. $I'(t)$ is the hypothetical decay curve the donor would have shown if it had lost its energy exclusively by energy transfer.

Fluorescence decay curve of donors in a collection of donor–acceptor pairs the distance between which is not unique

Suppose we irradiate an ensemble of donor–acceptor pairs, *A–B*, the distance between which is not unique, but is described by a normalized distribution function $f(r)$. The distance between one *A–B* pair to another *A–B* pair is assumed to be large, so that energy migration takes place within each pair only. Suppose also that the irradiation excites m donor chromophores and is of very short duration compared to the donor lifetime. Since the donor chromophores are excited randomly, the number of excited *A*'s that have a partner *B* chromophore in the range r to $r + dr$ is equal to $mf(r)dr$ at the moment of excitation ($t = 0$). This number will decay with time, with a first-order rate constant of $1/\tau + (1/\tau)(R_0/r)^6$, $1/\tau$ being the intrinsic rate constant of decay and $(1/\tau)(R_0/r)^6$ being the rate constant of energy loss to the neighboring acceptor. Let us define $mf(r,t)dr$ as the number of excited *A*'s that have a partner *B* chromophore in the range r to $r + dr$, and that have survived decay in the time range zero to t. $mf(r,t)dr$ is thus expressed by,

$$mf(r,t)dr = mf(r)\exp\left\{-\frac{t}{\tau} - \frac{t}{\tau}\left(\frac{R_0}{r}\right)^6\right\} dr \qquad [4]$$

The light intensity $I(t)$ of the fluorescence of the excited molecules A detected at time t is proportional to $\int_0^\infty mf(r,t)dr$. Thus,

$$I(t) = k\int_0^\infty f(r,t)dr = k\int_0^\infty f(r) \times \exp\left\{-\frac{t}{\tau} - \frac{t}{\tau}\left(\frac{R_0}{r}\right)^6\right\} dr \qquad [5]$$

where $k = m/\tau_0$, τ_0 being the radiative lifetime of A^*.

Fig. 1 illustrates the dependence of $I(t)$ on various hypothetical distributions of distances, $f(r)$, shown in Fig. 2. For the sake of simplicity, k was set equal to unity and the various distance distributions were chosen as parabolas of different mean distances and different scatter of distances. Each function $f(r)$ was normalized to unity, and thus assumed the form $a - {}^{16}/_{9}\,a^3(r - \mu)^2$ in the range $(\mu + 3/4a) > r > (\mu - 3/4a)$, a being the height and μ being the mean of the parabola. Obviously, the fluorescence decay curves of the donors are quite sensitive to variations in $f(r)$.

Interpretation of decay curves by procedures involving inverse Laplace transforms

Eq. **5** can be transformed into a mathematically simplified form. Let us define $I'(t) = I(t) \exp(t/\tau)$ and $y = (R_0/r)^6/\tau$. Additionally, define a new distribution function $g(y)$, so that $g(y)\mathrm{d}y = f(r)\mathrm{d}r$. Thus, $g(y)\mathrm{d}y$ is the fractional number of donor–acceptor pairs the distance between which falls in the corresponding range r to $r + \mathrm{d}r$. Introducing the new symbols into Eq. **5** one obtains:

$$I'(t) = I(t) \exp(t/\tau) = k\int_0^\infty f(r)\exp\left\{-\frac{t}{\tau}\left(\frac{R_0}{r}\right)^6\right\} dr$$

$$= k\int_\infty^0 g(y)\exp(-yt)dy = -k\int_0^\infty g(y)\exp(-yt)dy \qquad [6]$$

Thus, ignoring the scaling factor $-k$, $I'(t)$ *is the Laplace transform of* $g(y)$. In principle, $g(y)$ should therefore be obtainable from $I'(t)$ by procedures of inverse Laplace transforms.

Fig. 3 describes the functions $I'(t)$ corresponding to the various distribution functions $f(r)$ shown in Fig. 2. It is noteworthy that $I'(t)$, similarly to $I(t)$, is markedly affected by the shape of $f(r)$.

Since it is $f(r)$ rather than $g(y)$ that is of interest, the former may be obtained from the latter by the relations $y = (R_0/r)^6/\tau$ and $g(y)\mathrm{d}y = f(r)\mathrm{d}r$. Thus,

$$dy = -\frac{6R_0^6}{\tau r^7}\,dr \text{ and } f(r) = -\frac{6R_0^6}{\tau r^7}\,g(y) \qquad [7]$$

While the above approach is rigorous, it should be noted that in trying to derive $g(y)$, and thus $f(r)$, from $I'(t)$ through Eq. **6** one actually aims at resolving $I'(t)$ into a very large number of exponential decays. It has, however, been demonstrated that such a resolution demands an extremely high accuracy and precision for $I'(t)$ (6). Various numerical methods have been proposed to perform inverse Laplace transforms (6). The applicability of these methods to the interpretation of actual experimental data has yet to be tested.

Reconstruction of distribution functions from decay curves by the method of least squares

An approach that has proved feasible for the evaluation of $f(r)$ from $I(t)$ is to assume a plausible form for $f(r)$, insert the assumed function into Eq. **5**, and compare the calculated decay curve, $I_c(t)$, with the experimental curve $I(t)$. If the assumed function $f(r)$ contains enough adjustable parameters, it can be flexible enough to represent various possible distributions of distances between A and B. The parameters in the assumed $f(r)$ are then adjusted to yield the best fit between the calculated curve $I_c(t)$ and experimental curve $I(t)$. The quality of the experimental data put, of course, a limit on the finer details in the function $f(r)$ that can be evaluated [7].

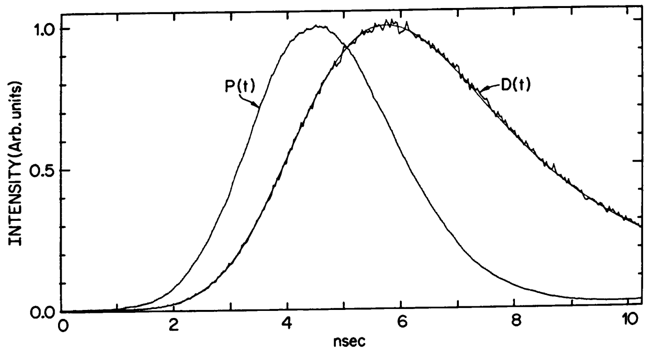

FIG. 4. Computer-simulated fluorescence response, $D(t)$, to an exciting flash, $P(t)$, of nonnegligible width. The noisy curve was calculated by Eq. **10**, assuming the distribution function $f(r)$ to be normalized with the parameters μ = 30 Å and a = 0.05. Random noise was added, equivalent to the Poisson-distributed counting error obtained when 10^4 photons were counted at the peak of $D(t)$. 400 Channels were used for the total time range. The full width at half-height of $P(t)$ was 3 nsec, while τ was chosen to be 4 nsec. The smooth $D(t)$ curve was obtained by fitting the noisy curve by the least-squares technique to a fluorescence response calculated by Eq. **10**. The values recovered for μ and a were 29.8 Å and 0.049, respectively.

As a criterion for the quality of fit between the calculated and experimental decay functions, $I_c(t)$ and $I(t)$, respectively, we used the root mean weighted square deviation, S,

$$S = \left[\frac{1}{N}\sum^{N} W_i(I(t_i) - I_c(t_i))^2\right]^{1/2} \qquad [8]$$

To evaluate S numerically, the time scale is divided into N equal intervals, t_i denoting the time of the ith interval. W_i is the statistical weight given to the ith experimental point†. A search for the parameters in the assumed distribution function that minimize S was performed by the REEP‡ or NLIN‡ computer programs for least-squares estimation of nonlinear parameters.

To illustrate this approach, let us imagine that the curves presented in Fig. 1 are experimental decay curves of fluorescence obtained from dilute solutions of donor–acceptor pairs separated according to distribution functions $f(r)$. To obtain $f(r)$ from the decay curves, $f(r)$ was assumed to be of the general shape of an inverted parabola, but nothing was presumed about the parameters of this parabola. Thus, the distribution functions were assumed to be of the form $a - {}^{16}/_{9}\,a^3(r - \mu)^2$ between the limits $(\mu + 3/4a), > r > (\mu - 3/4a)$, in which the mean, μ, and height at the maximum, a, were left as free pa-

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* The light intensity is customarily obtained in arbitary units. The experimental results may, however, be readily assigned the correct scale, since $I(t)$ should fulfill some simple relations, i.e., $I(0) = k$ and $1/m\int_0^\infty I(t)dt = \eta$, where η is the quantum yield of the fluorescence of the donor.

† $W_i = (1/\sigma_i^2)/(\Sigma 1/N\sigma_i^2)$ where σ_i^2 is the variance of the ith observation. If the noise in the decay curve is due to counting error when the photon counting technique is used, then $\sigma_i^2 \simeq n_i$, where n_i is the number of photons counted at the ith time interval.

‡ Shanno, D. F. (1967) *Share Program Library, SDA*, 3492; Marquardt, D. W. (1966) *Share Program Library, SDA*, 3094.

rameters. $I_c(t)$ thus assumed the form,

$$I_c(t) = \int_{\mu-3/4a}^{\mu+3/4a} \left[a - \frac{16}{9} a^3 (r-\mu)^2 \right] \times \exp\left\{ -\frac{t}{\tau} - \frac{t}{\tau}\left(\frac{R_0}{r}\right)^6 \right\} dr \qquad [9]$$

in which r and t are independent variables, $I_c(t)$ is the dependent variable, and a and μ are the parameters to be estimated. The values thus obtained for a and μ by minimization of S in Eq. **8** exactly equal to those used originally to construct the decay curves in Fig. 1.

It is of interest to note that the above method can be readily adapted to cases in which the duration of the exciting light pulse is not very short in comparison to the fluorescence decay time of the donor. In such a case, the experimentally obtained fluorescence response of the donor, $D(t)$, is a convolution of the exciting pulse, $P(t)$, and the true fluorescence decay of the donor, $I(t)$,

$$D(t) = \int_0^t P(t-s) I(s) ds \qquad [10]$$

The shape of $I(s)$ obviously depends on the parameters included in $f(r)$ (see Eq. **5**); here again, one is looking for the parameters included in $f(r)$ that yield the best fit between the experimentally obtained $D(t)$ and that calculated by Eq. **10**. Fig. 4 illustrates the above method for the evaluation of the parameters involved in $f(r)$ from the fluorescence response curve $D(t)$, when the curve involves a convolution with an excitation pulse $P(t)$ and, furthermore, contains some random noise. $D(t)$ was fitted to a function of the form shown in Eq. **10** with $f(r)$ assumed to be a parabola with adjustable parameters. The parameters obtained by the procedure of curve-fitting were in very good agreement with the correct parameters.

Discussion

The quantum yield of the light emitted by fluorescent molecules is proportional to the integral of the fluorescence decay curve with respect to time. Obviously, the study of the time course of fluorescence decay yields more information by far about the emitting system than the measurement of quantum yield. We have demonstrated above that the decay curve of the light emitted from the donor in a collection of macromolecules between the ends of which nonradiative energy transfer takes place yields useful information about the distribution of distances between the chain ends. Such information cannot be obtained from a single measurement of the total efficiency of energy transfer between the donor–acceptor pairs of chromophores attached to the ends of polymer chains.

Cantor and Pechukas (4) have recently proposed that the distribution of end-to-end distances of a polymer chain be evaluated from efficiency of energy transfer in a series of experiments in which the R_0 values for the donor–acceptor pairs are varied. It was suggested that different values of R_0 be obtained by use of different sets of chromophores, by partial quenching of the donor, or by change of solvent. It should be noted, however, that change of solvent may markedly affect the function $f(r)$ for the polymer; measurement of efficiency of energy transfer in one solvent may, therefore, be useless for the evaluation of $f(r)$ in another solvent. The suggested use of different sets of chromophores to obtain different R_0 values is experimentally a very lengthy and tedious procedure, even with modern techniques of synthesis. In practice, the number of determinations of efficiency of energy transfer is, therefore, expected to be limited, and the information obtained about $f(r)$ will be far more limited than that obtained from a single decay curve of donor fluorescence as suggested herewith. The accuracy now obtainable in the determination of fluorescence decay curves is remarkable indeed (8).

The dependence of the transfer of electronic excitation energy by the dipole–dipole mechanism on r^{-6} has been verified experimentally (2). However, the agreement between the experimentally found values of R_0 and the corresponding values calculated theoretically, according to Eq. **2**, is poor in various cases (5). An unequivocal explanation of the discrepancy between the measured and calculated values of R_0 has apparently not been presented. Therefore, it seems necessary to use experimentally determined values for R_0 for the pair of chromophores used. This value can be obtained, for example, by the concentration dependence of energy transfer between the chromophores when free in solution (9). One should be careful to avoid complications due to translatory diffusion of the chromophores in solution (13). It should be noted that if R_0 is in error, the shape of $f(r)$ will not be affected, but the units of r will not be correct.

The function $I'(t)$ defined as $I(t)e^{t/\tau}$ has a simple intuitive meaning, namely it is the hypothetical decay of the donor molecules due to energy transfer alone if no other decay processes took place. It can be readily shown that certain kinds of moments of $f(r)$ with respect to r can be obtained from $I'(t)$ by simple manipulation of this function. From Eq. **6** one obtains,

$$I'(t) = k \int_0^\infty f(r) \exp\left\{ -\frac{t}{\tau}\left(\frac{R_0}{r}\right)^6 \right\} dr \qquad [11]$$

Integration of $I'(t)$ with respect to time yields,

$$\int_0^\infty I'(t)dt = k \int_0^\infty \int_0^\infty f(r) \exp\left[-\frac{t}{\tau}\left(\frac{R_0}{r}\right)^6 \right] drdt = k\tau/R_0^6 \int_0^\infty r^6 f(r) dr \qquad [12]$$

The area under the $I'(t)$ curve yields the 6th moment of $f(r)$ with respect to the distance r. In contrast to the above integration of $I'(t)$ with respect to time, similar integration of $I(t)$ yields more complicated expressions involving $f(r)$. For example, the area under $I(t)$, which is equal to the quantum yield of the donor, is equal to $\int_0^\infty [(R_0^6)/(R_0^6+r^6)] f(r) dr$.

In principle, one can continue to obtain higher moments of $f(r)$. For example, the 12th moment is obtained by double integration of $I'(t)$ with respect to time:

$$\int_0^\infty \int_T^\infty I'(t)dtdT = k \int_0^\infty \int_T^\infty \left\{ \int_0^\infty f(r) \exp \left[-\frac{t}{\tau}\left(\frac{R_0}{r}\right)^6 \right] dr \right\} dtdT = \frac{k\tau}{R_0^6} \int_0^\infty \int_\tau^\infty r^6 f(r) \exp \left[-\frac{T}{\tau}\left(\frac{R_0}{r}\right)^6 \right] drdT = k\left(\frac{\tau}{R_0^6}\right)^2 \int_0^\infty r^{12} f(r) dr \qquad [13]$$

Corresponding integrations of experimentally obtained data may require lengthy, possibly unreliable, extrapolations to infinite time. This is especially true for the r^{12} or higher moments. Notwithstanding this limitation, approximate values for the various moments of $f(r)$ derived from $I'(t)$ may prove useful in some cases to obtain qualitative information about the distribution of end-to-end distances of polymers.

By expanding the exponent in the integral of Eq. **11** into a power series, one can readily show that the slope of $I'(t)$ with respect to t at $t = 0$ yields another moment of $f(r)$, namely $(R_0^6)/\tau \int_0^\infty (1/r^6) f(r) \mathrm{d}r$. This kind of moment is probably of little value in practice, since it will reach infinity unless $f(r)$ approaches zero faster than r^6 as r approaches zero.

We are grateful to Prof. Ephraim Katchalski for helpful discussions related to this work.

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