

# Energy Transfer. A System with Relatively Fixed Donor–Acceptor Separation

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The efficiency of electronic excitation energy transfer was measured between two donor and acceptor pairs, the first (A) with a *p*-methoxyphenylacetyl group as donor and a 1-naphthoyl group as acceptor, and the second (B) with a 1-naphthoyl group as donor and anthracene-9-carbonyl as acceptor. Attachment was to the hydroxyl groups of a "bisteroid" (XIV) resulting in a relatively fixed donor–acceptor separation of about 20 Å. The solvent used was dioxane. The energy transfer measurements were used, together with fluorescence and absorption spectral data of the donor and acceptor moieties, to calculate the donor–acceptor separation, *R*, according to the corrected Förster equation for dipole–dipole resonance transfer. The average values of *R* obtained assuming random donor–acceptor orientations were  $21.3 \pm 1.6$  Å. for pair A and  $16.7 \pm 1.4$  Å. for pair B. The average donor–acceptor separation obtained from measurements of molecular models, allowing for donor and acceptor rotation around bonds at the attachment sites, was  $21.8 \pm 2.0$  Å. for A and  $21.5 \pm 2.0$  Å. for B, taking a linear average; and  $19.2 \pm 2.0$  Å. for A and  $19.4 \pm 2.0$  Å. for B, taking a  $(1/R^6)^{-6}$  average. Considerations based on steric factors and transition moment polarization directions predict a somewhat higher value for B. The calculated and measured separations for pair A agree to within experimental uncertainties. The somewhat low calculated separation between the groups of pair B was most likely due to nonrandom orientations tending to align the interacting dipoles and increase transfer, but may also have been due to an additional contribution to the energy transfer from an exciton process.

## Introduction

Measurement of the transfer of electronic excitation energy between molecules can, in principle, be used to obtain information about the separation between these molecules. Knowledge of this separation can in turn be used to study interactions between molecules and structural alterations of macromolecules. The relation of the energy transfer to donor–acceptor separation, orientation, and spectral properties, and to solvent conditions, depends on the interaction energy between the energy donor and energy acceptor transition dipoles.<sup>1</sup> When this interaction energy is much greater than the vibrational band width of the corresponding transitions ( $10^3$  to  $10^4$  cm.<sup>-1</sup>), the interaction can be considered to be between two fixed dipoles and exciton transfer should result. This type of energy transfer depends on  $1/R^3$ , where *R* is the donor–acceptor separation. When the interaction energy is much less than an individual vibronic band width (10 to 100

cm.<sup>-1</sup>), fixed dipole-induced dipole resonance transfer would be predicted. Such resonance energy transfer depends on  $1/R^6$ . However, the exact values of these limits are not known, and the behavior of the energy transfer expression at intermediate interaction energies is difficult to predict. Therefore, we considered it desirable to measure the energy transfer with fluorescence techniques using a model compound, under conditions such that the above parameters were known, at a relatively weak interaction energy. Such experiments allow a check of the most plausible expression governing the process or, in a sense, a calibration of the energy transfer process as a basis for further investigations.

To be suitable for this type of study, a model compound must satisfy certain requirements. First, the donor and acceptor groups must be such that energy transfer between them is, in fact, possible. For weak donor–acceptor interaction energies, there must occur absorption transitions in the energy acceptor at the same wave lengths as there occur emission transitions in the energy donor; *i.e.*, there must be an overlap between the donor fluorescence spectrum and the acceptor absorption spectrum. For strong donor–acceptor interaction energies, spectral overlap is not as important, and only relative spectral proximity is needed.<sup>1</sup> Also, for maximum sensitivity in these measurements, the donor and acceptor should be separated by a known distance at which the energy transfer is near 50%. Intuitively, this means that the energy transfer must be large enough to measure but not so large that the transfer efficiency is little affected by a change in the transfer probability. Mathematically, this means that the change in the energy transfer efficiency with the fractional change in donor–acceptor separation will be a maximum.

The compounds chosen for our studies satisfied these criteria. Two donor–acceptor pairs were used, each donor–acceptor pair being joined by the same rigid decacyclic system (*vide infra*). In the first pair the energy donor was a *p*-methoxyphenylacetyl group and the energy acceptor was a 1-naphthoyl group. In the second, 1-naphthoyl was the donor and anthracene-9-carbonyl was the acceptor. There is good overlap between the donor fluorescence spectrum and the acceptor absorption spectrum in both pairs.

For weak interactions, the critical distance at which the energy transfer can be expected to be 50% efficient can be shown, using equations to be discussed later, to be near 20 Å. for both donor–acceptor pairs. At such a separation, the interactions between the donors and acceptors used here are indeed weak. The interaction energy  $\Delta U$  is given as

$$\Delta U = \frac{K|\bar{\mu}_1||\bar{\mu}_2|}{n^2R^3} \quad (1)$$

(1) Th. Förster in "Comparative Effects of Radiation," M. Burton, J. Kirby-Smith, and J. Mazel, Ed., John Wiley and Sons, Inc., New York, N. Y., 1960.

Table I

Compounds	M.p., °C. <sup>a</sup>	[α] <sup>25</sup> D, <sup>b</sup> deg.	λ <sub>max</sub> , <sup>c</sup> mμ	ε	Pertinent infrared absorption bands, cm. <sup>-1</sup>	Formula	Analyses, %			
							Found		Calcd.	
							C	H	C	H
I	123-123.5	-19	228.5 <sup>d</sup> 276.5 283	13700 <sup>d</sup> 2030 1785	1725, 1670, 1610, 1590 <sup>e</sup>	C <sub>30</sub> H <sub>38</sub> O <sub>4</sub>	77.85	8.15	77.9	8.3
II	179-180	-8	295	7200	1715, 1665, 1650, 1590, 780 <sup>f</sup>	C <sub>32</sub> H <sub>36</sub> O <sub>3</sub>	82.3	7.85	82.0	7.75
VI	230-231	-32	276.5 283	2 × 1975 2 × 1730	1740, 1730, 1710 <sup>g</sup>	C <sub>61</sub> H <sub>78</sub> O <sub>8</sub>	78.0	8.35	78.0	8.35
VII	331-332	-23	295	2 × 7220	1710, 1590, 1580, 1500 <sup>g</sup>	C <sub>65</sub> H <sub>74</sub> O <sub>6</sub>	82.15	7.85	82.1	7.85
VIII	>350 <sup>h</sup>	-43	295	46	1735, 1720, 1705 <sup>f</sup>	C <sub>47</sub> H <sub>66</sub> O <sub>6</sub>	77.65	9.15	77.65	9.15
XIV	>350 <sup>i</sup>	-58	295	52						
IV	189-190				1740, 1620, 1610, 1580, 1515 <sup>f</sup>	C <sub>62</sub> H <sub>80</sub> O <sub>8</sub>	78.05	8.7	78.1	8.45
XIII	165-167		231 <sup>d</sup> 275 <sup>d</sup> 282 <sup>d</sup>	20500 <sup>d</sup> 3200 <sup>d</sup> 2700 <sup>d</sup>	1745, 1715, 1670, 1635, 1600, 1590 <sup>e</sup>	C <sub>39</sub> H <sub>46</sub> O <sub>6</sub>	77.2	7.6	77.7	7.6
IXa + b	279-280		276.5 283	1745 1540	3605, 1745, 1740, 1730, 1720, 1605 <sup>e</sup>	C <sub>62</sub> H <sub>76</sub> O <sub>6</sub>	78.65	8.9	78.95	8.9
Xa + b	307-308		295	7440	1710 <sup>g</sup>	C <sub>64</sub> H <sub>68</sub> O <sub>6</sub>	81.0	8.45	81.35	8.6
XIa + b	242-245		284 296	7810 7520		C <sub>63</sub> H <sub>76</sub> O <sub>7</sub>	80.35	7.85	80.05	8.1
XIIa + b	...		296 346 363 382.5	7895 6095 8940 8085	1720, 1715, 1705, 785, 730, 680 <sup>f</sup>	C <sub>69</sub> H <sub>76</sub> O <sub>6</sub>	82.5	7.55	82.75	7.65
XV	110-113	-17	276.5 283	1860 1630	1720, 1610, 1580, 810 <sup>f</sup>	C <sub>36</sub> H <sub>54</sub> O <sub>3</sub>	80.55	9.8	80.85	10.2
XVI	155-156	-12	295	7540	1710, 1595, 1575 <sup>f</sup>	C <sub>38</sub> H <sub>52</sub> O <sub>2</sub>	83.9	9.45	84.4	9.7
XVII	201-202	+28	330 346 363 382.5	3080 6100 9130 8230	1715 <sup>f</sup>	C <sub>42</sub> H <sub>64</sub> O <sub>2</sub>	85.0	9.05	85.35	9.2

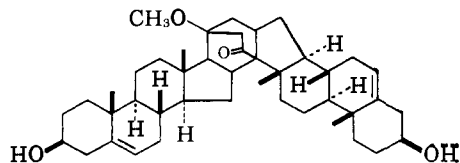
<sup>a</sup> Uncorrected, *in vacuo* when over 250°. <sup>b</sup> In ca. 1% solution in *p*-dioxane. <sup>c</sup> In *p*-dioxane unless stated otherwise. <sup>d</sup> In methanol. <sup>e</sup> In carbon tetrachloride. <sup>f</sup> In Nujol. <sup>g</sup> In chloroform. <sup>h</sup> Reported<sup>3</sup> m.p. 312-313°. <sup>i</sup> Reported<sup>23</sup> m.p. 312°.

where  $\bar{\mu}_1$  and  $\bar{\mu}_2$  are the interacting dipole strengths,  $K$  is an orientation factor

$$K = \frac{\bar{\mu}_1 \cdot \bar{\mu}_2}{|\bar{\mu}_1| |\bar{\mu}_2|} - 3 \left( \frac{\bar{\mu}_1 \cdot \bar{n}}{|\bar{\mu}_1|} \right) \left( \frac{\bar{\mu}_2 \cdot \bar{n}}{|\bar{\mu}_2|} \right) \quad (2)$$

where  $\bar{n}$  is the normal between the two dipoles,  $n$  the solvent index of refraction, and  $R$  the donor-acceptor separation. If the approximation  $2.303 \int \epsilon(\nu) d\nu = 2.5 \times 10^{38} \nu_{1m} |\bar{\mu}|^2$  is used,<sup>2</sup> where  $\epsilon$  is the extinction coefficient,  $\nu$  the frequency, and  $\nu_{1m}$  the mean absorption frequency, and, using  $n^2 \approx 2$  (e.g., dioxane),  $K \leq 2$ , and  $R \approx 20$  Å., then the interaction energy  $\Delta U$  for the first donor-acceptor pair  $\leq 3$  cm.<sup>-1</sup> and that for the second donor-acceptor pair  $\leq 6$  cm.<sup>-1</sup>.

For the rigid framework to which the donor and acceptor groups were attached, we chose a decacyclic system (shown below) first synthesized by Morita,



Slomp, and Jensen.<sup>3</sup> This nearly symmetrical molecule provides a large and fixed separation (about 18.5

(2) G. M. Barrow, "Introduction to Molecular Spectroscopy," McGraw-Hill Book Co., Inc., New York, N. Y., 1962, p. 72.

(3) K. Morita, G. Slomp, and E. V. Jensen, *J. Am. Chem. Soc.*, **84**, 3779 (1962).

Å.) between two potential attachment sites, the two hydroxyl groups, to which donor and acceptor groups were joined *via* ester linkages. The oxygen-oxygen distance is fixed because of the fused ring nature of the "bisteroid" nucleus, but some uncertainty exists in the exact separation because of the possibility of rotation of the donor and acceptor around the bonds at the attachment sites. Taking this rotation into consideration, the donor-acceptor separations obtained from measurements on Dreiding molecular models were near 20 Å.

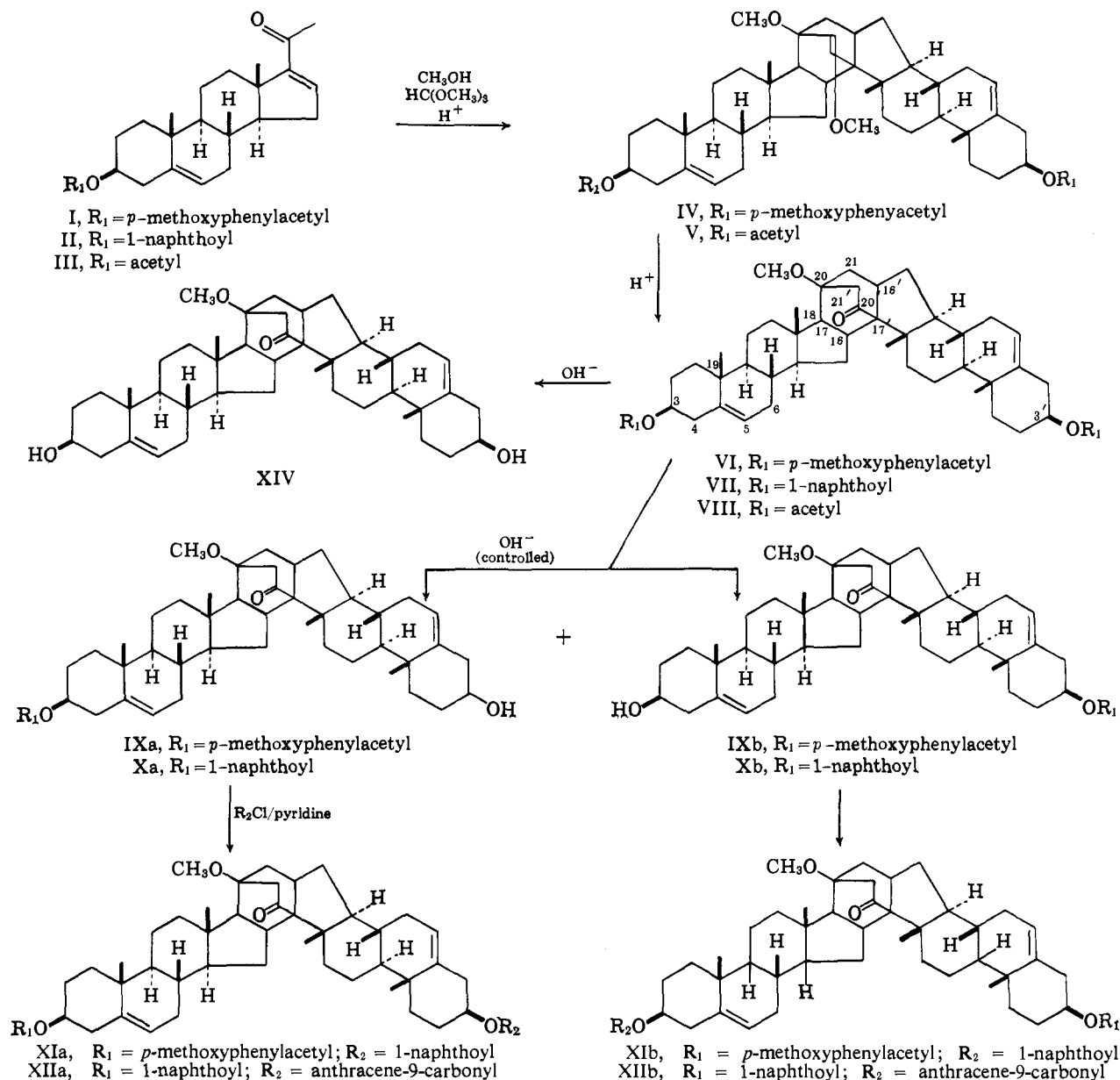
Previous investigators have shown that it is possible to use concentrated solutions of free donors and acceptors and measure the transfer either by sensitized fluorescence<sup>4</sup> or by fluorescence depolarization.<sup>5</sup> However, with such systems, some arbitrary estimate must be made of the donor-acceptor distance of closest approach, and complicated effects due to high concentrations (e.g., association) can occur. Some workers have studied donors and acceptors attached to the same molecule. In some of these experiments, there was only a statistical average of donor per acceptor, and the exact donor and acceptor positions were not known.<sup>6,7</sup> In another system the donor and acceptor were connected by flexible chains whose length variation introduced uncertainties.<sup>8</sup> Finally, other work

(4) G. Weber, *Biochem. J.*, **75**, 335 (1960).

(5) G. Weber, *Trans. Faraday Soc.*, **50**, 552 (1954).

(6) G. Weber and F. W. J. Teale, *Discussions Faraday Soc.*, **27**, 134 (1959).

(7) V. Shore and A. Pardee, *Arch. Biochem. Biophys.*, **62**, 355 (1956).



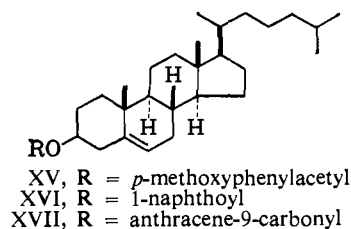
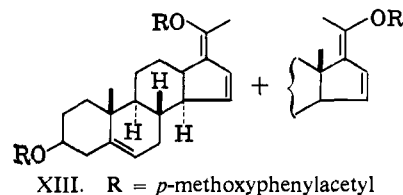
has been reported<sup>9</sup> with systems where the donor and acceptor were held so close that transfer was nearly 100%, and nothing could be said about the dependence of transfer on separation except that the separation was below a certain limit.

The system used in the present work is considered to be superior to others used in similar attempts to verify the electronic energy transfer theory. It allows measurement of the electronic energy transfer between single donor-acceptor pairs at a uniform donor-acceptor separation at very low optical densities.

### Experimental

To synthesize compounds such as XIa containing one donor and one acceptor moiety, the following approach was adopted. An ester of 16-dehydropregn-5-en-3 $\beta$ -ol-20-one (e.g., I) was "dimerized"<sup>3</sup> to the "bisteroid" IV, and then further transformed to the more stable ketone diester VI. Partial ester hydrolysis followed by careful separation gave *inter alia* a mixture of isomeric monoesters IXa and IXb. This mixture

could not be resolved and was converted by acylation to a mixture of diesters XIa and XIb<sup>10</sup> containing one



donor and one acceptor moiety. The latter isomeric mixture similarly could not be resolved and was used as such in the physical measurements.

(8) O. Schnepf and M. Levy, *J. Am. Chem. Soc.*, **84**, 172 (1962).

(9) S. Shifrin, *Biochim. Biophys. Acta*, **81**, 205 (1964).

(10) Thereafter collectively referred to as XI; similar designation for other a,b pairs.

Table II. Nuclear Magnetic Resonance Spectra<sup>a</sup>

Assignment	Compound				
	I <sup>b</sup>	VII <sup>b</sup>	VIII <sup>c</sup>	XIII <sup>c, d</sup>	
C-18, -19 (and -18', -19')	9.13 (3), 8.97 (3)	9.18, 9.03, 8.92	9.22, 9.05 (6), 8.97 (6)	9.08, 9.03, 8.96	
CH <sub>3</sub> COO-			7.97 (6)		
C-21	7.85 (3)	7.4	7.75-7.44 (10)	7:75	
C-4, -4', -7, -7'					
ArCH <sub>2</sub> COO-	6.60 (2)				
CH <sub>3</sub> O-	6.26 (3)	6.78 (3)	6.76 (3)	6.24	
				6.20	
C-6 (and -6')	4.72 (1)	4.5 (2)	4.7		
C-16	3.48 (1)				
Aromatic	3.4-2.8 (4)	2.8-0.8 (14)			

<sup>a</sup> Chemical shift in  $\tau$  (with respect to tetramethylsilane); number of protons given in parentheses. <sup>b</sup> In carbon tetrachloride. <sup>c</sup> In deuteriochloroform. <sup>d</sup> N.m.r. indicated that XIII is a mixture of geometric isomers.

**Materials.** Physical constants and elementary analyses of compounds synthesized are summarized in Tables I and II. In view of the ease with which steroids cocrystallize, thin layer chromatography (over silica, using ethyl acetate-benzene (1:5) unless stated otherwise) was used extensively.

**16-Dehydropregn-5-en-3 $\beta$ -ol-20-one *p*-Methoxyphenylacetate (I) and Its Enol *p*-Methoxyphenylacetate (XIII).** 16-Dehydropregn-5-en-3 $\beta$ -ol-20-one (11 g.) was treated with 25 g. of *p*-methoxyphenylacetyl chloride in 600 ml. of pyridine at room temperature overnight. On removal of pyridine and working up, a gum was obtained which was separated by chromatography over alumina to give 5.0 g. of I (31%) as needles from ethanol-water, and 3.7 g. of XIII (17%), needles from benzene-ethanol.

**The Dimeric Ketone Di-*p*-methoxyphenylacetate VI.** To 530 mg. of 16-dehydropregn-5-en-3 $\beta$ -ol-20-one *p*-methoxyphenylacetate (I) dissolved in 4 ml. of trimethyl orthoformate was added 3.3 ml. of a 0.6% solution of concentrated sulfuric acid in anhydrous methanol. After 2 hr. at room temperature, the solution was evaporated *in vacuo*, and the residue distributed between ether and aqueous sodium bicarbonate. Chromatography of the ether-soluble material over alumina gave 260 mg. (48%) of needles consisting of a mixture of the dimeric enol ether IV and of the dimeric ketone VI. Pure enol ether IV crystallizing as plates from benzene-ethanol was obtained from the mixture by chromatography over silica. Conversion of the mixture to pure ketone VI was effected by treatment of 680 mg. with 40 ml. of 1% concentrated sulfuric acid in dioxane at room temperature for 90 min. The yield of the ketone VI, plates from benzene-ethanol, was 93%.

**Partial Ester Hydrolysis of the Dimeric Ketone Di-*p*-methoxyphenylacetate VI.** A solution of 1.26 g. of VI in a mixture of 40 ml. of tetrahydrofuran, 30 ml. of methanol, and 2.68 ml. of 1 *N* aqueous sodium hydroxide (1 equiv.) was stirred at 0°. After 1 hr. when the ratio of diester:monoester:diol was estimated (thin layer chromatography) as 3:2:1, the solution was made just acid to phenolphthalein. Organic solvent was removed and the precipitated solid chromatographed over alumina. *n*-Hexane-benzene eluted recovered starting material, while elution with chloroform-benzene yielded 240 mg. of a mixture of mono-*p*-methoxyphenylacetates IXa and IXb crystal-

lizing as minute needles from benzene-methanol, and having  $R_f$  0.3 (silica/ethyl acetate-benzene (1:3)).

***p*-Methoxyphenylacetate-1-Naphthoate Mixture XIa and XIb.** A solution of 105 mg. of the mono-*p*-methoxyphenylacetates IXa and IXb in pyridine was treated with 0.5 g. of 1-naphthoyl chloride at room temperature for 24 hr. The solution was poured into water and the precipitated solid filtered and washed. After removal of 245 mg. of 1-naphthoic anhydride by crystallization (chloroform-methanol), the product was chromatographed over alumina to yield 64 mg. (51%) of crystals of the mixture XIa and XIb.

**16-Dehydropregn-5-en-3 $\beta$ -ol-20-one 1-Naphthoate (II).** 16-Dehydropregn-5-en-3 $\beta$ -ol-20-one (19.2 g.) in 200 ml. of pyridine was treated with 50 g. of 1-naphthoyl chloride at room temperature overnight. Concentration and addition of water precipitated a solid. Fractional crystallization from benzene-methanol removed 1-naphthoic anhydride and yielded 13.6 g. (48%) of II.

**The Dimeric Ketone Di-1-naphthoate VII.** Acid-catalyzed condensation of II as in the case of I gave 31% yield of VII, needles from chloroform-methanol.

**Partial Ester Hydrolysis of the Dimeric Ketone Di-1-naphthoate VII.** This was carried out as in the case of VI. A mixture of mono-1-naphthoates Xa and Xb was obtained as small needles from benzene-methanol, having  $R_f$  0.3 (thin layer chromatography).

**1-Naphthoate-Anthracene-9-carboxylate Mixture XIIa and XIIb.** A solution of 340 mg. of the mono-1-naphthoate mixture Xa and Xb and of 0.72 g. of anthracene-9-carbonyl chloride (m.p. 97-99°) in 5 ml. of pyridine was heated under reflux. An additional 0.80 g. of the acid chloride was added in two portions at 15-min. intervals, and the reaction was terminated after a total of 45 min. by the addition of water. The precipitated solid was washed with water and methanol and dried. Crystallization from benzene removed 0.87 g. of anthracene-9-carboxylic anhydride, and the remaining material was chromatographed twice over 15 g. of neutral alumina (grade III). The 1-naphthoate-anthracene-9-carboxylate mixture XIIa and XIIb which was eluted with *n*-hexane-benzene (2:1) was obtained as a very pale yellow precipitate (35 mg., 77%) by the addition of methanol to a benzene solution. It was finally chromatographed a third time and reprecipitated three times.

*The Dimeric Ketone Diol XIV.* A solution of 310 mg. of the di-1-naphthoate VII in a mixture of 30 ml. of tetrahydrofuran, 5 ml. of methanol, and 2 ml. of 1 *N* aqueous sodium hydroxide was heated under reflux for 3 hr. Removal of organic solvents and addition of water precipitated the diol (195 mg., 93%) which crystallized from chloroform-methanol as plates. Circular dichroism (dioxane)  $[\theta]$  was  $1.7 \times 10^3$  at 290  $m\mu$ .

*The Dimeric Ketone Diacetate VIII.* Crude dimeric enol ether diacetate V<sup>3</sup> (510 mg.) was dissolved in 10 ml. of dioxane, and 0.4 ml. of concentrated sulfuric acid was added with stirring. Water was added after 45 min. and the precipitated solid crystallized from benzene-methanol. Circular dichroism (dioxane)  $[\theta]$  was  $2.1 \times 10^3$  at 290  $m\mu$ .

*Cholesteryl p-Methoxyphenylacetate (XV).* Cholesterol (0.5 g.) was treated with excess of *p*-methoxyphenylacetyl chloride in pyridine at room temperature overnight. The chromatographed (alumina) product crystallized as plates (220 mg.) from ether-methanol.

*Cholesteryl 1-Naphthoate (XVI).* This was prepared as in the case of XV in 56% yield. It crystallized as plates from chloroform-methanol.

*Cholesteryl Anthracene-9-carboxylate (XVII).* A solution of 300 mg. of cholesterol and 440 mg. of anthracene-9-carbonyl chloride in 5 ml. of pyridine was heated under reflux for 4 hr. Reflux was continued for a further 4 hr. after the addition of 750 mg. of the acid chloride. The solution was poured into water and the precipitated solid collected. From this the benzene-insoluble anthracene-9-carboxylic anhydride was removed, and chromatography over alumina afforded 290 mg. (64%) of XVII, pale yellow needles from chloroform-methanol.

*Methods.* Individual donor and acceptor spectra were obtained from measurements on the corresponding esters of either cholesterol (XV-XVII) or of 16-dehydro-pregn-5-en-3 $\beta$ -ol-20-one (I and II). The solvent used was *p*-dioxane of either Fisher certified reagent grade or Matheson Coleman and Bell spectroscopic grade.

*Instruments.* (1) Absorption measurements were performed on a Cary Model 15 recording spectrophotometer. Blanks were run to correct for base-line variation with wave length.

(2) Fluorescence spectra were obtained using a Zeiss spectrofluorometer. The light source was an XB501 Osram 450-w. xenon lamp, and the phototube was an RCA 1 P28. The excitation and emission monochromators were Zeiss M4QIII prism monochromators. An FM4 Zeiss fluorescence attachment, which contained a cell holder for four square cells, was used. Excitation was through one of the upright sides of the cell, while emission was read at right angles to excitation through the bottom of the cell. Solvent blanks were measured, and phototube gain was periodically adjusted to keep constant the readings on a standard solution (otherwise the variation was ~5%).

(3) Optical rotatory dispersion measurements (in dioxane) were obtained using a Cary Model 60 recording spectropolarimeter. Circular dichroism measurements were kindly performed by Dr. S. Beychok of the College of Physicians and Surgeons, Columbia University, using a Roussel-Jouan dichrograph.

The fluorescence spectra obtained on the Zeiss spectrofluorometer were corrected for relative number of incident photons as a function of the wave length of the exciting light (relative input efficiency), and relative response of the detecting system as a function of wave length of emission (relative output efficiency). Quantum counters of  $10^{-2}$  *M* solutions of 8-aminonaphthalene-1,5-disulfonic acid in water ( $265 m\mu < \lambda < 365 m\mu$ ) and rhodamine B in ethylene glycol ( $365 m\mu < \lambda < 440 m\mu$ ) were used to determine input efficiency.<sup>11,12</sup> Output efficiency was determined from these data and readings in which a piece of aluminum foil placed at the cell position was used to reflect the light directly from one monochromator to the other. When obtaining spectra, low solution optical densities ( $0.03 \pm 0.02$ ) were used to minimize self-absorption and other complications possible with concentrated solutions.

The absolute quantum yields of the steroid esters containing the two donor groups were determined by comparing their fluorescence yields with those of solutions of tyrosine (in the case of the *p*-methoxyphenylacetyl ester) and tryptophan (in the case of the 1-naphthoyl ester). These amino acids have fluorescence emission spectra with maxima less than 5  $m\mu$  away from those of the corresponding donor group, and their quantum yields (0.21 for tyrosine and 0.195 for tryptophan) have been determined.<sup>13</sup> The spectra were all corrected for relative output efficiency, but this enters as a second-order effect owing to the similarity between donor and amino acid spectra.

The total band width of excitation was computed from the particular slit width used and dispersion values supplied by the monochromator manufacturer. The total absorption was corrected for the variation of input efficiency over this band width.

To determine the energy transfer efficiency, the solution containing the donor-bisteroid-acceptor compound was excited at a wave length where both the donor and acceptor had appreciable absorption. The resulting emission spectrum was assumed to be the sum of emission from the donor and acceptor. Comparisons of the relative contributions from each moiety with those expected on the basis of measurements of isolated donor or acceptor steroid ester fluorescence, when corrected for absorption differences, could be used to determine both the efficiency of transfer to the acceptor and the efficiency of transfer from the donor. The latter value was determinable only by an extrapolation procedure to be described later.

Additional experiments were also performed which helped to characterize the energy transfer in the system described in this paper.<sup>14</sup>

(11) G. Weber and F. W. J. Teale, *Trans. Faraday Soc.*, **53**, 646 (1957).

(12) G. Weber and F. W. J. Teale, *ibid.*, **54**, 640 (1958).

(13) F. W. J. Teale and G. Weber, *Biochem. J.*, **65**, 476 (1957).

(14) These experiments involved studies of energy transfer (1) as a function of concentration of the donor-bisteroid-acceptor; (2) as a function of added free donor or acceptor (as steroid esters) in solutions of donor-bisteroid-acceptor. (3) Energy transfer measurements were also made on solutions containing only free donor and free acceptor. Energy transfer was independent of variables involved in 1 and 2 and was not observed in 3. These experiments demonstrated that there was no significant amount of energy transfer between donor and acceptor attached to different steroid molecules.

Experiments were carried out to study the effect of chloroform, a fluorescence quencher, on both *p*-methoxyphenylacetyl fluorescence

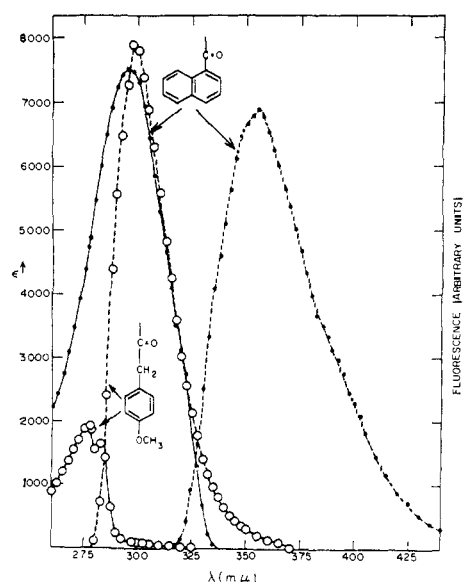


Figure 1. The absorption spectra of *p*-methoxyphenylacetyl group  $\circ-\circ-\circ$ , and 1-naphthoyl group,  $\bullet-\bullet-\bullet$  (see text for explanation); the fluorescence spectra of *p*-methoxyphenylacetyl group (with excitation at 275  $m\mu$ ),  $\circ-\circ-\circ$ , and 1-naphthoyl group (with excitation at 290  $m\mu$ ),  $\bullet-\bullet-\bullet$ . Both fluorescence spectra are in arbitrary units.

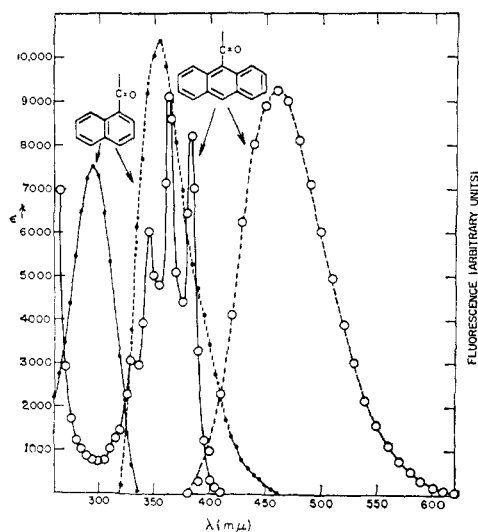


Figure 2. Absorption spectra of 1-naphthoyl group,  $\bullet-\bullet-\bullet$ , and anthracene-9-carbonyl group,  $\circ-\circ-\circ$  (see text for explanation); fluorescence spectra of 1-naphthoyl group (with excitation at 290  $m\mu$ ),  $\bullet-\bullet-\bullet$ , and anthracene-9-carbonyl group (with excitation at 365  $m\mu$ ),  $\circ-\circ-\circ$ . Both fluorescence spectra are in arbitrary units.

## Results and Discussion

Following absorption of light, an excited molecule in solution usually loses vibrational (and sometimes electronic) energy until it reaches the thermal vibrational level distribution of the first excited electronic

and energy transfer to 1-naphthoyl. Energy transfer efficiency and donor fluorescence in the presence of energy transfer were quenched to the same extent by chloroform. This result, combined with the influence of energy transfer on the efficiency with which chloroform quenched the donor fluorescence, supports one of the principal assumptions of Förster's theory, namely that energy transfer competes with fluorescence but does not follow it. All these experiments rule against any appreciable occurrence of donor fluorescence followed by acceptor absorption.

state. From here, further dissipation of energy is possible, either by fluorescence, or, under suitable conditions, by the transfer of excitation energy to another molecule. Energy transfer occurring before this state is reached would have to be of a strong exciton nature.

According to Förster's theory, the efficiency of energy transfer,  $T$ , which equals (quanta transferred/quanta absorbed by the donor), is given by

$$T = \frac{(R_0/R)^6}{1 + (R_0/R)^6} \quad (3)$$

where  $R$  is the donor-acceptor separation and  $R_0$  is the separation at 50% transfer efficiency. The corrected form of Förster's equation<sup>15</sup> for  $R_0$  is

$$R_0 = \sqrt[6]{\frac{9000 \ln 10 K^2 Q J}{128 \pi^3 n^4 N}} \quad (4)$$

Here,  $Q$  is the donor fluorescence quantum yield in the absence of energy transfer,  $n$  is the solvent refractive index, and  $N$  is Avogadro's number.  $K$ , the orientation factor discussed earlier, =  $\cos AB - 3 \cos A \cos B$  where  $AB$  is the angle between the donor and acceptor transition dipoles, and  $A$  or  $B$  is the angle between the respective group and the line of centers between the two groups.

$$J = \int I_\lambda \epsilon_\lambda \lambda^4 d\lambda \quad (5)$$

and is the overlap integral between the normalized donor fluorescence,  $I_\lambda$ , and the acceptor extinction coefficient,  $\epsilon_\lambda$ .  $\int I_\lambda d\lambda = 1$  by definition of normalization. The fluorescence of the donor is used as an estimate of the donor emission transition moment (which is one of the factors determining the transfer) but *not* as an indication that the energy transfer is due to fluorescence with reabsorption.

In the following, we compare the donor-acceptor separation,  $R$ , obtained from energy transfer measurements (using Förster's equation for calculation of  $R$ ) with the separation determined directly by measurements on Dreiding molecular models.

**Spectra.** The donor and acceptor fluorescence and absorption spectra are shown in Figures 1 and 2. In correcting for the steroid moiety absorption (amounting to only a few per cent of the total absorption in this spectral region), the extinction coefficient of a steroid ester was assumed to be the sum of that due to the particular steroid component and that due to the donor or acceptor moiety. The extinction coefficients of the combined donor-biesteroid-acceptor molecules equaled the sum of those of the constituent components to within experimental error. This excludes strong donor-acceptor coupling ( $>10^3 \text{ cm.}^{-1}$ ).

The fluorescence emission spectra for both donors were corrected for apparatus output response. The

(15) In two recent papers of Förster<sup>16,17</sup> the expression for  $R_0$  contains a  $\pi^6$ . However, the original derivation<sup>18,19</sup> was checked and found to have an algebraic error; our extension<sup>20</sup> of Förster's original derivation also yielded a different factor, namely  $\pi^3$ . This correction has been independently reported by Ketskemeti.<sup>21</sup>

(16) Th. Förster, *Radiation Res. Suppl.*, 2, 326 (1960).

(17) Th. Förster, *Discussions Faraday Soc.*, 27, 7 (1959).

(18) Th. Förster, "Fluoreszenz Organischer Verbindungen," Vandenhoeck and Ruprecht, Göttingen, 1951, p. 85.

(19) Th. Förster, *Ann. Physik*, 2, 55 (1948).

(20) S. A. Latt, M.D. Dissertation, Harvard Medical School, 1964.

(21) I. Ketskemeti, *Z. Naturforsch.*, 17a, 666 (1962).

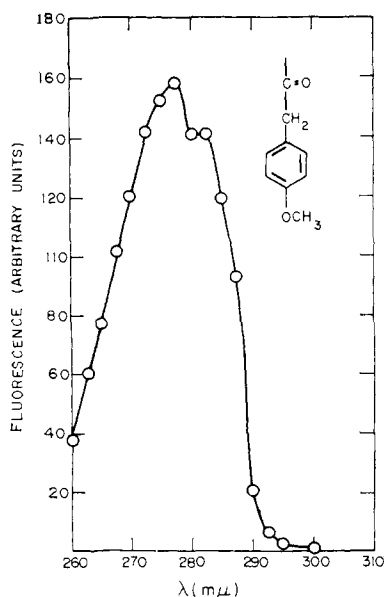


Figure 3. Excitation spectrum of *p*-methoxyphenylacetyl group (emission read at 300  $m\mu$ ).

overlap between the donor fluorescence and acceptor absorption spectra is apparent from the figures.

The corrected excitation spectra of all three moieties are shown in Figures 3, 4, and 5. Values for  $\lambda$  below 270  $m\mu$  have a large uncertainty owing to the extremely low lamp output below that wave length. The excitation spectra (obtained using solutions of low optical density) are nearly proportional to the corresponding absorption spectra. This result is consistent with there being a constant quantum yield of fluorescence over the entire absorption band, a finding especially important in the case of the 1-naphthoyl and anthracene-9-carbonyl groups (both of which are used as energy acceptors), since it is tacitly assumed in the experimental determination of transfer efficiency.

**Overlap Integral,  $J$ .** The values of the overlap integral were calculated for both donor-acceptor pairs according to eq. 5: for *p*-methoxyphenylacetyl-bisteroid-1-naphthoyl (XI),  $J = 4.56 \times 10^{-15}$ ; for 1-naphthoyl-bisteroid-anthracene-9-carbonyl (XII),  $J = 7.87 \times 10^{-15}$  ( $J$  values in  $10^{-3} \text{ cm.}^6/\text{mole}$ ).

**Quantum Yields,  $Q$ .** The fluorescence quantum yields of the donors were determined as described in the Experimental section: for *p*-methoxyphenylacetyl (in compound I),  $Q = 0.225$ ; for 1-naphthoyl (in compound XVI),  $Q = 0.106$ . No significant difference was observed between the quantum yields of the donors attached to single steroids (given above) and those of the same groups attached to bisteroids (e.g., compounds IX and X). The quantum yield values given above are probably accurate to within 15%.

**Index of Refraction,  $n$ .** Values given by Foss, Kang, and Schellman<sup>22</sup> for  $n$  of *p*-dioxane at several wave lengths throughout the ultraviolet and visible regions were used to plot a curve of  $n$  vs.  $\lambda$  from which

(22) J. Foss, Y. Kang, and J. Schellman, "Dispersion of Refractive Index of Various Solvents," unpublished data; see S. P. Colowick and N. O. Kaplan, Ed., "Methods in Enzymology," Vol. VI, Academic Press, New York, N. Y., 1963, p. 955.

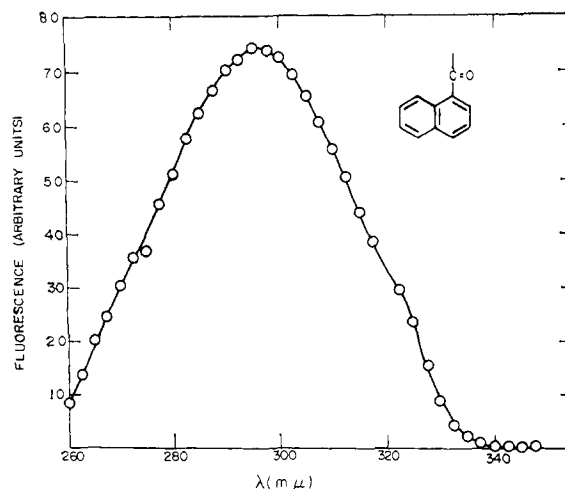


Figure 4. Excitation spectrum of 1-naphthoyl group (emission read at 375  $m\mu$ ).

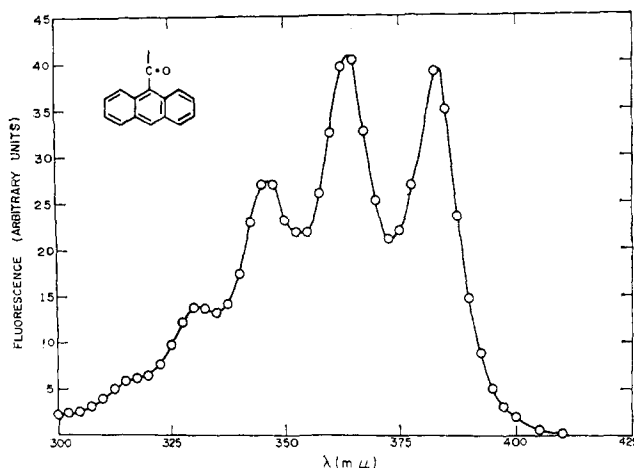


Figure 5. Excitation spectrum of anthracene-9-carbonyl group (emission read at 490  $m\mu$ ).

values of  $n$  at desired wave lengths were determined:  $n_{295m\mu} = 1.456$ ;  $n_{360m\mu} = 1.439$ .

**The Orientation Factor,  $K^2$ .** The average value of  $K^2$ , assuming random donor-acceptor orientations, is  $2/3$ . The range of orientations about the hydroxyl attachment sites for the *p*-methoxyphenylacetyl (and even the 1-naphthoyl groups) would be expected to be extensive enough for this value to be a reasonable estimate for the compound involving these two groups as donor and acceptor. However, the bulky anthracene group probably will be more restricted in its orientation than will the *p*-methoxyphenylacetyl group, and thus a nonrandom orientation distribution may be expected for the 1-naphthoyl-anthracene-9-carbonyl pair.

**Determination of  $R_0$ .** The values of  $R_0$  were calculated from the above data and the corrected formula given at the beginning of this section.  $R_0$  for compound XI ( $22.8 \pm 0.9 \text{ \AA.}$ ) was calculated using  $n_{\text{dioxane}}$  at 295  $m\mu$ ;  $R_0$  for compound XII ( $22.0 \pm 0.8 \text{ \AA.}$ ) was calculated using  $n_{\text{dioxane}}$  at 360  $m\mu$ .  $\bar{K}^2$  was assumed to be  $2/3$ . The uncertainties were calculated assuming  $J \pm 10\%$  and  $Q \pm 15\%$ , and thus  $R_0 \pm 3.8\%$ .

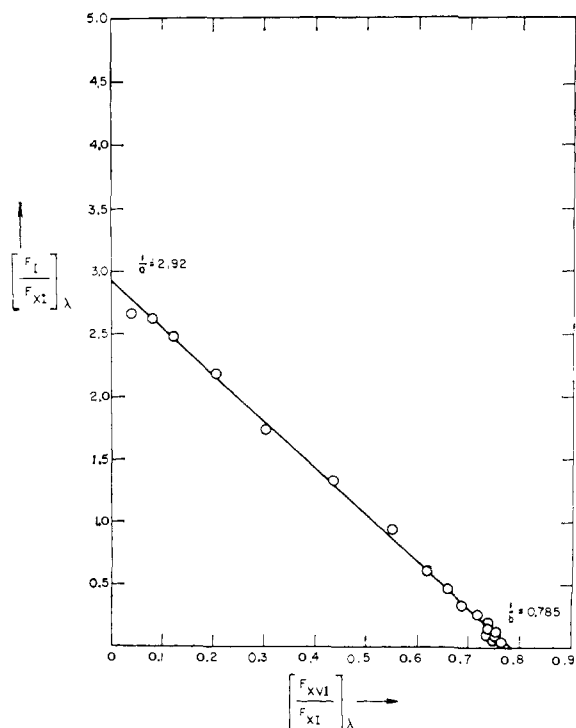


Figure 6. A plot of the ratio of the fluorescences of compounds I and XVI referred to compound XI; for nomenclature, see text.

*R/R<sub>0</sub> Transfer Experiments.* (1) *p*-Methoxyphenyl-acetyl-Bisteroid-1-Naphthoyl Compound XI. Excitation was at 275 mμ with a band width of 11 mμ. Emission was read with a constant 0.6-mm. (3–5 mμ) slit width. Corrections were made for the variation of the lamp output over the excitation band width. A correction was made for the steroid moiety absorption. Both transfer efficiency to the acceptor, *T*<sub>2</sub>, and quenching efficiency of the donor, *T*<sub>1</sub>, were calculated.

The assumption of this procedure is that the donor-bisteroid-acceptor fluorescence (*F*<sub>XI</sub>) at any wave length λ can be expressed in terms of the donor and acceptor fluorescence (*F*<sub>I</sub> and *F*<sub>XVI</sub>, respectively) as

$$F_{XI\lambda} = aF_{I\lambda} + bF_{XVI\lambda} \quad (6)$$

or

$$1 = a \frac{F_{I\lambda}}{F_{XI\lambda}} + b \frac{F_{XVI\lambda}}{F_{XI\lambda}} \quad (7)$$

A plot of *F*<sub>I</sub>/*F*<sub>XI</sub> vs. *F*<sub>XVI</sub>/*F*<sub>XI</sub> yields intercepts 1/*a* and 1/*b*. In the absence of energy transfer, *a* should be equal to the ratio of the absorptions of the donor group in the two solutions I and XI; similarly, *b* should be equal to the ratio of the absorptions of the acceptor in the solutions XVI and XI. Energy transfer will increase *b* and decrease *a*. A representative plot of *F*<sub>I</sub>/*F*<sub>XI</sub> vs. *F*<sub>XVI</sub>/*F*<sub>XI</sub> is shown in Figure 6.

Optical density of

XI (at 275 mμ)	<i>T</i> <sub>2</sub>	<i>T</i> <sub>1</sub>
0.0742	0.659	0.654
0.0647	0.623	0.610
0.0284	0.505	0.512
0.0148	0.585	0.647
	$\bar{T}_2 = 0.593$	$\bar{T}_1 = 0.606$
		$\bar{T} = 0.60$

There is no significant difference between *T*<sub>2</sub> and *T*<sub>1</sub>. The uncertainty in the individual determinations of the transfer efficiencies is about 15% for *T*<sub>2</sub> and about 10% for *T*<sub>1</sub>. The uncertainty in the average value of *T* is less than 10% and thus, at *T* = 0.60, the uncertainty in (*R*<sub>0</sub>/*R*)<sup>6</sup> is less than 25%. No consistent concentration dependence of energy transfer was apparent.

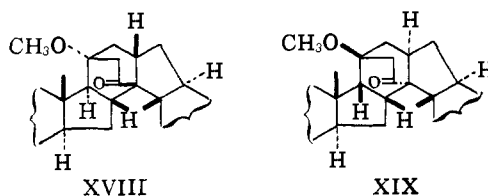
From eq. 3, using *T* = 0.60, one obtains (*R*<sub>0</sub>/*R*)<sup>6</sup> = 1.5 and *R*<sub>0</sub>/*R* = 1.07. Since *R*<sub>0</sub> = 22.8 ± 0.9 Å., then *R* is calculated to be 21.3 ± 1.6 Å.

(2) *1-Naphthoyl-Bisteroid-Anthracene-9-carbonyl Compound XII*. A procedure similar to that described above was used, with excitation at 322.5 mμ instead of 275 mμ.

Optical density of XII (at 322.5 mμ)	<i>T</i> <sub>2</sub>	<i>T</i> <sub>1</sub>
0.0191	0.850	0.829
0.0093	0.846	0.844
	$\bar{T}_2 = 0.848$	$\bar{T}_1 = 0.837$
		$\bar{T} = 0.84$

Again *T*<sub>2</sub> and *T*<sub>1</sub> are effectively equal. The uncertainty in the individual transfer efficiency determinations is less than 10% for *T*<sub>2</sub> and less than 5% for *T*<sub>1</sub>. The uncertainty in  $\bar{T}$  is about 5%, and thus, at *T* = 0.84, the uncertainty in (*R*<sub>0</sub>/*R*)<sup>6</sup> is less than 30%. Using  $\bar{T}$  = 0.84, one calculates (*R*<sub>0</sub>/*R*)<sup>6</sup> = 5.25, and thus *R*<sub>0</sub>/*R* = 1.32. Since *R*<sub>0</sub> equals 22.0 Å., *R* is calculated to be 16.7 ± 1.4 Å.

*Measurement of R from Molecular Models.* Estimation of the donor-acceptor separation, *R*, requires a knowledge of the complete stereochemistry of the "bisteroid" XIV. Considerations based on reaction mechanisms (an acid-catalyzed and stereospecific Diels-Alder addition<sup>23</sup> followed by a *trans* cyclization) predicted two most likely configurations XVIII and XIX.<sup>24</sup> Of these we favor XIX for two reasons. The



first is based on a consideration of the addition of two molecules of 16-dehydropregn-5-en-3β-ol-20-one (as the methyl enol ether) (see ref. 3) in the least hindered manner. The second reason is derived from the measured optical properties of compounds synthesized. On extending the octant rule<sup>25</sup> to the bicyclo[2.2.2]-octanone system, one may predict a small negative Cotton effect for configuration XVIII and a small positive one for XIX. Experimentally, a positive rotation was shown by compounds XIV and VIII in both optical rotatory dispersion and circular dichroism<sup>26,27</sup> measurements. In the sequel, measurements of *R* refer to a Dreiding molecular model of the "bisteroid" with complete stereochemistry as shown in XIX.<sup>28</sup>

(23) R. B. Woodward and T. J. Katz, *Tetrahedron*, **5**, 70 (1959), and literature cited therein.

(24) Professor E. V. Jensen, personal communication.

(25) W. Moffitt, R. B. Woodward, A. Moscovitz, W. Klyne, and C. Djerassi, *J. Am. Chem. Soc.*, **83**, 4013 (1961).

(26) L. Velluz and M. Legrand, *Angew. Chem.*, **73**, 603 (1961).

(27) M. Legrand and J. Mathiew, *Bull. soc. chim. France*, 1967 (1961).



Calculation of the average distance from the center of the donor moiety to the center of the acceptor moiety was based on a model in which the attached groups (of known dimension) could pivot around their attachment sites. Two types of averages were taken. The first was a linear average, simply  $1/2$  the sum of the likely minimum and maximum separations of the donor and acceptor. The second was an average of  $(1/R^6)^{-6}$  calculated by assuming all separations between the two limits equally possible. The second average is a better mathematical representation of the separation effect on transfer. The statistical weighting due to the solid angle increment at a given extension (minimal at complete extension) was neglected as was any weighting due to steric considerations (which would have an opposite effect of favoring extended positions).

The linear averages are 21.8 Å. for XI and 21.5 Å. for XII. The  $(1/R^6)^{-6}$  averages were 19.2 Å. for XI and 19.4 Å. for XII. Uncertainties for all values are 2.0 Å. (to the nearest Å.).

The calculated and measured separations are tabulated in Table III. Uncertainties in measured separations are estimated to the nearest Å.

Table III

Compound	$\bar{K}^2$	$R_{\text{calcd}}$ , Å.	$R_{\text{measd}}$ (from Dreiding models), Å.
XI	$2/3$	$21.3 \pm 1.6$	$21.8 \pm 2.0$ (linear av.) $19.2 \pm 2.0$ ( $[1/R^6]^{-6}$ )
XII	$2/3$	$16.7 \pm 1.4$	$21.5 \pm 2.0$ (linear av.) $19.4 \pm 2.0$ ( $[1/R^6]^{-6}$ )

The donor-acceptor separation,  $R$ , in compound XI calculated from spectral data and the energy transfer measurements (considering only resonance transfer) agrees with the  $R$  obtained directly from molecular models to within experimental uncertainties. However,  $R_{\text{calcd}}$  (using the same assumptions) on the basis of measurements on compound XII is less than that determined geometrically; *i.e.*, here, the observed energy transfer was greater than that predicted.

The most likely explanation for this discrepancy between the predicted and observed transfer in compound XII is that the value of the average orientation factor is greater than the estimate of  $2/3$  which was used to calculate the predicted separation. Since both the naphthalene and (especially) the anthracene groups can be expected to have restricted movement about their attachment sites, a value of  $\bar{K}^2$  different from

(28) The oxygen-oxygen separations as measured on models having all other possible configurations fall within 2 Å. of the corresponding separation in the case of configuration XIX.

$2/3$  is a good possibility. Since the major long wavelength transition for both the naphthalene and anthracene groups is polarized along the short axis of the molecule,<sup>29,30</sup> steric restrictions causing these groups to extend outward from their attachment sites would tend to align the transitions with the line between their centers and would thus increase  $\bar{K}^2$ . For  $\bar{K}^2 = 4/3$ , the experimentally calculated  $R$  ( $18.5 \pm 1.5$  Å.) is close to the directly measured  $R$ .

At least three additional explanations of the experimental results with compound XII can be suggested. First, it is possible that transfer of an exciton type occurred between the components of the compound. This would occur only if the dipole interaction energies were at the lower limit of that needed for strong interactions. Secondly, there may have been van der Waals attraction between the anthracene moiety and the steroid nucleus, tending to decrease  $R$  and thus increase the measured energy transfer efficiency. Finally, the uncertainties may actually be greater than the estimates given.

### Conclusion

The electronic excitation energy transfer in dioxane solution occurring between the two donor-acceptor pairs (*p*-methoxyphenylacetyl-1-naphthoyl and 1-naphthoyl-anthracene-9-carbonyl) attached to the hydroxyl groups of a bisteroid XIV at about a 20-Å. separation can be interpreted as being principally, if not totally, due to dipole-dipole resonance transfer. However, a small amount of exciton transfer can not be excluded. The close correspondence between the measured donor-acceptor separations and those calculated using the  $1/(\text{separation})^6$  dependence of transfer predicted by the Förster equations (3 and 4) indicates that measurement of the transfer of electronic excitation energy between moieties can be used to estimate the separation (of the order of 20 Å.) between such moieties and thus is potentially a powerful tool for investigating interactions between certain molecular groups and changes in the microstructure of macromolecules.

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(29) The naphthalene transition referred to here has a maximum at 295  $m\mu$  and is transversely polarized. There is also a much weaker transition near 325  $m\mu$  with a longitudinal polarization.<sup>30</sup>

(30) H. H. Jaffé and M. Orchin, "Theory and Applications of Ultraviolet Spectroscopy," John Wiley and Sons, Inc., New York, N. Y., 1962, Chapter 13.