Table 2. Rate of hydrolysis of X-Y-p-nitroanilides (pNA) with X-prolyl dipeptidyl-aminopeptidase

| Substrate | Enzyme activity μmole/min mg protein (37 °C) | % |
|---|---|---|
| Gly-Pro-pNA | 11.02 | 100 |
| Gly-Leu-pNA | 0.000 | 0.000 |
| Gly-Sarcosine-pNA | 0.009 | 0.080 |
| Gly-Gly-pNA | 0.002 | 0.016 |
| Gly-Hyp-pNA | 0.201 | 1.82 |
| Gly-Ala-pNA | 0.176 | 1.60 |
| Ala-Gly-pNA | 0.011 | 0.10 |
| Ala-Ala-pNA | 1.194 | 10.83 |

Homogeneous enzyme from human submaxillary gland was used as enzyme. Activities were measured at pH 8.7 in 71 mM glycine-NaOH buffer and at a substrate concentration of 1.4 mM. The values are the mean of duplicate experiments.

lary glands in order to examine the specificity of the N-terminal amino acid, and found that glycylproline p-nitroanilide had the highest activity among the substrates at the optimum pH (8.7), followed by p-nitroanilides of alanine, lysine, arginine, glutamic acid, and aspartic acid in a decreasing order of activity[6]. Since the homogeneous enzyme from human submaxillary gland did not hydrolyze glycylphenylalanine β-naphthylamide at all[5], the enzyme was supposed to be specific for the 2nd amino acid proline. However, the purified enzyme from porcine kidney was found to hydrolyze not only X-Pro-Y, but also X-Ala-Y[3,4]. Therefore, in order to examine the specificity of the 2nd amino acid, we have synthesized new chromogenic substrates, p-nitroanilides of the dipeptides, Gly-Pro, Gly-Leu, Gly-Sarcosine, Gly-Gly, Gly-Hyp, Gly-Ala, Ala-Gly and Ala-Ala.

These compounds were synthesized in a tosylate form by coupling Boc-Gly or Boc-Ala N-hydroxysuccinimide ester with several amino acid p-nitroanilides used in this study in N,N-dimethylformamide followed by the treatment with p-toluenesulfonic acid in acetic acid at room temperature. Amino acid p-nitroanilides used as the starting material were synthesized by coupling corresponding carbobenzoxy amino acids with p-nitroaniline applying phosphorus oxychloride method[7]; the carbobenzoxy group was removed by the treatment with 25% HBr-acetic acid at room temperature.

Analytical data for the final products were summarized in table 1. X-Prolyl dipeptidyl-aminopeptidase in human submaxillary gland was purified to a nearly homogeneous form from the materials obtained at autopsy by a procedure described previously[5]. The enzyme activity was assayed by directly measuring the liberated p-nitroaniline by the method described previously[6]. Incubation mixture contained 75 μmoles glycyne-NaOH buffer (pH 8.7), 1.5 μmoles of each X-Y-p-nitroanilide, and enzyme plus water to 1.05 ml. Incubation was carried out at 37 °C for 30 min.

The results are shown in table 2. Among various substrates having the sequence of Gly-Y-p-nitroanilide, Gly-Pro-p-nitroanilide was hydrolyzed almost specifically. Only Gly-Hyp-p-nitroanilide and Gly-Ala-p-nitroanilide had slight but significant (2%) activity. However, Ala-Ala-p-nitroanilide had about 11% of the activity. The results suggest that the dipeptidyl-aminopeptidase is highly specific for the 2nd amino acid proline, but hydroxyproline and alanine can also be the 2nd amino acid.

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## Energy transfer from the second excited singlet state of spirobifluorene

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*Summary.* Energy transfer from the second excited state of spirobifluorene is examined by polarization spectroscopy. Evidence against spiroconjugation and evidence in favor of a coulombic mechanism is reviewed and discussed. This coulombic interaction is categorized as an intramolecular energy transfer caused by the 'extrinsic factors' of the Weber nomenclature.

Bis-(2,2'-biphenylene)-methane (common name spirobifluorene, SBF) with its spirolinked planes has been used as a model compound in studying intrachromophoric energy transfer (ET)[2]. The 1 excited singlet state of fluorene (F) is reported to be long axis polarized while the 2 excited singlet is short axis polarized[3]. When the flurenic moieties are joined via the spirocarbon linkage to form SBF, the 1 excited states of the dimeric components posess dipoles that are perpendicularly oriented, and thus no dipole-dipole interaction would be expected. But, if the chromophores should be excited to the 2 excited singlet state, where the dipoles are oriented in a parallel fashion, the possibility of ET occurring through a dipolar interaction exists.

Energy transfer between the favorably oriented 2 excited singlet dipoles would be expected to produce an excitonic splitting of the degenerate levels. This excitonic splitting is difficult if not impossible to measure because it is a radiationsless decay from a perturbed 2 singlet and hydrocarbons usually emit from the lowest vibronic level of the lowest excited single state (Kasha's Rule). However, an indirect indication of whether ET is occurring might be found by measuring the polarization of the resulting singlet state emission. Depolarization of the fluorescence of SBF as compared to F would offer strong evidence for the occurrence of intramolecular ET.

If ET is to occur through a coulombic mechanism, the dipoles must be oriented in such a way that the projection

of one upon the other is relatively large, or equivalently, that the angle of their intersection is small. And if the energy is transferred from the 1 to the 2 chromophore, a depolarization of the resulting fluorescence of the 2 chromophore should occur. This in turn should result in a significant change in polarization parameters.

The excitation and emission spectra of F ($5 \times 10^{-5}$ M) are shown in figure 1, A; similar spectra for SBF ($1 \times 10^{-6}$ M) are found in figure 1, B. The solvent used was methylcyclohexane: isopentane (1:5) (MI) at 77 °K. The polarization of fluorescence of F and SBF in MI at 77 °K excited at 306 nm (1 excited singlet) is shown in figure 2, A; the same compounds excited at 285 nm (2nd excited singlet) are shown in figure 2, B.

The polarization spectra of F excited at 306 nm and 285 nm are essentially equivalent. But the polarization of SBF at the same excitation wavelengths is quite different beginning at about 340 nm and extending to 390 nm, indicating a different method of deactivation of the 1st excited level of SBF. That the polarized light is observed means that the oscillators are essentially parallel to the exciting light vectors. This, plus the fact that $S_1$ is highly depolarized in SBF, implies that at least part of the fluorescent energy is derived from the 2nd excited singlet.

To eliminate the possibility that the interaction is intramolecular energy 'leakage' across the spirocarbon atom via spiroconjugation (and therefore not coulombic) is important. The 7-nm bathochromic shift of the lowest energy peaks in the absorption spectra of SBF as compared to F was originally determined by Hofer[4] and again by Hass[5]. A similar shift in the peaks of an amino-

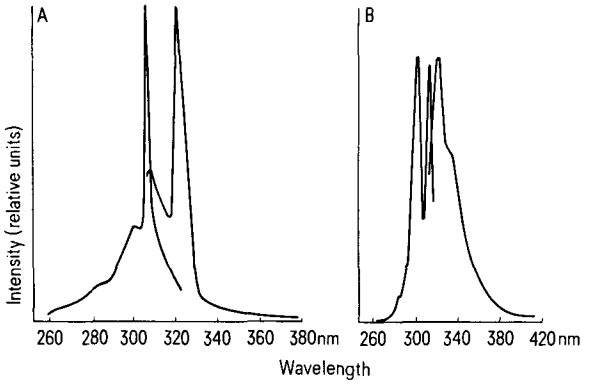


Fig. 1. Excitation and emission of *A* fluorene ($5 \times 10^{-5}$ M) and *B* SBF ($1 \times 10^{-6}$ M) in methylcyclohexane:isopentane (1:5) at 77 °K.

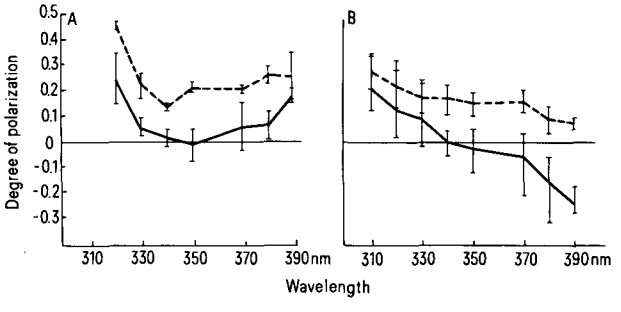


Fig. 2. Fluorescence polarization of fluorene and SBF in MI at 77 °K: *A* Excitation at 306 nm; *B* excitation at 285 nm. Vertical bar shows range of the experimental data. ····· fluorene; —— SBF.

nitro substituted SBF has been observed[2], suggesting a mechanism involving either a 'variable solvation effect' or a substitution effect in the 9 (spirocarbon atom) position.

The absorption data of Eaborn and Shaw[6] on 9-substituted fluorenes show that electron donating groups, such as amino or phenyl groups, cause a bathochromic shift comparable in magnitude to that seen in SBF. Similar shifts are also found in the 260 nm bands of the F and SBF curves[5]. Furthermore, data[5] indicate that the solvation effect that occurs between the polar (EPA[7]) and nonpolar (MI) solvents, may affect $\varepsilon_{max}$ rather than the $\lambda_{max}$. However, if a solvation mechanism were to be applied, the cases involved would be nonpolar solute interacting with nonpolar solvent (MI) or nonpolar solute interacting with polar solvent (EPA) (cases I and II of the Bayliss-McRae categorization); both of these instances produce a bathochromic shift[8]. Thus, both the substitution and solvation data qualitatively argue against spiroconjugation. Furthermore, Heilbronner[9] reported a hypsochromic spectral shift due to spiroconjugation in a symmetrical heterocycle. There is no measurable widening or splitting of absorption bands[4,5] implying that the coupling between bands is relatively small and the energy of absorption is localized in the chromophore that absorbed the energy. All this evidence indicates that the transfer of energy from one F moiety to the other in SBF probably does not involve spiroconjugation.

According to Weber[10] the change in position of the emission dipole from the time of excitation to the time of emission (depolarization) can result from 1 of 2 phenomena: brownian movement or ET. The ET possibilities in turn can be of 2 types: coupling of 2 electronic dipoles or the coupling of an electronic dipole with a vibrational dipole. Since brownian movement can be ruled out because the molecule is rigidly clamped by the solvent matrix, 1 of the 2 ET mechanisms remains to explain the depolarization phenomenon.

Weber's expression relates the dipole components of absorption and emission:

$$\frac{1}{p} - \frac{1}{3} = \left(\frac{5}{3}\right)\left[\frac{2}{3\cos^2\lambda - 1}\right]\left[\frac{2}{3\langle\cos^2\omega\rangle - 1}\right],$$

where p = measured polarization; $\lambda$ = angular separation of the absorption and emission oscillators; $\omega$ = angle between the emission dipoles.

If there is no change in the absorption and emission oscillator orientations in the time required for both phenomena to occur, then the depolarization from the maximum value of $^1/_2$ is due to 'intrinsic' causes. Further

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loss of polarization can be caused by 'extrinsic' factors such as migration of excitation (ET) and brownian movement[10]. Thus, the $\lambda$ term of the equation is the 'intrinsic factor' while the term containing $\omega$ is the 'extrinsic factor'. The calculated values of $\lambda$ for SBF are uniformly 12–15° higher than that of F in the excitation range of 270 nm to 305 nm where F emission is monitored at 306 nm and SBF at 324 nm. Also the average value of $\omega$ in F going from excitation at 306 nm to excitation at 285 nm is calculated to be 25°. On the other hand, the SBF data (excitation at 285 nm) show a higher $\omega$ value (about 35°) in the 320–350 nm range and very high values (37°–78°) in the 370–390 nm range. We interpret these data to mean that the higher wavelength region of the SBF fluorescence band excited at 285 nm is more susceptible to polarization changes than the lower energy part of the band.

The extrinsic contribution to depolarization increases by a factor of 2 in going from 330 to 340 nm by a factor of 3 in going from 330 to 380 nm. The additional depolarization from extrinsic causes must occur through ET between the neighboring intramolecular chromophores. Thus to Weber's 2 categories of extrinsic contributions to depolarization (intermolecular ET via dipole interactions and brownian movement) can be added a 3rd category: intramolecular ET via dipole interactions, which plays an important role in the increasing of depolarization in SBF.

## Differential banding induced in polytene chromosomes of *Drosophila melanogaster* stained with acridine orange

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*Summary.* Experiments carried out on polytene chromosomes of *Drosophila melanogaster* showed differential staining of certain areas when cytological preparations were exposed to light, or treated with formaldehyde, and subsequently stained with acridine orange. Some hypotheses are discussed regarding the involvement of some proteic fractions which, in addition to DNA, could pay a part in such banding.

It is known that eukaryotic chromosomes exhibit patterns of transverse fluorescent banding when stained with some acridine derivates[2]; the initial interest in such banding, as simplifying the recognition of particular chromosomes, was followed by that regarding the linear structure of eukaryotic chromosomes due to possible interaction of chromosomal DNA with fluorochromes. It is in fact known that some of these compounds, when bound to specific polynucleotide DNA sequences, enhance their fluorescence, characterizing specific base compositions. Quinacrine, for example, was shown to be an indicator of base compositions in polynucleotide segments: AT-rich sequences enhance while GC-rich sequences quench Q- fluorescence[3, 4], even if the specific interspersion of GC base pairs in AT-rich segments seems to play a role in quenching of in vitro fluorescence of this compound[5]. Hoechst 33258, an alkaline bi-benzimidazol derivative, is another indicator of AT-rich polynucleotide sequences; the fluorescence in vitro of this compound is enhanced by both AT- and GC-rich segments, though the AT-rich sequences increase the fluorescence more than GC[6, 7]. It ist still uncertain, however, if it is possible to make the same assumptions about the behavior of such fluorochromes, either with DNA in solution or when it is part of complex structures like eukaryotic chromosomes[8, 9].

In contrast to the above-named compounds, acridine orange (a.o.) fluorescence is not increased by specific base sequences, since for this compound both $(dA)_n$ $(dT)_n$ and $(dG)_n$ $(dC)_n$ enhance fluorescence in vitro[10]. Chromosomes stained with this fluorochrome show bright yellow-green fluorescence all along their length. Because the a.o.-DNA bond seems to be intercalating, the emission of yellow-green fluorescence could be considered to be the result of the a.o.-DNA complex when all the spaces between pairs of adjacent bases of the nucleid acid in its double helix state are saturated with fluorochrome molecules[11, 12]. There is, however, emission of fluorescent light with a wavelength characteristic of red-orange when the dye molecules interact with each other. In this connection, it is important to emphasize that treatments producing depolymerizations, in addition to denaturation of DNA, can also cause the fluorescence to change from yellow-green to red when the nucleic acid is bound to a.o.[13].

In thus seems possible to differentiate, with reasonable certainty, double helix polynucleotide sequences producing yellow-green fluorescence, from single helix sequences emitting red fluorescence. Because of the similar behavior of the DNA in fixed chromosomes and in solution[11], one can consider the a.o. as an indicator for characterizing form and dimensions of the nucleic acid in chromosomes. In this paper we describe the a.o. differential staining obtained after light and/or formaldehyde treatment on polytene chromosomes of *Drosophila melanogaster*. Some hypotheses on the meaning of the fluorescent banding are proposed.

*Materials and methods.* 3rd instar larvae of *Drosophila melanogaster* (Canton S) raised at the Zoology Department of the University of Wisconsin were used. The salivary glands were extracted in Ringer solution and were then treated with 45% acetic acid for 3 min. After squashing, the siliconized coverslips were removed with a razor blade from slides immersed in liquid nitrogen; fixation was effected in 95% ethyl alcohol for 10 min. Slides were stained with 0.01% acridine orange in Sorensen phosphate buffer M/15, pH 7.1 for 15 min. The excess dye was removed by a 30 min treatment in the same buffer solution. Preparations were then mounted in a drop of phosphate buffer, sealed with fingernail polish and exposed to the light of a 14 W sterilizing mercury lamp (General Electric) for times varying from a few min to 10 days. Control slides were contemporaneously kept in the dark for the same times as those exposed to light. Some preparations were treated with 4% formaldehyde for 10 min before staining. Slides were observed daily.