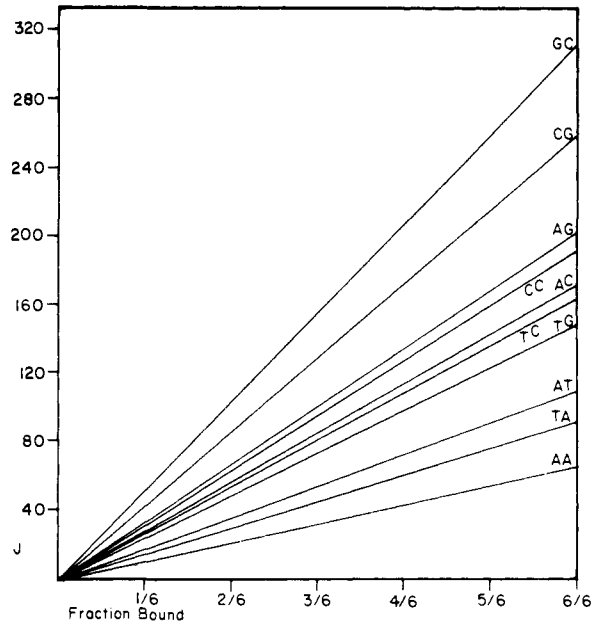

Figure 2. CD change upon binding of actinomycin for each first neighbor.

dAGT], poly[dAT:dAT], poly[dGC:dGC], and poly[dAG:dCT]) in the native form and at approximately 12 levels of actinomycin concentration. Actinomycin has a nearly negligible CD (<2% of the complex CD) and the approximate free actinomycin concentration was maintained in the reference cell. Because of problems in defining a consistent measure of saturation we have looked at the CD spectra to find such a reference. Examination of the spectra shows that the region of the spectrum from 220 to 275 nm shows a monotonic change during binding. In order to obtain a scalar quantity which reflects the extent of binding we have subtracted the native spectrum from the spectra taken in the presence of actinomycin to give difference spectra. We then take the square root of the sum of the squares of the elements in this difference spectrum from 220 to 275 nm, measured at 2.5 nm intervals, as our scalar parameter, J. When J for a given DNA is plotted vs. total actinomycin concentration, a clear picture of saturation results. Using plots of this type, the particular actinomycin concentration which gives a fraction of bound sites of any value can be determined. We have chosen to use spectra ranging from a fraction bound of 0 to 1 in intervals of $\frac{1}{6}$. Seldom did we have a measured spectrum corresponding to one of these values of fraction bound. However, the spectra at those particular values of fraction found usually lay in a small interval between two measured spectra. Knowing the fraction bound for the measured spectra and also the desired value of the fraction bound we have taken an appropriate linear combination of the two measured spectra at the ends of the interval containing the desired value. In this way we have produced a set of seven spectra for each DNA for which the fraction bound ranges from 0 to 1 at regular intervals of $\frac{1}{6}$. A typical set of curves for *E. aerogenes* DNA is shown in Figure 1.

We have applied eq 2 to each of the sets of 11 DNA CD spectra at the seven values of fraction bound. Solving these equations results in seven T matrices which give the CD contribution of the first-neighbor units at the different levels of fraction bound. The T matrix resulting from the native CD spectra (0 fraction bound), is subtracted from the other T matrices generating difference T matrices. The parameter J is then evaluated as described previously for each first-neighbor unit at each level of fraction bound. The results of this procedure are shown in Figure 2 for actinomycin D. The lines shown are least-squares lines for each first-neighbor unit and these lines were forced to pass through the origin. The individual points have not been indicated because the scatter is quite large and the plot becomes very confusing. The probable errors of the slopes in Figure 2 are listed in Table I.

Table I. Slopes and Probable Errors in Figure 2

X_pY	Slope	Probable error of slope
AA	64.9	1.63
AC	171	23
AG	203	27
TA	92.6	2.7
TC	163	31
TG	148	31
CC	192	56
GC	310	61
AT	109	2.6
CG	258	59

We have separated the first-neighbor units into three groups. The first-neighbor units GpC and CpG are very favorable binding sites for actinomycin D whereas ApG, CpC, ApC, TpC, and TpG appear to be less attractive sites and ApT, TpA, and ApA are unfavorable sites. The perturbations on the latter group are probably due to second nearest neighbor interactions. These conclusions are in agreement with the general observation that a G-C base pair is required for actinomycin binding but they are not consistent with the x-ray results of Sobell.[5] We have found both CpG and GpC to be favorable binding sites whereas Sobell indicates that while GpC should bind strongly CpG should be much less active. The data of Wells[3] et al. which indicate that actinomycin binds most strongly to poly [dGC:dGC] is often sited as evidence for the Sobell model. However, it is also consistent with our findings.

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The Formation of a Semiquinone Form of DeazaFAD Bound to D-Amino Acid Oxidase[1]

Sir:

Deazaflavins have recently been used as flavin analogues to study the mechanism of both nonenzymic[2] and enzymic[3]

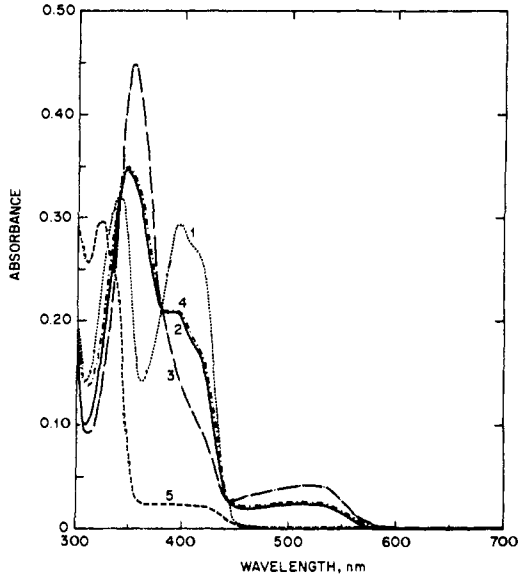

Figure 1. Effect of EDTA-light irradiation on deazaFAD-containing D-amino acid oxidase: (curve 1) oxidized deazaFAD-containing D-amino acid oxidase (24 μM with respect to deazaFAD) under an argon atmosphere in 0.1 M sodium pyrophosphate buffer pH 8.4 containing 0.1 M EDTA; (curve 2) same as curve 1, after 15 min of irradiation with an Ektagraphic Model AF-2 slide projector using a 300-W quartzline lamp at a distance of 20 cm; (curve 3) same as curve 2, but after 30 min of irradiation; (curve 4) same as curve 3 but after a small amount of air was introduced into the sample. The sample was then made fully aerobic and a spectrum identical with 1 was obtained. D-Alanine was then added to a final concentration of 20 mM and curve 5 was obtained 60 min later.

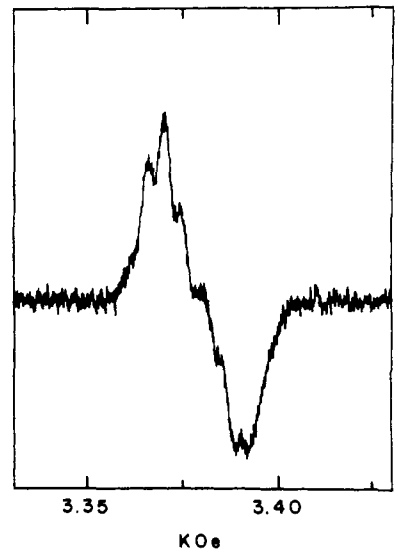

Figure 2. Free radical signal generated upon EDTA-light irradiation. DeazaFAD D-amino acid oxidase (100 μM) in 0.1 M pyrophosphate buffer, pH 8.4 containing 0.1 M EDTA, was irradiated with the Ektagraphic slide projector described above in a Varian flat cell mounted in the EPR cavity. Prior to irradiation the baseline was flat and after 30 min of irradiation, the light was turned off and the EPR spectrum was recorded. The Varian E-4 EPR spectrometer was coupled with a PDP-11 minicomputer for signal averaging eight spectra which were recorded with the following instrument parameters: modulation amplitude, 2.5 G; power, 2 mW; temperature, 25 °C; gain, 1.0×10^4; scan rate, 8 min; and time constant, 0.3 s. The amount of radical was estimated by comparing the integrated area of this spectrum with that obtained with a standard solution of native flavodoxin which had been fully converted to its semiquinone form by irradiation in the presence of EDTA. A g value of 2.02 was obtained for the deazaFAD semiquinone.

flavin reactions. Although the similarities in chemical properties between flavins and deazaflavins have been described[4] a stable semiquinone form of a deazaflavin has not been previously reported.[5] We now wish to report the formation of the semiquinone form of 5-deazaFAD bound to D-amino acid oxidase.[6]

Figure 1 shows the changes in the absorption spectrum produced by irradiation of deazaFAD-containing D-amino acid oxidase at pH 8.1 in the presence of 0.1 M EDTA under anaerobic conditions. It is noted that this procedure produces a species characterized by a broad long-wavelength absorption band in the region of 450–575 nm, and a second absorption maximum at 384 nm. Spectrally this species appears similar to the anionic or "red" semiquinone form of D-amino acid oxidase which exhibits absorption maxima at 370, 400, and 490 nm[7] and indeed exhibits a reddish pink color by visual inspection. The conversion of the oxidized deazaflavin to this new species does not appear to involve any spectrally discernible intermediates, as isosbestic points are maintained at 445, 383, and 345 nm. Evidence that the new spectral species represents enzyme-bound flavin was obtained by demonstrating that after air reoxidation of the new spectral species, the oxidized deazaFAD could be reduced by D-alanine, a reaction which only occurs with enzyme-bound deazaflavin.[6]

In order to demonstrate that the spectrum obtained upon EDTA-light irradiation is indeed the deazaflavin semiquinone, a similar experiment was conducted in which the deazaFAD-containing enzyme was irradiated in an EPR flat cell designed for aqueous samples and the appearance of a free radical signal was monitored with time. As shown in Figure 2 a free radical signal was indeed generated by this procedure and corresponded to 32% of the deazaflavin in the semiquinone form. The somewhat low yield of radical obtained in this experiment may in part be due to a combination of factors: (1) Only a 75% yield of radical is observed with the native FAD form of this enzyme.[7] (2) Some protein denaturation occurred during the 2-h period needed to accumulate the EPR data. Protein denaturation was often observed after EDTA-light radiation and presumably reflects the instability of the protein in the semiquinone form. It is noteworthy to point out the appearance of hyperfine structure in the EPR spectrum of the deazaFAD radical, as such hyperfine structure is not generally observed with enzyme-bound flavin radicals.[7]

Attempts to produce deazaFAD D-amino acid oxidase semiquinone by dithionite titration produced only the fully reduced species. Likewise, neither dithionite titration nor EDTA-light irradiation could produce a deazaflavin semiquinone form of free deazaFAD at pH 8.1, deazaFMN *N*-methylglutamate synthetase at pH 8.3, deazaFAD glucose oxidase at pH 5.8, 9.2, and 10.3 or deazaFMN flavodoxin at pH 5.8. Dithionite titration resulted in full reduction of the deazaflavin, whereas EDTA-light irradiation resulted in a 30–50% reduction of the flavin.

It is not clear at present why deazaflavin semiquinones are not generally observed with deazaflavin-containing proteins, nor do we understand the inability of dithionite to produce the semiquinone of deazaFAD-containing D-amino acid oxidase. However, the results of EDTA-light irradiation of deazaFAD-containing D-amino acid oxidase do illustrate that a semiquinone form of deazaflavin can be produced albeit under ideal conditions. Edmondson et al.[2c] suggested that their inability to detect a semiquinone form of deazaFMN bound to the Shethna flavoprotein reflected the requirement for nitrogen at the 5-position of the isoalloxazine ring; however, the fact that we have detected a stable deazaflavin semiquinone makes this hypothesis untenable.

Reoxidation of the semiquinone form of deazaFAD is too rapid to measure on a recording spectrophotometer. This can be contrasted to the absence of detectable air reoxida-

tion of fully reduced deazaFAD bound to D-amino acid oxidase.[6] Although the mechanism of the oxygen dependent reoxidation of reduced flavins is not fully understood,[8] it has been suggested that this reaction proceeds via the intermediate formation of the flavin semiquinone.[8a] Thus, the lack of oxygen reactivity of reduced deazaflavins[6] might be attributed to a slow oxidation of fully reduced deazaflavin to the deazaflavin semiquinone.

The results of this study further support the use of deazaflavins as models for elucidating the mechanism of action of flavoproteins, since it has now been demonstrated that like normal flavins, deazaflavins can exist in three chemical states, oxidized, reduced, and semiquinone. The importance of flavin semiquinones as intermediates in flavoprotein reactions is still a controversial issue.[4] The present results serve to illustrate that deazaflavin semiquinones are also potential intermediates in deazaflavoprotein catalyzed reactions.

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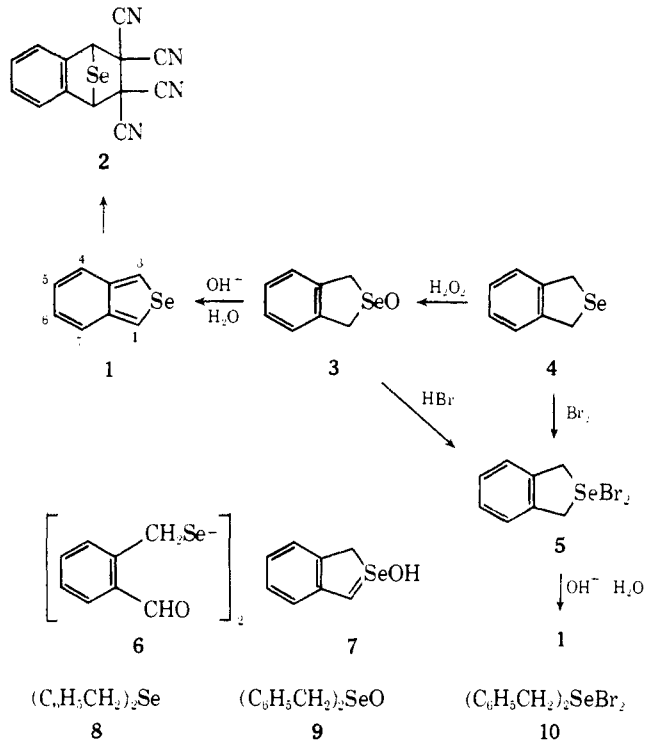
Benzo[*c*]selenophene. A Base-Catalyzed Selenoxide Dehydration

Sir:

The highly reactive *o*-quinonoid heterocycles benzo[*c*]furan[1] and benzo[*c*]thiophene[2] have been the objects of considerable chemical interest, both from the synthetic and the theoretical points of view. The selenium analogue of these compounds, benzo[*c*]selenophene (**1**), has hitherto remained unknown, even in the form of substitution products. We now describe the first synthesis of the unstable benzo[*c*]selenophene (**1**), as well as the first instance of a base-catalyzed dehydration of a selenoxide.

Dibenzyl selenoxide (**9**) is readily prepared in high yield either by the hydrogen peroxide oxidation of dibenzyl selenide (**8**)[3] or by treatment of dibromide **10** with aqueous alkali.[4] By contrast, oxidation of 2-selenaindane (**4**) has yielded only an anomalous product[5] now recognized as 2,2′-diformyldibenzyl diselenide (**6**),[6] whereas treatment of the corresponding dibromide **5** with alkali was stated to effect



debromination to the original selenide **4**.[5] Since the reductive debromination of a selenide dibromide by base has no precedent, we reexamined this reaction with the following results.

Dibromide **5** was destroyed within a few minutes when rubbed with cold aqueous 15% NaOH. When the resulting milky emulsion was stirred at 0 °C with hexane, benzo[*c*]selenophene (**1**) was slowly liberated and extracted into the organic phase. Heterocycle **1**, which has a pronounced naphthalene-like odor, polymerized upon attempted isolation in the pure state but was found to be fairly stable as a dilute hexane solution. Its ultraviolet absorption spectrum is very similar to that of benzo[*c*]thiophene,[7] compared to which it shows a small (ca. 7 nm) bathochromic shift: λ_{max} (hexane) 273, 286, 291, 298, 302 sh, 305 sh, 312, 323, 328, 336 sh, 340, 344 sh, 353, 357, 362 sh nm. The rate of formation of **1**, as monitored by uv, increases with alkali concentration, and the reaction is best carried out using cold 40% NaOH for 2 h.

Benzo[*c*]selenophene (**1**) reacts rapidly with tetracyanoethylene in benzene (32% based on dibromide **5**) to give the highly crystalline adduct **2**, mp 220 °C dec.[8] The mass spectrum of this adduct is in full accord with the assigned structure, showing major peaks at *m/e* 310 (M, 8%), 230 (M − Se, 7%), 128 (TCNE, 66%), 182 (M − TCNE, 99%), and 102 (M − TCNE − Se, 100%).

The NMR spectrum of **1** in $CDCl_3$ showed the following peaks: δ 8.40 (s, 2 H, H_1 and H_3), 7.33–7.54 (m, AA′ part of AA′BB′, 2 H, H_4 and H_7), and 6.77–7.02 (m, BB′ part of AA′BB′, 2 H, H_5 and H_6).[9] These peaks vanished immediately upon addition of TCNE, and adduct **2** crystallized from the solution.

Since the generation of selenophene **1** from dibromide **5** can be rationalized without requiring the intermediacy of selenoxide **3**, we examined the oxidation of selenide **4** with 1 equiv of neutral H_2O_2 in cold methanol. Immediate dilution of the reaction mixture with water and CCl_4 extraction yielded only traces of starting material from the organic phase. The aqueous phase contained selenoxide **3**, since addition of HBr caused the immediate precipitation of dibromide **5** (50% pure material after ether washing). A similar-