Interactions of a Fluorescent Active-Site-Directed Inhibitor of Thrombin: Dansylarginine *N*-(3-Ethyl-1,5-pentanediyl)amide†

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ABSTRACT: Dansylarginine *N*-(3-ethyl-1,5-pentanediyl)amide is a specific and potent inhibitor of thrombin. The apparent avidity and specificity of the inhibitor coupled with the fluorescence properties of the dansyl moiety suggested to us that the compound could be extremely useful as a probe for the study of thrombin and its interactions. The synthesis of the inhibitor was accomplished by coupling 4-ethylpiperidine to dansylarginine previously activated with *N,N'*-carbonyldiimidazole. Although the compound is not susceptible to hydrolysis catalyzed by thrombin, it competitively inhibits the thrombin-catalyzed hydrolysis of either synthetic substrates or fibrinogen with an apparent K_i of about 10^{-7} M. When the inhibitor is bound to thrombin, marked changes in the fluorescence properties of the dansyl moiety are observed, including a threefold increase in intensity and lifetime and a decrease in the depolarization of the excitation signal. Using measurements of fluorescence intensity and polarization to study binding, a dissociation constant of 4.3×10^{-8} M and a stoichiometry of 1 mol of inhibitor per mol of thrombin were found. The enhancement of fluorescence intensity concomitant with binding provides a continuous monitor of the conversion of prothrombin to thrombin as catalyzed by the "prothrombinase" complex. This permits the precise analysis of the kinetics of thrombin formation. In addition, thrombin-catalyzed feedback reactions which can occur during prothrombin activation are inhibited, and therefore the interpretation of the kinetics of the activation process are greatly simplified. In general the present results indicate that dansylarginine *N*-(3-ethyl-1,5-pentanediyl)amide is an ideal probe of the catalytic function and numerous interactions of thrombin.

There are several well-characterized inhibitors of thrombin; some are natural products while others have been chemically synthesized. Members of the latter group interact either covalently or noncovalently, but irrespective of the specific mode of binding, their effects are usually predicated on either or both of the following facts—thrombin is a serine protease, and its substrate specificity is akin to that of trypsin. Because enzymes of this general description are common, it is difficult to find specific inhibitors of thrombin unless one relies not only on the primary substrate binding determinants but also on the presumptive substrate subsites in designing the inhibitors.

Much effort has been expended in attempts to synthesize inhibitors of this last-mentioned type (Geratz & Tidwell, 1977) and recently Okamoto et al. (1975, 1976) and Hijikata et al. (1976) have described the remarkable potency and specificity of DAPA[1] as one such inhibitor of thrombin. Unfortunately, the authors did not provide a description of synthetic methods. Yet it is apparent from their data, and from the known fluorescent properties of the dansyl moiety, that this molecule could be an invaluable tool in the study of physicochemical interactions that involve thrombin.

In this paper are described a simple synthesis for, and the physical and chemical properties of, DAPA. Detailed specifically is the nature of its interaction with thrombin as elucidated by studies of thrombin kinetics and by changes in fluorescence parameters of the dansyl moiety which occur upon ligand binding.

Materials and Methods

Materials. Dansylarginine was obtained from Pierce Chemical, Rockford, IL. When subjected to high performance liquid chromatography (LC) on a column of C18 Bondapak in a Waters Associates apparatus, the material eluted as a single peak and was therefore used without further purification. *N,N'*-Carbonyldiimidazole was purchased from Sigma and was purified by sublimation as follows: the material was heated to 90 °C at a pressure of 20 μm until about 10% of the mass had sublimed. Heating was then continued at 115 °C at the same pressure until approximately all but 10% of the material remaining from the previous step had sublimed. That portion of the sublimate collected by heating at 115 °C was retained and stored desiccated at 22 °C in the dark. A sample of 4-ethylpiperidine, homogeneous as deduced by gas–liquid chromatographic analysis, was the gift of Dr. William Sowers, Reilly Tar Chemical, Indianapolis, IN. Dimethyl sulfoxide (Me_2SO), acetonitrile, and ethyl acetate were purchased from Burdick and Jackson, Muskegon, MI. Me_2SO was dried over molecular sieves (no. 4) prior to use. Heptane was obtained from Fisher Chemicals. Tosylarginine methyl ester (TAME) was obtained from Fox Chemical Company, Los Angeles, CA. The thrombin substrate, Bz-Phe-Val-Arg-*p*-nitroanilide (S2160), was obtained from Ortho Diagnostics, Raritan, NJ. All other reagents were of analytical grade.

Proteins. Human α-thrombin was prepared by the method of Downing et al. (1975). Bovine α-thrombin was prepared from Parke-Davis topical thrombin by the method of Lundblad et al. (1975) using gradient elution. Bovine β-thrombin was obtained by incubation of topical thrombin in 0.025 M sodium phosphate buffer, pH 6.5 at 37 °C for 12 h, followed by isolation according to Lundblad et al. (1975). Bovine factor V was isolated according to Nesheim et al. (1979). Prior to use the factor V was diluted to 0.1 mg/mL in 0.15 M NaCl, 0.02 M Tris-HCl, pH 7.4, and activated by incubation with thrombin (1.0 NIH unit per mL final concentration) for 1 min at 37 °C. Bovine prothrombin and bovine factor X were prepared as described by Bajaj & Mann (1973). Factor Xa was prepared from isolated factor X with insolubilized Russell's

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[1] Abbreviations used: DAPA, dansylarginine *N*-(3-ethyl-1,5-pentanediyl)amide; $NaDodSO_4$, sodium dodecyl sulfate.

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viper venom as described by Downing et al. (1975). It was assayed as described by Bajaj & Mann (1973) and had a specific activity of 863 units per mg based on a bovine plasma standard. The molecular weight and extinction coefficient ($E^{1\%}_{1cm}$) of both human and bovine α-thrombin were taken as 37 400 (Mann & Elion, 1978) and 19.5 (Winzor & Scheraga (1974), respectively. The same extinction coefficient was assumed for β-thrombin. Bovine fibrinogen was prepared from bovine plasma by precipitation in the cold with ethanol according to the NIH procedure, *Minimum Requirements for Dried Thrombin* (1946).

Preparation of DAPA. To 0.6 mL of dry Me_2SO at 22 °C were added 75 mg of dansylarginine and 180 mg of *N,N'*-carbonyldiimidazole. After 10 min, 4-ethylpiperidine (180 μL) was added and incubation was continued at 22 °C for 4 h, after which 2 mL of 0.15 M NaCl was added. The mixture was then extracted with 3.0 mL and then 1.0 mL of ethyl acetate. The combined ethyl acetate extracts were backwashed three times with water. The ethyl acetate was then removed with a stream of nitrogen resulting in an oil which was put under vacuum for 2 to 3 h (20 μm) to remove traces of water. The residue was taken up in 3 mL of ethyl acetate, and heptane (6 mL) was added with stirring, resulting in the appearance of a solid to semisolid material, the precipitation of which could be facilitated by chilling to 0 °C. When the liquid phase had clarified, it was decanted and the remaining solid was washed with 5 mL of heptane. Residual solvent was removed by a stream of nitrogen. The relatively dry residue was then taken up in 10 mL of 0.15 M HCl and added over a period of approximately 30 min at 22 °C to a 1.0 × 10 cm column of XAD-2 hydrophobic resin. Nonhydrophobic materials, including excess HCl, were removed by washing the column with 100 to 200 mL of H_2O. Dansylarginine 4-ethylpiperidine hydrochloride was then eluted at 22 °C from the column with approximately 40 mL of acetonitrile:H_2O (4:1, v/v). The majority of the acetonitrile was removed by rotary flash evaporation and the resulting aqueous solution was lyophilized to dryness, resulting typically in 75 mg of dry, water-soluble, yellowish powder. Overall yields were 70%.

Physical Characterization of DAPA. DAPA preparations were assessed for purity by high pressure liquid chromatography using a Waters Associates LC equipped with a C18 Bondapak reverse phase column. Chromatograms were developed over 10 min with a linear gradient (program 6) starting with 0.01 M sodium acetate (pH 4.5):methanol (9:1, v/v) and ending with 0.01 M sodium acetate (pH 4.5):methanol (1:9, v/v). Eluted components were detected by absorption at 254 nm. Absorption spectra of DAPA were recorded in H_2O using a Cary 219 recording spectrophotometer. Measurements of fluorescence intensity, spectra, and polarization were made using an SLM series 8000 spectrofluorimeter. Fluorescence lifetimes were measured with an SLM subnanosecond lifetime instrument. Proton nuclear magnetic resonance spectra were recorded on a Bruker 270-MHz NMR spectrometer by Mr. R. Thrift at the Freshwater Biology Institute, University of Minnesota, Navarre, MN. Elemental analyses of the synthesized DAPA were performed in duplicate by Chemalytics, Tempe, AZ.

Interaction of DAPA with Thrombin. (a) Kinetic measurements using synthetic substrates were performed by adding aliquots of a thrombin solution to buffered solutions of substrates and DAPA in 0.05 M Tris-HCl, pH 8.0, followed by measurement of initial velocities with a Beckman Acta C-III recording spectrophotometer. TAME assays were recorded at 247 nm, and S2160 assays were recorded at 405 nm. In some instances, 1% poly(ethylene glycol) 6000 was included to prevent adsorption of thrombin to glass (Wasiewski et al., 1976). Clotting assays were performed by a modified NIH procedure described by Mann et al. (1971) and Lundblad et al. (1976). To determine inhibitory effects of DAPA with fibrinogen as substrate, a standard curve was established by recording clotting times when serial dilutions of thrombin were used. The effect of DAPA was then assessed by adding fixed quantities of thrombin solution to fibrinogen solutions containing various concentrations of inhibitor and recording the clot time. Residual thrombin activity at each inhibitor concentration was then determined from the standard curve.

(b) The binding of DAPA to thrombin was examined by fluorescence techniques. Titrations were performed both by the addition of ligand to protein and vice versa. The interactions were monitored by both fluorescence intensity and polarization where the signal originated from the dansyl moiety of DAPA. The experiments were performed at 22 °C in 0.02 M Tris, 0.15 M NaCl buffer, pH 7.4. Fluorescence polarization was measured using the SLM instrument operated in the T-format (Weber & Bablouzian, 1966); λ_{ex} was 350 nm and the signal was recorded at 515 nm. Monochromators were placed in front of both photomultiplier tubes and by this means the depolarizing effects of Rayleigh scattering or the polarizing effects of Raman scattering (which is maximal at 397 nm for λ_{ex} of 350 nm) were eliminated. Fluorescence lifetimes were measured using the phase modulation technique of Spencer & Weber (1969). Light at the excitation wavelength of 330 nm was isolated by use of an excitation monochromator (slit widths of 8 and 0.5 nm, respectively) and a Corning 7-54 filter whereby stray light effects were minimized. To eliminate the appearance of stray and scattered excitation light in the fluorescence emission, a combination filter (comprising Corning 3-70 and 3-144 filters) was used.

In order to calculate the stoichiometry and affinity of the DAPA–thrombin interaction from fluorescence intensity data, aliquots of a stock solution of bovine thrombin (0.72 mg/mL, 1.93×10^{-5} M) were added sequentially to a solution of 4.24×10^{-7} M DAPA in 0.02 M Tris-HCl, 0.15 M NaCl, pH 7.4, 22 °C. By this procedure the thrombin concentration was varied from 0 to 7.7×10^{-7} M. After each addition of protein, the total energy emitted between 490 and 560 nm was determined by integrating the DAPA emission spectrum between those wavelengths (λ_{ex}, 350 nm, excitation and emission slits at 2 and 1 nm, respectively).

The dissociation constant and stoichiometry were calculated from data plotted according to the equation shown below. P_0 and L_0 are the nominal concentrations of protein and ligand, respectively; f and b are fraction-free and fraction-bound ligand, respectively; n is the number of ligand binding sites per protein molecule; and K_d is the dissociation constant for a single binding site.

$$\frac{1}{f} = \frac{n}{K_d}\frac{[P_0]}{b} - \frac{L_0}{K_d}$$

This equation is formally identical with that of Scatchard (1949) as described by Tanford (1961) except that b and f refer explicitly to bound and free ligand rather than protein. In addition, the equation has been rearranged so that the independent variable (P_0/b) contains the nominal concentration of the component (protein) varied in the experiment. This modification made it possible to obtain K_d and n from linear plots of fluorescence intensity data under the experimental conditions presently chosen in which protein was added in increments to a fixed quantity of ligand rather than vice

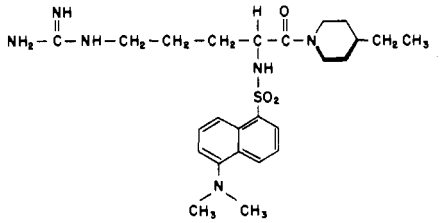

FIGURE 1: Chemical structure of DAPA. The compound was synthesized by coupling 4-ethylpiperidine to dansyl-L-arginine previously activated with *N,N'*-carbonyldiimidazole. DAPA was isolated by extraction and precipitation with organic solvents followed by desorption from a hydrophobic resin.

versa. This approach was chosen to eliminate the problem of variable levels of background fluorescence which would have accompanied titration with DAPA. To calculate b and f, the fluorescence intensity observed at saturation (F_2) had to be known. Since F_2 is approached only asymptotically, the "best" value for this parameter was found by an iterative procedure which was performed in the following manner. First the greatest intensity observed in the titration was taken as the saturation value and was used as a parameter for calculating the ordered pairs, $(1/f, P_0/b)$, from the remaining titration data. These pairs were then subjected to linear regression analysis followed by repetition of the process for each of numerous increments in the value of F_2. The value of F_2 which maximized the correlation coefficient of the ordered pairs $(1/f, P_0/b)$ was taken as the "best" value and was used in subsequent calculations of f and b.

Use of DAPA to Monitor the Formation of Thrombin Active Sites during Prothrombin Activations. Varying amounts of prothrombin (10–80 μg/mL, (1.39–11.1) × 10^{-7} M, final concentration) were prepared in 0.02 M Tris-HCl, pH 7.4, 0.15 M NaCl, 0.13 mM cephalin, 2.5 mM $CaCl_2$, and 2.0 × 10^{-6} M DAPA. Bovine factor Xa was then added to a final concentration of 1.0 unit/mL (1.2 μg/mL, 2.7 × 10^{-8} M). The prothrombin conversion reactions were initiated by the addition of thrombin-activated factor V (factor V_a) to a final concentration of 5.0 units/mL (1.2 × 10^{-8} M). The change in fluorescence intensity resulting from the interaction of DAPA with newly formed thrombin was monitored with time using an Aminco fluorocolorimeter equipped with a chart recorder. The primary filter was a Corning 7-60. A Corning 4784 and a Wratten 2A were used together as secondary filters. All experiments were carried out at 22 °C.

Results

Chemical and Physical Characterization of DAPA. The structural formula of DAPA is shown in Figure 1. Thin-layer and high pressure liquid chromatographic methods were used to assess the purity of the synthetic product. Thin-layer chromatography on polyamide in the solvent system acetonitrile:0.02 M sodium acetate, pH 4.5 (9:1, v/v), yielded a single spot with R_f value of 0.86; in this system, the starting material, dansylarginine, chromatographed to R_f 0.38. By high pressure liquid chromatography, the material was adjudged to be greater than 95% pure. The chemical identity of the material was examined by NMR and elemental analysis. NMR results indicated major proton resonances at 0.761, 0.884, 1.481, 2.236, and 2.819 ppm (resonances derived primarily from the 4-ethylpiperidine moiety) and in the region 7.114–8.441 ppm (resonances of the aromatic dansyl moiety) which are consistent with the proposed structure of the molecule. Elemental analysis was performed in duplicate for the elements hydrogen, carbon, nitrogen, sulfur, and chlorine; oxygen was calculated by difference. The average of two

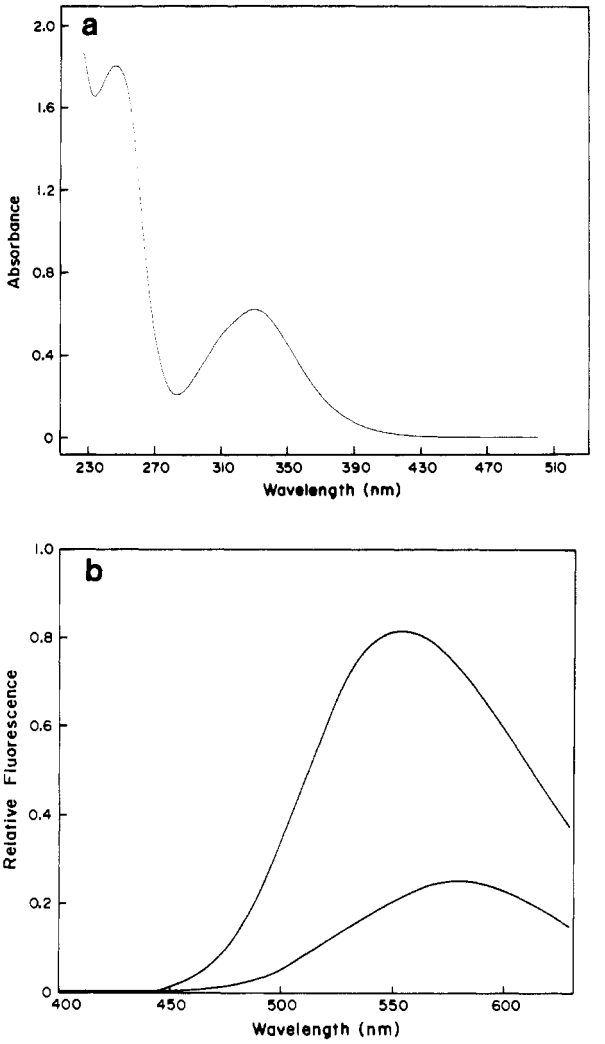

FIGURE 2: Absorption and fluorescence emission spectra of DAPA. (a) Absorption spectra of DAPA·HCl dissolved in water. (b) Corrected fluorescence emission spectra of DAPA. The upper and lower spectra were measured in the presence and absence of thrombin, respectively. Concentration of DAPA was 3.5 × 10^{-6} M in both instances and concentration of thrombin was 8.3 × 10^{-6} M. λ_{ex} was 350 nm. These spectra were recorded using EMI 9815 photomultiplier tubes and are truncated above 640 nm because the correction factors beyond this wavelength were too large to be reliable.

analyses permitted the calculation of the empirical formula, $C_{24.4}H_{38.6}O_{3.7}N_{6.32}S_{1.06}Cl_{1.00}$. The corresponding theoretical formula is $C_{25}H_{39}O_3N_6S_1Cl_1$. The results of the directly measured elements indicate that the empirical and theoretical formulas are identical within the precision of the measurements. Elemental analysis indicated that the synthesized derivative is the monohydrochloride salt. The ultraviolet spectrum of DAPA in water is shown in Figure 2a. The compound exhibited a maximum absorbance of 330 nm with a calculated millimolar extinction coefficient (1-cm path length) of 4.01. Fluorescence emission spectra of DAPA in aqueous buffered solution with and without excess thrombin (8.3 × 10^{-6} M) are shown in Figure 2b. The DAPA concentration was 3.5 × 10^{-6} M in both instances. The excitation wavelength was 350 nm. Fluorescence emission wavelength maxima were 578 and 555 nm in the presence and absence of thrombin, respectively. The fluorescence intensity of the dansyl moiety increased by a factor of ~3 upon the interaction of DAPA and thrombin.

The fluorescence lifetimes of DAPA in the presence and absence of thrombin and in media of varying polarity are

Table I: Summary of Fluorescence Properties of DAPA[a]

conditions	λ_{max} (emission nm)	τ_ϕ (ns)	τ_m (ns)
0.15 M NaCl, 0.02 M Tris, pH 7.4	578	4.39	4.61
buffer as above with excess thrombin	555	9.61	9.65
methanol	538	12.54	11.82
dimethylformamide	528	15.86	15.21
dioxane	502	17.07	16.43

[a] For lifetime data, excitation wavelength was 330 nm, excitation monochromator slits were set at 1 nm, and a Corning 7-54 filter was used in the excitation beam. To isolate the emission bands, a Corning 3-144 and 37-70 filter combination was employed. Values of λ_{max}(emission) were obtained from corrected spectra.

shown in Table I. In buffered aqueous solutions the fluorescence lifetime (τ_ϕ) observed was 4.39 ns. Under similar conditions in the presence of excess thrombin, a value of 9.61 ns was observed. Lifetime values observed in various solvents ranged from 4.39 ns in the most polar medium (H_2O) to 17.1 ns in the least polar (dioxane).

Interaction of DAPA with Thrombin: Enzyme Kinetics. In Figure 3 (a,b) are shown inverse plots of the activity of human α-thrombin in the presence and absence of DAPA. The substrate of Figure 3a was TAME; that of Figure 3b was S2160. The approximate thrombin concentrations used were 0.15 mg/mL (4.0×10^{-9} M) and 0.040 (1.1×10^{-9} M) with TAME and S2160, respectively. In both instances the results obtained in the absence of inhibitor are indicated by the lower line; those obtained at respective DAPA concentrations of 7.0×10^{-8} and 1.4×10^{-7} M are indicated by the middle and upper lines, respectively. The units on the vertical axis are the reciprocals of absorbance changes per minute at 247 nm (TAME) and 405 nm (S2160). The experiments were carried out at 22 °C in 0.02 M Tris-HCl, pH 8.2, 1% PEG-6000. In both instances linear relationships between the reciprocals of velocity and substrate concentration were obtained. All data extrapolated to the same point in the vertical axis, suggesting that with both TAME and S2160 as substrates the inhibition of human α-thrombin by DAPA is competitive.

"Apparent" K_i values were calculated from the slopes of the lines given in Figures 3a and 3b and were as follows. With TAME as substrate, K_i values of 4.8×10^{-8} and 6.0×10^{-8} M were found at DAPA concentrations of 7.0×10^{-8} and 1.4×10^{-7} M, respectively. An apparent K_i value of 1.0×10^{-7} M at both inhibitor concentrations was found when S2160 was used as substrate. Inhibition of bovine α-thrombin with fibrinogen as substrate was determined by clotting as described in Materials and Methods. The fibrinogen concentration was held constant at about 3.0 mg/mL and the DAPA concentration was varied. A 50% reduction in clotting activity was observed at a DAPA concentration of 1.2×10^{-7} M. In similar experiments reported previously (Prendergast et al., 1977) with human α-thrombin, 50% inhibition of clotting activity was observed at 1.5×10^{-7} M DAPA.

In experiments using a constant concentration of S2160 (5×10^{-7} M) the extent of inhibition of both the α and β forms of bovine thrombin was studied at several concentrations of DAPA. β-Thrombin is a derivative of α-thrombin formed by limited proteolysis of the latter (Lundblad et al., 1975; Kingdon et al., 1977). Compared with the α form, β-thrombin has minimal activity toward fibrinogen, partial activity toward tripeptide substrates such as S2160, and full activity toward simple amino acid derivatives such as TAME (Workman et al., 1977). Despite the differences in the capacities of the two enzymes to cleave different substrates, no differences were detected in the susceptibility of the enzymes to inhibition by DAPA. In both instances 50% inhibition was observed at an inhibitor concentration of 9×10^{-8} M and the extent of inhibition at all concentrations of inhibitor was similar for both enzymes. A summary of the inhibitory properties of DAPA

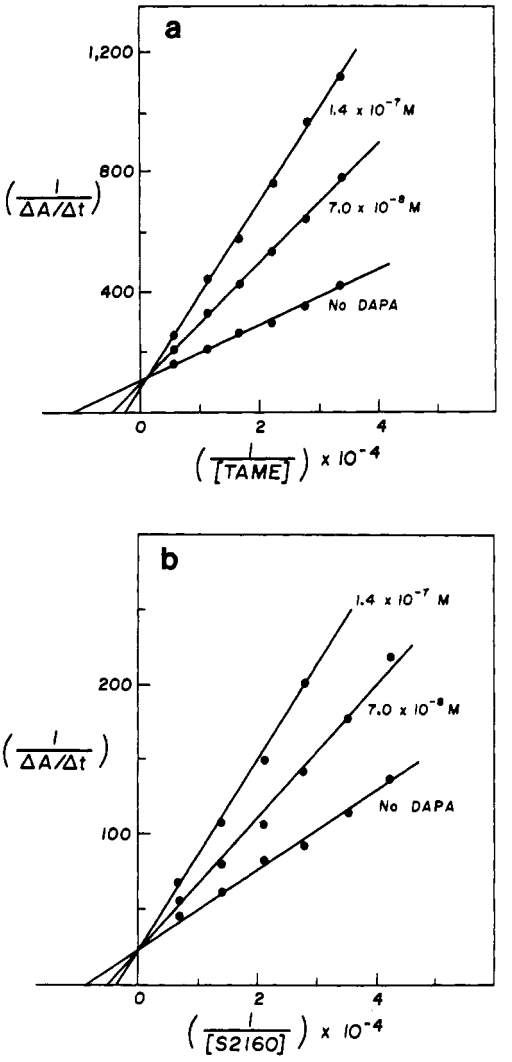

FIGURE 3: Effects of DAPA on thrombin-catalyzed hydrolysis of TAME (a) and S2160 (b). The reciprocals of the rates of change of absorbance (vertical axes) are plotted vs. reciprocals of concentrations of substrate (horizontal axes). Concentrations of DAPA in respective experiments are recorded on the graphs. Approximate concentrations of thrombin used in these experiments were 4×10^{-9} and 1.1×10^{-9} M with TAME and S2160, respectively.

Table II: Summary of the Inhibitory Properties of DAPA[a]

enzyme	substrate	"apparent" K_i (M)
α-thrombin (h)	S2160	1.0×10^{-7}
α-thrombin (h)	TAME	5.4×10^{-8}
α-thrombin (h)[b]	fibrinogen	1.5×10^{-7}
α-thrombin (b)	fibrinogen	1.2×10^{-7}
factor Xa (b)	S2222	$>1.0 \times 10^{-4}$ [c]

[a] Source of the enzyme is indicated by h for human or b for bovine. The substrates S2160 and S2222 are peptide *p*-nitroanilides specific for thrombin and factor Xa, respectively. TAME is tosyl-L-arginine methyl ester. Inhibition of thrombin by DAPA appeared to be competitive. [b] Taken from Prendergast et al. (1977). [c] This value is somewhat equivocal in that no more than 10-20% apparent inhibition of factor Xa could be observed in the presence of DAPA at concentrations of 10^{-4} M.

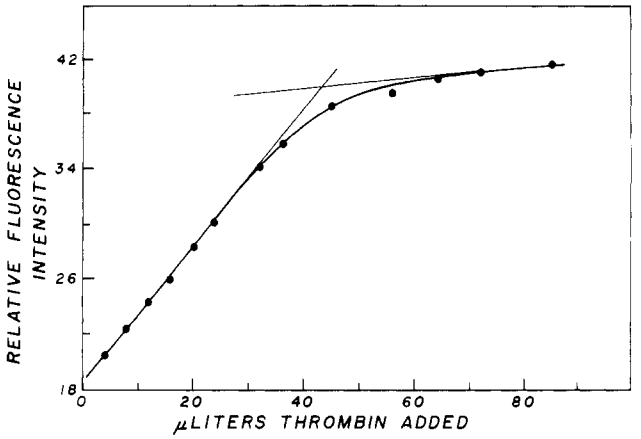

FIGURE 4: Increase of fluorescence intensity of DAPA titrated with thrombin. Two milliliters of DAPA (4.2×10^{-7} M) in 0.02 M Tris-HCl, 0.15 M NaCl, pH 7.4, was titrated with a stock solution of thrombin (1.5×10^{-5} M). Relative fluorescence intensity values (vertical axis) are plotted against microliters of thrombin solution added (horizontal axis). Integrated intensities were measured from 490 to 560 nm; λ_{ex} was 350 nm. The intersection of the extrapolated line occurs at a thrombin:DAPA molar ratio of 0.98.

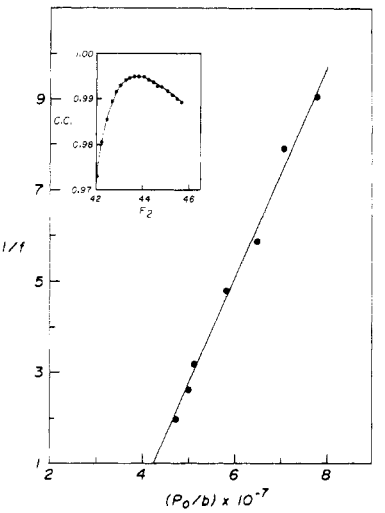

FIGURE 5: Determination of the affinity and stoichiometry of the binding of DAPA to thrombin. Data from the titration of DAPA with thrombin (see Figure 4) is plotted according to the equation described in Materials and Methods. Values of the reciprocals of fraction-free DAPA are indicated by the vertical axis; values of the ratio of thrombin to fraction-bound DAPA are on the horizontal axis. From this plot a dissociation constant of 4.3×10^{-8} M was determined. The stoichiometry of binding was found to be 1.11 mol of DAPA per mol of thrombin. In the inset are plotted the linear correlation coefficients of titration data (vertical axis) vs. assigned value of the asymptote toward which titration data was assumed to be approaching (see Materials and Methods).

toward α-thrombin and factor Xa is given in Table II.

Experiments were performed to determine whether or not thrombin catalyzes the hydrolysis of DAPA. Thrombin at a final concentration of 2 NIH units/mL was added to a 10^{-4} M solution of DAPA in 0.02 M Tris-HCl, pH 7.4 at 22 °C. At various times during the next 24 h, aliquots were removed and analyzed by chromatography on polyamide thin-layer sheets developed in acetonitrile:0.05 M sodium acetate, pH 4.5 (9:1, v/v). No hydrolysis of the inhibitor was detected for the duration of the experiment. Thus, DAPA does not inhibit thrombin as a competitive substrate.

DAPA displayed little if any potential to inhibit factor Xa. At inhibitor concentrations up to 10^{-4} M, the rate of the factor Xa catalyzed hydrolysis of the synthetic substrate S2222 was depressed by no more than 10–20%. Under comparable conditions, the thrombin catalyzed hydrolysis of the synthetic substrate S2160 was inhibited by more than 99.5%. This selectivity of DAPA for thrombin over factor Xa was also demonstrated in a prothrombin activation system. Prothrombin (0.1 mg/mL) was incubated at 37 °C for 15 min with factor Xa (1.0 units/mL), 2.5 mM $CaCl_2$, 0.13 mM cephalin, factor Va (5.0 units/mL), and 0.02 M Tris-HCl, pH 7.4, 0.15 M NaCl in both the presence and absence of 10^{-4} M DAPA. The reaction mixtures were then analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis. When DAPA was absent, the expected products, thrombin, prothrombin fragment 1, and prothrombin fragment 2, were observed. In the presence of 10^{-4} M DAPA, however, only the products of the factor Xa catalyzed cleavages, thrombin and fragment 1·2, appeared; the thrombin-catalyzed cleavage of the latter to fragment 1 and fragment 2 was prevented.

Interaction of DAPA with Thrombin: Fluorescence Measurements. Both fluorescence intensity and polarization measurements were used to monitor the interaction of DAPA with thrombin.

Fluorescence intensity values observed when aliquots of a stock solution of thrombin were added sequentially to a 4.24×10^{-7} M solution of DAPA are depicted in Figure 4. The units on the vertical axis are relative intensity values, and those on the vertical axis are total microliters of stock thrombin solution added. The fluorescence intensity initially increased sharply as thrombin was added to the solution containing DAPA but eventually approached a plateau. The end point of the titration is indicated by the intersection of the extrapolated lines in Figure 4 and occurred at a thrombin to DAPA molar ratio of 0.98, suggesting a 1:1 binding stoichiometry.

The data of Figure 4 were also used to calculate the dissociation constant as well as stoichiometry of the interaction of DAPA with thrombin. The calculations were made by procedures and equations described in Materials and Methods, and the results are presented graphically in Figure 5. The units on the horizontal axis are ratios of the nominal concentration of thrombin to fraction-bound DAPA, while the units on the vertical axis are the reciprocal of fraction-free DAPA. The line drawn through the indicated points was determined by least-squares linear regression analysis. A dissociation constant (K_d) of 4.30×10^{-8} M was calculated from the slope of the line. Using the ratio of the slope to the vertical intercept, multiplied by the nominal DAPA concentration, a value of 1.11 was calculated for the number of DAPA binding sites per thrombin molecule. Shown as an inset of Figure 5 is the plot obtained by the iterative procedure used to find the "best" value of fluorescence intensity at saturation (F_2). The linear correlation coefficient of experimental data (vertical axis) is plotted against assigned values of F_2 (horizontal axis). The value of 43.85 obtained for F_2 by this procedure was used in all calculations of fractional saturation.

Polarization experiments were performed by adding DAPA to a solution of thrombin. In Figure 6 are depicted the results of an experiment in which sequential aliquots of a 1.7×10^{-4} M solution of DAPA were added to a 1.93×10^{-6} M buffered solution of bovine α-thrombin. Fluorescence polarization values remained essentially constant over the initial part of the titration and thereafter decreased with increasing concentrations of DAPA. The equivalence point of the titration is indicated by the intersection of the dashed lines at point A, corresponding to a DAPA to thrombin molar ratio of 0.85. This value is in reasonable agreement with the values obtained with fluorescence intensity measurements, either by direct

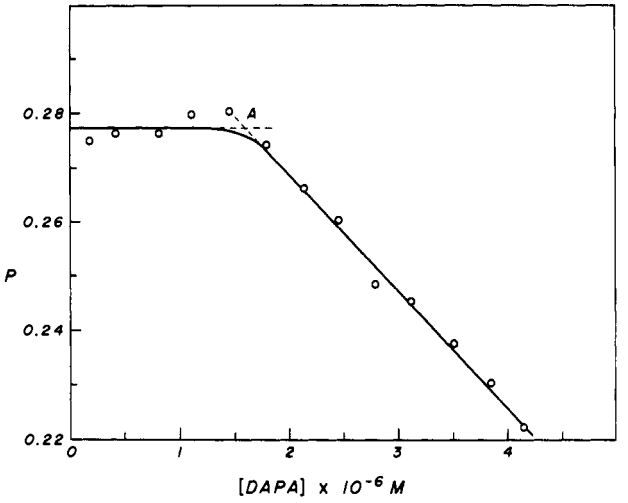

FIGURE 6: Fluorescence polarization signals observed upon titration of thrombin with DAPA. Aliquots of DAPA were added to a 1.93 × 10^{-6} M solution of thrombin in 0.02 M Tris-HCl, 0.15 M NaCl, pH 7.4 at 22 °C, to give concentrations of DAPA as indicated by the horizontal axis. With each addition the polarization of the fluorescence signal was measured, values of which are indicated by the vertical axis (λ(excitation), 350 nm; λ(emission), 515 nm). The intersection of the extrapolated lines, indicated by point A, occurred at a DAPA to thrombin molar ratio of 0.85.

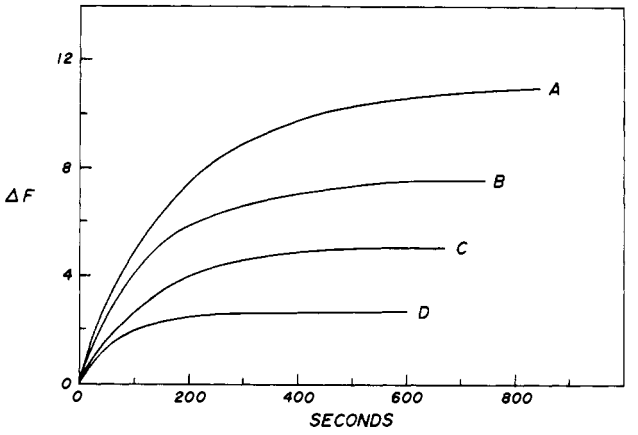

FIGURE 7: Fluorescence intensity upon activation of protrombin in the presence of DAPA. Prothrombin at concentrations of 80 (A), 60 (B), 40 (C), and 20 (D) μg/mL was activated by factor Xa in the presence of Ca^{2+}, phospholipid, factor Va, and 2.0 × 10^{-6} M DAPA. The change in fluorescence (vertical axis) is plotted against time (horizontal axis) after the initiation of the reaction by factor Va. Intensity measurements were made with a filter fluorometer.

inspection of the fluorescence intensity of titration data (0.98) or by calculation (1.11). All of these values indicate a binding stoichiometry of 1 mol of DAPA per mol of thrombin. The polarization data also indicate that the rotational mobility of DAPA bound to thrombin is highly restricted compared with its mobility in solution.

Interaction of DAPA with Thrombin: Use of DAPA to Monitor the Formation of Thrombin Active Sites during the Activation of Prothrombin. The specificity of DAPA for thrombin, the enhancement of fluorescence intensity that accompanies binding, and its lack of inhibitory action on factor Xa suggested that DAPA could be used to monitor thrombin formation during the activation of prothrombin. Experiments were performed in which various amounts of prothrombin were activated with factor Xa in the presence of activated factor Va, Ca^{2+}, cephalin, and DAPA in molar excess over prothrombin. The course of the reaction was monitored by fluorescence intensity measurements. The results of such

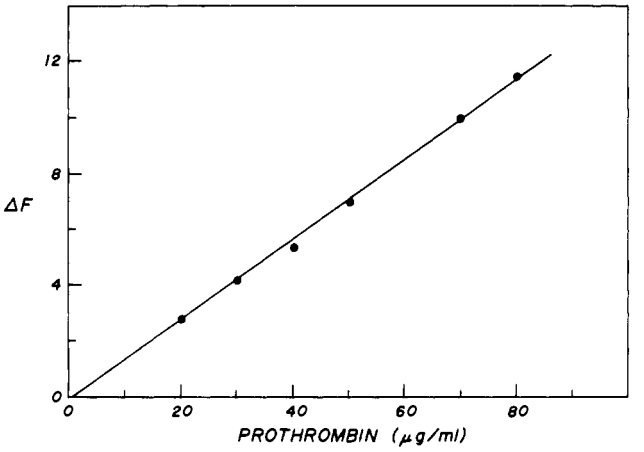

FIGURE 8: Fluorescence changes observed after complete activation of prothrombin. The change in fluorescence observed at plateau (ΔF), i.e., after complete activation of prothrombin, is indicated by the vertical axis. The initial concentrations of prothrombin are indicated by the horizontal axis. Reaction conditions were as described in the legend of Figure 7.

experiments are shown in Figure 7. The units on the horizontal axis are seconds after the initiation of reaction by the addition of activated factor V (factor Va). The units shown on the vertical axis are relative fluorescence intensity. The initial concentrations of prothrombin in the respective experiments were 80 (A), 60 (B), 40 (C), and 20 (D) μg/mL. The concentrations of all other components were identical in the four experiments. In all instances the fluorescence values (ΔF) initially increased rapidly and then approached a plateau, indicating completion of the reaction. Control experiments were carried out in the absence of DAPA in which thrombin measurements were made by removing aliquots of the reaction mixture at regular intervals and performing clotting assays according to the NIH procedure as modified by Mann et al. (1971). The time course of the increase in fluorescence intensity was identical with that of thrombin production (as measured by clotting assay), showing that the fluorescence measurements reliably indicated the formation of thrombin.

The maximum change in fluorescence intensity attained in activation experiments varied with the amount of prothrombin initially present. This change (vertical axis) was plotted against initial concentrations of prothrombin (horizontal axis). The result (Figure 8) is a straight line passing through the origin, further indicating that fluorescence intensity measurements provide a reliable monitor of prothrombin activation.

Discussion

Of several approaches attempted for the preparation of DAPA, the one which was most suitable involved the coupling of 4-ethylpiperidine to dansylarginine previously activated with *N,N'*-carbonyldiimidazole. The synthesis was completed in about 4 h at room temperature and typically resulted in yields of 70%. The reaction produced few side products and therefore the isolation of the desired compound required only simple extractions and precipitations with organic solvents and desorption from a hydrophobic resin. This permitted the isolation of DAPA of high purity without the need for complex separation techniques.

The properties of DAPA and its interaction with thrombin were studied both by investigations of the effect of the compound on the kinetics of thrombin and by measurements of fluorescence intensity, polarization, and lifetimes of the dansyl moiety in both the absence and presence of the enzyme. The studies of the kinetics of thrombin indicated that inhibition

by DAPA is competitive with an apparent K_i of 10^{-7} M, a value in good agreement with concentrations of DAPA required to give 50% inhibition (I_{50}) reported by Okamoto et al. (1975, 1976). The inhibition is specific in that the effects of DAPA on factor Xa (as shown presently) or trypsin, reptilase, and plasmin (Okamoto et al., 1975, 1976) are negligible in comparison with effects on thrombin. Interestingly, DAPA inhibits β-thrombin as effectively as α-thrombin, even though the two proteins exhibit marked differences in their respective capacities to catalyze the hydrolysis of certain synthetic substrates and fibrinogen (Workman et al., 1977).

Changes in fluorescence properties associated with the binding of DAPA to thrombin include a marked increase in intensity and polarization and a similar increase in lifetime. Changes in intensity and polarization were used to directly measure the dissociation constant and stoichiometry of the DAPA–thrombin interaction. The stoichiometry of binding was found to be 1 mol of DAPA per mol of the thrombin, and a value of 4.3×10^{-8} M was found for the dissociation constant, in good agreement with the K_i values of 10^{-7} M determined from kinetics.

Although the primary emphasis of the work presented in this paper was on the preparation and use of DAPA as a probe for the formation and various interactions of thrombin, the changes in fluorescence signals and kinetic parameters accompanying the interactions of DAPA or its analogues could also provide information on the catalytic site and factors determining the substrate specificity of the enzyme. For instance, the "blue" shift in the emission spectra of bound DAPA indicates that the environment of the binding site for the probe is less polar than the surrounding medium; the increases in the lifetime and intensity of fluorescence suggest that bound DAPA is measurably removed from dynamic quenching effects of components of the bulk solution; and the marked change in fluorescence polarization indicates that the probe is quite rigidly held at the binding site. The similarity of the two lifetime measurements (τ_ϕ and τ_m) observed when DAPA is bound to thrombin suggests that the guanidino group is fixed, as in a binding "pocket", and is not capable of partially quenching the fluorescence of the dansyl moiety. This is in contrast to the differences observed in τ_ϕ and τ_m when DAPA is in free solution (see Table I). (Although not shown here, NMR data suggest that the guanidino moiety significantly interacts with the naphthyl ring system in free solution.)

The changes in the fluorescence properties (intensity, lifetime, and polarization) concomitant with binding are clearly exploitable as signals with which to monitor the interactions of the DAPA–thrombin complex itself and the interactions of thrombin with the other molecules. Since DAPA binds in a noncovalent, freely reversible manner and with a well-defined stoichiometry, the uncertainties regarding structural perturbations caused by the probe are minimized. This is to be contrasted with the inevitable uncertainties associated with the use of covalently bound probes with regard to probe location and potential perturbations of structure that are difficult to define. That thrombin does not catalyze the hydrolysis of DAPA implies that the probe will remain unaltered over the course of experiments and thus may be used in studies where equilibrium, rather than transient, conditions are required.

A specific example of the value of DAPA as a probe is afforded by its use to monitor the kinetics of the activation of prothombin, a complex reaction involving the factor Xa catalyzed hydrolysis of prothrombin to yield thrombin and prothrombin fragment 1·2, followed by the thrombin-catalyzed cleavage of the latter to prothrombin fragments 1 and 2. The rate of the process is increased greatly by both Ca^{2+} and phospholipid and is further enhanced by the protein cofactor, factor Va. Thus, involved in the conversion of prothrombin to thrombin are numerous interactions between protein, Ca^{2+}, and lipid. Because of the large number of such interactions, the process is characterized by complex kinetics. Further complexities can arise from product (thrombin)-catalyzed feedback reactions such as the cleavage of the primary substrate, prothrombin, to fragment 1 plus the secondary substrate, prethrombin 1, the kinetics of activation of which are quite different from those of prothrombin. Other potential thrombin-catalyzed feedback events include the activation and possible subsequent inactivation of factor V. The products of the feedback reactions, when formed in situ, can alter kinetics of "prothrombinase" in a manner difficult to assess quantitatively. By eliminating the feedback reactions, DAPA simplifies the interpretation of the kinetics of prothrombin activation. The inhibition of the feedback reactions also makes possible the preparation and isolation of prothrombin fragment 1·2 so that its effects on the activation process can be studied. In addition, the change in fluorescence intensity associated with the interaction of DAPA and thrombin provides a continuous monitor of the time course of prothrombin conversion, thereby generating data in greater abundance and with higher precision than obtainable by, for example, thrombin assays performed at discrete intervals.

It is interesting to speculate on the factors which determine the remarkable specificity and potency of DAPA. The arginine moiety presumably interacts electrostatically with a carboxylate group in the binding pocket. The dansyl group is analogous to the tosyl moiety of TAME and presumably binds to a nonpolar site. But the unique properties of DAPA appear to derive from the 4-ethylpiperidine moiety of the molecule. Evidence for this assertion is gleaned from the data of Okamoto et al. (1975, 1976) who showed that the 4-ethylpiperidine moiety conferred both greater potency and specificity when compared with sundry other esters and amide derivatives of dansylarginine. Interestingly, the enantiomer (dansyl-D-arginine *N*-(3-ethyl-1,5-pentanediyl)amide) is not inhibitory (Okamoto et al., 1975, 1976). Intuitively, these features suggest that the hydrophobicity and conformation of the 4-ethylpiperidine group promote interaction with a second hydrophobic site. A second possibility is that the 4-ethylpiperidine moiety confers a unique conformation on DAPA which promotes markedly enhanced binding of the arginine and dansyl groups. In any event, the effects of DAPA illustrate that other inhibitors of, or substrates for, thrombin may be derived from the dansylarginine moiety. Accordingly, continued investigations of the properties of such arginine derivatives, especially those containing groups such as dansyl or possibly others which exhibit changes in spectral properties upon binding, might well aid in the elucidation of those features of the thrombin molecule which contribute to its substrate specificity and effectiveness as a catalyst.

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Isolation and Identification of Previtamin D_3 from the Skin of Rats Exposed to Ultraviolet Irradiation†

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ABSTRACT: The process of the photolytic activation of vitamin D precursor(s) in the skin has been elucidated by a detailed analysis of the products formed after ultraviolet light exposure. The photolytic product isolated from the skin of rats exposed to ultraviolet irradiation was identified as previtamin D_3 by several criteria including its (a) characteristic ultraviolet absorption spectrum, (b) mass spectrum, and (c) thermal isomerization to vitamin D_3, which itself was identified also by mass spectroscopy. Vitamin D_3 per se was not formed by ultraviolet irradiation—vitamin D_3 arises exclusively from the thermal conversion of previtamin D_3. Detectable amounts of lumisterol$_3$ or tachysterol$_3$ were not seen.

As early as 1890, Palm and his colleagues (Palm, 1890) made the observation that sunlight played an important role in the maintenance of healthy bone. Subsequently, a number of investigators demonstrated that both animals and children with rickets could be cured by exposure to either mercury-vapor quartz lamps or sunlight (Huldshinsky, 1919; Goldblatt & Soames, 1923a,b; Steenbock & Black, 1924, Hess et al., 1925). These observations provided the impetus for the isolation and chemical characterization of the $\Delta^{5,7}$-diene sterols, which, after exposure to ultraviolet light, are photochemically converted to antirachitic substances characterized as 9,10-secosteroids with a 5,6-cis triene system (Askew et al., 1931; Windaus and Boch, 1936). Ergosterol (a plant $\Delta^{5,7}$-diene sterol) and 7-dehydrocholesterol (an animal $\Delta^{5,7}$-diene sterol) were isolated, purified, and exposed to ultraviolet irradiation. From the irradiation reactions were isolated ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3), respectively, from among the other photoisomeric products (Windaus et al., 1932, 1936; Askew et al., 1931).

Because 7-dehydrocholesterol is found in appreciable quantities in rat, pig, and human skin, it was assumed that this $\Delta^{5,7}$-diene sterol is photochemically converted to vitamin D_3 when skin is exposed to sunlight. In fact, several investigators provided chromatographic evidence (Okano et al., 1977) as well as structural evidence that includes an ultraviolet-absorption spectrum and mass spectrum (Esvelt et al., 1978) for the presence of vitamin D_3 in rat and human skin (Rauschkolb et al., 1969) exposed to ultraviolet irradiation.

However, when $\Delta^{5,7}$-diene sterols are irradiated with ultraviolet light in a quartz vessel, vitamin D is not one of the photolytic products. Instead, lumisterol, tachysterol, and

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