Biochem. J. (1970) **116**, 341–348
Printed in Great Britain

Depolarization of the Intrinsic and Extrinsic Fluorescence of Pepsinogen and Pepsin

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(*Received* 8 *July* 1969)

1. The effects on the intrinsic tryptophan emission anisotropy of pepsin and pepsinogen solutions produced by (*a*) changes in temperature, (*b*) increases in viscosity with added glycerol at constant temperature and (*c*) decreases in lifetime through collisional quenching by potassium iodide were measured at several excitation wavelengths. The rotational-relaxation times calculated from results provided by method (*b*) approximate to the theoretical values for the two proteins, on taking hydration and shape factors into account, on the basis of random orientation of the tryptophan groups within the macromolecules. Differences between the results provided by methods (*b*) and (*c*) are attributable to inter-tryptophan resonance-energy-transfer depolarization, and the anomalous values recorded in method (*a*) can be attributed to the temperature-dependence of the limiting anisotropies. 2. Two different monomeric conjugates of pepsin, each containing one extrinsic fluorescent group per macromolecule, gave widely different relaxation times. This difference may arise from a specific orientation of the emission dipole in the enzyme. In active-site-labelled pepsin (1-dimethylamino-naphthalene-5-sulphonylphenylalanine–pepsin) this orientation would be approximately parallel to the symmetry axis of the equivalent ellipsoid, whereas in the other conjugate (1-dimethylaminonaphthalene-5-sulphonyl-pepsin) the orientation may be roughly normal to this direction, or some independent rotation of parts of the protein molecule is possible.

The apparent relaxation time ρ_h measured by the method of fluorescence depolarization (Weber, 1953*a*) for a monodisperse protein of known molecular weight can be compared with ρ_0, the value calculated for the equivalent anhydrous sphere. In those examples where $\rho_h > \rho_0$, hydration and shape factors have been invoked to reconcile the two values (Weber, 1953*b*; Steiner & McAlister, 1957*a*,*b*; Green, 1964; Brewer & Weber, 1966), and independent rotation of those parts of the macromolecule that contain the fluorescent group has been suggested in those cases where $\rho_0 > \rho_h$ (Edelhoch & Steiner, 1966; Johnson & Richards, 1962; Johnson & Mihalyi, 1965). The relaxation times of the rotating subunit and of the whole macromolecule can be measured independently (Wahl, 1969). Thermal activation of such independent rotations may be revealed by depolarization measurements made at constant temperature by the addition of a viscous solute (Wahl & Weber, 1967; Green, 1964; Frattali, Steiner & Edelhoch, 1965). In non-spherical macromolecules the contributions of the principal relaxation times to the experimental value depends on the number and orientation of the absorption and emission dipoles in relation to the macromolecular axes. When several similar fluorescent groups are present random orientation may be assumed with some confidence. On the other hand, many examples of specific reactions are known where preferred orientation of a single fluorescent group is highly probable (Hartley & Massey, 1956; White, 1964; Winkler, 1965). Affinity-labelling of enzyme active sites would certainly lead to one unique position for the fluorochrome (Haugland & Stryer, 1967). In addition to these factors, the experimental determination of the relaxation time may be complicated by a dependence on temperature or viscosity of the fluorescence lifetime or the limiting polarization, parameters that have been generally considered constant. When changes in quantum yield occur, the possibility that the lifetime also changes must be considered and corrections applied to the polarization data. Where the absorption-polarization spectrum is complex, the observed polarization depends strongly on the excitation wavelength, and very small absorption shifts may considerably alter the limiting polarization. In the present work fluorescence-polarization measurements were made for the intrinsic tryptophan

groups of pepsinogen and pepsin, pepsin with a single DNS residue linked to the *N*-terminus and pepsin affinity-labelled at the single active site with a DNS-phenylalanine diazoketone reagent. The observed relaxation times are interpreted in terms of the orientation of the emission dipoles in the macromolecules.

THEORY

Effects of preferred orientation on polarization. In terms of emission anisotropy (Jablonski, 1960) the depolarization equation of Perrin (1929) can be expressed as:

$$\frac{A_0}{A} = 1 + \frac{3\tau}{\rho} \qquad (1)$$

where A and A_0 are the anisotropies observed in the presence and absence of rotations respectively, τ is the mean actual lifetime and ρ the relaxation time. For a spherical macromolecule:

$$\rho = \frac{3V\eta}{RT} \qquad (2)$$

where R is the gas constant, V the molar volume, T the absolute temperature and η the viscosity of the medium.

Eqn. (1) as applied by Weber (1953*b*) to protein conjugates implies that the macromolecule is spherical, or if non-spherical has randomly oriented equivalent emission dipoles, so that ρ is the harmonic mean of the principal relaxation times. The general equation for dipoles having preferred orientation with respect to the axes $X = Y$, Z, of an ellipsoid of revolution has also been given by Perrin (1934, 1936) and more recently by Memming (1961). If the absorption and emission oscillators are coincident, and placed at angle α to Z the symmetry axis [this approximation is justified when the limiting anisotropy approaches the technical limiting value at the absorption edge (0.36); in the fluorescent conjugates reported here, the limiting anisotropy was 0.3, so that the angle β between the absorption and emission dipoles, given by $\cos\beta = \sqrt{[(5A+1)/3]}$, was about 20°; as shown in Fig. 5(*a*) of Weber & Anderson (1969) the decrease in relative anisotropy has a maximum value of 20% for $\beta = 20°$, $\alpha = 45°$ and axial ratio 4, and decreases with α; thus a finite value for β produces an overestimate of the angle α], the observed fractional anisotropy is:

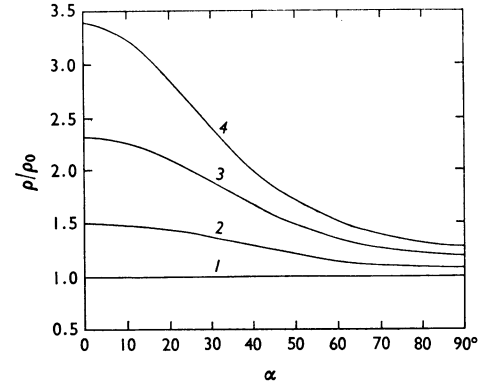

Fig. 1. Variation of the ratio ρ/ρ_0 for prolate ellipsoids of revolution of axial ratio 2, 3 and 4 (curves *2*, *3* and *4*) with the angle α between the absorption-emission dipole and the symmetry axis. ρ is the observed rotational relaxation time, and ρ_0 is the relaxation time for an equivalent sphere (curve *1*).

$$\frac{A}{A_0} = \frac{(2-3\sin^2\alpha)^2}{4+24D_X\tau} + \frac{3\sin^4\alpha}{4+16D_Z\tau+8D_X\tau} + \frac{12\sin^2\alpha(1-\sin^2\alpha)}{4+4D_Z\tau+20D_X\tau} \qquad (3)$$

D_X and D_Z are the rotational diffusion coefficients, so that:

$$D_X = D_Y = \frac{RT\phi_X}{8V\eta} \quad \text{and} \quad D_Z = \frac{RT\phi_Z}{8V\eta}$$

where ϕ_X and ϕ_Z are coefficients determined by the axial ratio X/Z of the ellipsoid (Perrin, 1934). Substituting for D_X and D_Z in eqn. (3) gives:

For spherical macromolecules $D_X = D_Y = D_Z$, $\phi_X = \phi_Y = \phi_Z = 4/3$, $\sin^2\alpha = 2/3$, so that eqn. (4) reduces to the simple form given in eqn. (1), putting $\rho = 3V\eta/RT$. Two of the terms in eqn. (4) disappear for $\alpha = 0$, when:

$$\frac{A_0}{A} = 1 + \frac{3RT\tau}{V\eta}\phi_X \qquad (5)$$

For other orientations in non-spherical cases the plot of A_0/A versus $T\tau/\eta$ will be non-linear, but the departure from non-linearity will be very small for axial ratios less than 5. The variation with α of the average relaxation time, calculated from eqn. (4) as the initial slope of the plot of $1/A$ versus $T\tau/\eta$, for axial ratios 1, 2, 3 and 4, is shown in Fig. 1. As would be expected the ratio between the maximum ($\alpha = 0$) and minimum ($\alpha = \pi/2$) values of ρ increases markedly with axial ratio.

MATERIALS

Porcine pepsinogen and pepsin were twice- or thrice-crystallized material from several sources (Sigma Chemical Co., St Louis, Mo., U.S.A., Mann Laboratories Inc., New York, N.Y., U.S.A., and Worthington Biochemical Corp., Freehold, N.J., U.S.A.) and were used without further purification. Glycerol, L-phenylalanine, DNS chloride and KI were AnalaR-grade materials from British Drug Houses Ltd., Poole, Dorset, U.K. Other reagents were general purpose reagent grade.

Preparation of DNS-pepsin. Pepsin (70mg) was dissolved in 0.1M-sodium phosphate buffer, pH7 (9ml), and

$$\frac{A}{A_0} = \frac{(2-3\sin^2\alpha)^2}{4+\frac{3RT\tau}{V\eta}\phi_X} + \frac{3\sin^4\alpha}{4+\frac{RT\tau}{V\eta}(2\phi_Z+\phi_X)} + \frac{12\sin^2\alpha(1-\sin^2\alpha)}{4+\frac{RT\tau}{2V\eta}(\phi_Z+5\phi_X)} \qquad (4)$$

cooled to 5°C. DNS chloride (6mg) in 1ml of acetone was added with stirring. After 24h at 5°C the solution was filtered and dialysed against buffer for 6h at 5°C. To remove free DNS acid, the solution was then passed down a column (1cm×4cm) of Dowex 2 equilibrated at pH7. After centrifugation at 100000*g* for 15min the conjugate was ready for use.

Preparation of active-site-labelled pepsin. By following the general procedure of Delpierre & Fruton (1966), L-phenylalanine was made to react with DNS chloride and the carboxyl moiety then converted into a diazoketone group ($CO \cdot CHN_2$) as follows. L-Phenylalanine (0.5g), DNS chloride (0.5g), water (40ml), acetone (30ml) and solid $NaHCO_3$ (1g) were shaken together at 20°C for 3h. After decantation, the solution was acidified with 2M-HCl to pH2 and extracted with chloroform (2×30ml). The organic phase was evaporated *in vacuo* and the residue dissolved in acetone (15ml). Water was added until a cloudiness appeared, after which the suspension was kept at 4°C to allow crystallization of DNS-L-phenylalanine to take place. The DNS-L-phenylalanine (0.1g) in diethyl ether (3ml) was cooled to 0°C. Thionyl chloride (0.2ml) was added and immediately a white precipitate formed. The mixture was shaken for 10min at 20°C and the acid chloride isolated by filtration, followed by a wash with cold diethyl ether and drying *in vacuo*. The acid chloride (0.1g) was dissolved in ethereal diazomethane (10ml, approx. 0.4M) at 0°C and left overnight at 20°C. The solid DNS-L-phenylalanine diazoketone derivative was isolated by removal of diethyl ether and excess of diazomethane *in vacuo*.

Pepsin (7mg) was dissolved in 0.01M-sodium acetate buffer, pH5.5 (8ml). To this was added 0.01M-$CuCl_2$ (1ml) and the solution cooled to 4°C. A saturated solution of the diazoketone in acetone (1ml) was added dropwise, with stirring. The mixture was kept for 24h at 4°C and then the precipitated excess of the diazoketone removed by centrifugation at 100000*g* for 30min. The conjugate was further purified by use of a column (10cm×1cm; void volume approx. 5ml) of Sephadex G-25 equilibrated with 0.01M-sodium acetate buffer, pH5.5. The conjugate emerged in the 2ml after the void volume. More-concentrated solutions were prepared by freeze-drying salt-free solutions of conjugate.

METHODS

Absorption spectra were obtained with a Cary 14 recording spectrophotometer. Fluorescence-excitation and fluorescence-emission spectra were determined with a double monochromator instrument of conventional design, employing Bausch and Lomb 500mm 1200 lines/mm gratings. Fully corrected spectra were obtained as described by Teale & Weber (1957). Quantum yields were determined by using a glycogen standard (Weber & Teale, 1957). Fluorescence lifetimes were measured with a 20MHz phase and modulation fluorimeter, and also by a nanosecond flash lamp and sampling oscilloscope (Mackey, Pollack & Witte, 1965; Birks & Munro, 1967). Emission anisotropies were determined with a direct-reading modification of the instrument of Weber (1956), which was also capable of continuously recording values of emission anisotropy or fluorescence polarization. For pepsin and pepsinogen the emission filters were Chance OX1+Schott WG3, whereas for DNS fluorescence Ilford 406 filters were employed. Viscosities of glycerol–water mixtures relative to water were measured in an Ostwald viscometer.

RESULTS

By observing the intrinsic tryptophan emission ($\lambda_{max.}$ 342nm), the rotational-relaxation times of native pepsin and pepsinogen given in Table 1 were determined in 0.1M-acetate buffer, pH5.5, in three ways.

(*a*) By varying the solution temperature between 0 and 40°C. The mean lifetime was an inverse function of temperature (Fig. 3), and linear plots were obtained by plotting $1/A$ versus $T\tau/\eta$. Lifetime of fluorescence was independent of the excitation wavelength in the range 270–305nm. Compatibility of the values obtained by phase shift and by demodulation of fluorescence, and also direct inspection of the decay curves, revealed little heterogeneity in this parameter.

(*b*) At different constant temperatures by the

Table 1. *Relaxation times of pepsin and pepsinogen at 20°C in aqueous buffer*, pH5.5

In 0.1M-acetate buffer, pH5.5, at 20°C both proteins had similar fluorescence lifetimes (τ 4.6ns) and absolute quantum yield (q 0.395).

Excitation wavelength (nm)	Relaxation time (ns)					
	Pepsin			Pepsinogen		
	(*a*) 0–40°C	(*b*) 0–50% glycerol	(*c*) 0–0.5M-KI	(*a*) 0–40°C	(*b*) 0–50% glycerol	(*c*) 0–0.5M-KI
270	46±4	46±3	22±2	67±4	55±4	30±2
280	67±4	43±4	20±1	67±4	53±4	27±2
290	27±3	44±3	25±1	28±3	51±4	35±3
295	21±2	46±3	37±2	24±2	54±4	41±3
300	20±2	43±2	44±2	23±2	50±5	47±5
305	22±2	44±2	44±2	24±2	48±3	46±5

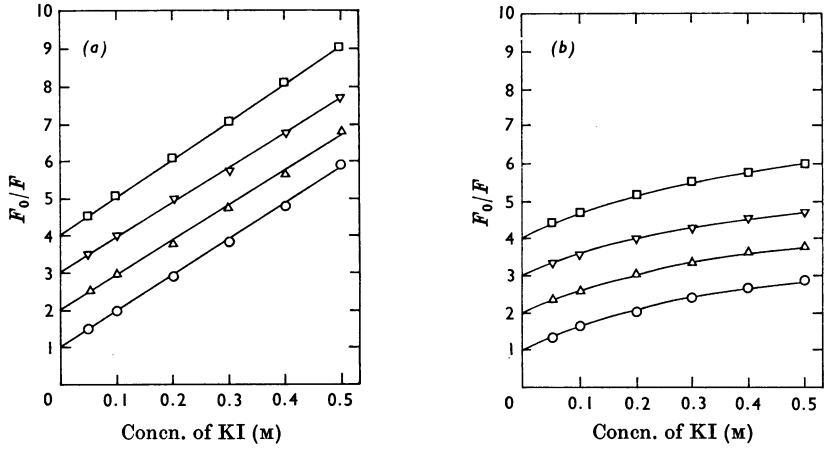

Fig. 2. Stern–Volmer plots for the quenching of the tryptophan emission of (*a*) pepsin and (*b*) pepsinogen by aqueous KI at different excitation wavelengths: ○, 270nm; △, 280nm; ▽, 290nm; □, 300nm. To separate the plots for the three longer wavelengths the ordinate scale has been displaced downwards 1 unit successively. The ordinate shows the inverse fractional fluorescence intensity, F_0/F.

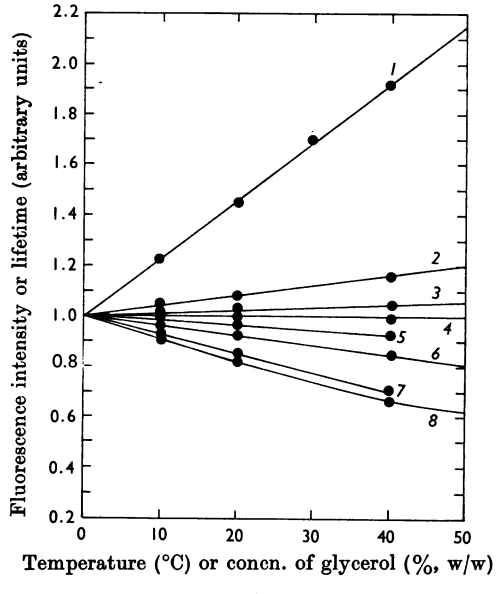

Fig. 3. Relative changes in fluorescence intensity F or lifetime τ produced by change in temperature or glycerol concentration for tryptophan emission of pepsinogen and pepsin, and for visible fluorescence in DNS-pepsin and DNS-L-phenylalanine–pepsin. Curve *1*, DNS-pepsin, F with glycerol; curve *2*, DNS-L-phenylalanine–pepsin, τ and F with glycerol; curve *3*, DNS-pepsin, τ with glycerol; curve *4*, τ and F of pepsin and pepsinogen with glycerol; curve *5*, DNS-L-phenylalanine–pepsin, τ and F with temperature; curve *6*, DNS-pepsin, τ with temperature; curve *7*, DNS-pepsin, F with temperature; curve *8*, τ and F of pepsin and pepsinogen with temperature.

addition of glycerol up to 50% (w/w). No changes in emission spectrum, quantum yield or fluorescence lifetime were produced by glycerol (Fig. 3), but a selective absorption increase characteristic of tyrosine was observed. Linear plots of $1/A$ versus $1/\eta$ were obtained by this method.

(*c*) At constant temperature and viscosity by the addition of potassium iodide, an efficient quencher of the tryptophan fluorescence. Stern–Volmer plots (Stern & Volmer, 1919) for potassium iodide quenching at several different excitation wavelengths within the absorption bands of the enzymes and the zymogen are shown in Fig. 2. In both cases the rates of decrease in quantum yield and mean lifetime were almost identical, but pepsinogen showed, in contrast with pepsin, a residual fluorescence (approx. 30%) apparently arising from a tryptophan moiety inaccessible to the iodide ions. The quenching efficiency was unchanged by high concentrations of potassium chloride, so that specific charge interactions could be discounted. At maximum quenching the residual fluorescence band in both proteins showed a small shift (2–3nm) towards shorter wavelengths, revealing some heterogeneity in emission. Linear plots of $1/A$ versus τ were obtained.

In Fig. 3 the relative changes in fluorescence intensity, F, or lifetime, τ, caused by changes in temperature or glycerol concentration for the tryptophan emission of pepsinogen and pepsin and for the visible fluorescence of DNS-pepsin and DNS-L-phenylalanine–pepsin are shown. The changes in τ were used to correct the polarization

data for the effects of temperature and glycerol concentration.

By using methods (*a*), (*b*) and (*c*), measurement of emission anisotropies were made at wavelengths 270, 280, 290, 295, 300 and 305nm. The relaxation times at 20°C calculated from the experimental results are listed in Table 1, together with the mean lifetimes and absolute quantum yields. Polarization spectra of 10μM-tryptophan and pepsin in aqueous glycerol solutions, and of pepsin in aqueous potassium iodide solutions, were continuously recorded between 260 and 305nm. Graphical extrapolations to zero lifetime or infinite viscosity were made, by using the measured lifetime and viscosity values, at excitation wavelengths chosen to resolve details in the spectra shown in Fig. 4.

Absorption spectrophotometry showed that the conditions used to make pepsin react with DNS chloride gave a conjugate containing a maximum of one fluorochrome moiety, probably linked to the *N*-terminal isoleucyl residue (F. W. J. Teale, unpublished work). Since pepsin is rapidly inactivated at neutral pH (Edelhoch, 1957), the conjugate showed little (approx. 3%) proteolytic action. Similar 1:1 stoicheiometry was obtained in the affinity-labelling of the enzyme with the substrate analogue diazoketone, where the addition of 1mol/mol of pepsin completely inactivated the enzyme. Both the DNS-pepsin conjugates had fluorescence maxima at 517nm, though with different fluorescence lifetimes and quantum yields, shown in Table 2. The conjugates were shown to be monomeric by ultracentrifugation and by gel filtration on Sephadex G-75. The sedimentation coefficients and the elution volumes were similar to the values given by native monomeric pepsin. The relaxation times of the DNS conjugates were measured by methods (*a*) and (*b*). The fluorescence lifetimes were dependent on the glycerol concentration, and decreased markedly with temperature increase (Fig. 3). Linear plots of $1/A$ versus T/η at 0, 20 and 35°C were obtained, but in plotting $1/A$ versus $T\tau/\eta$ for method (*a*) the DNS-pepsin conjugate showed at low ionic strength (0.01M buffer) an abrupt decrease in relaxation time at about 18°C. The relaxation times calculated at 20°C for the two DNS conjugates in aqueous buffer are listed in Table 2.

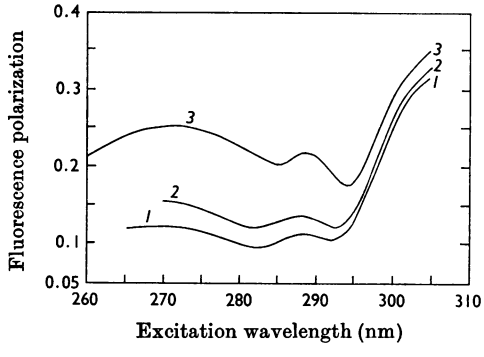

Fig. 4 Polarization spectra of: curve *1*, pepsin, polarization extrapolated to infinite viscosity; curve *2*, pepsin, polarization extrapolated to zero lifetime; curve *3*, tryptophan in glycerol at 0°C.

DISCUSSION

The position of the fluorescence maximum in both pepsin and pepsinogen (342nm) is characteristic of tryptophan in a relatively polar environment (Teale, 1960), so that the emitting residues are mainly of the 'exposed' type. Energy transfer to tryptophan from tyrosine is known to be negligible (Badley & Teale, 1969), and the average quantum yield has been calculated as 0.395 in both proteins. This value is compatible with the observed mean lifetime (4.6ns) in both native proteins, and the processes responsible for the differences in yield between free and protein-bound tryptophan must operate during the lifetime of the singlet excited state. Possible mechanisms could be collisional quenching, charge transfer, resonance-energy transfer or exciplex formation (Longworth, 1968). The five or six tryptophan residues shown to be present by spectrophotometric assay (Arnon & Perlmann, 1963; Edelhoch, 1967) must moreover have comparable yields and lifetimes, if they emit independently, but the possibility of energy transfer from residues differing widely in quantum yield from the ultimate emitter cannot be overlooked. The rotational-relaxation times measured

Table 2. *Relaxation times at* 20°C *of DNS-pepsin and DNS-*L*-phenylalanine–pepsin*

Conjugate	Relaxation time (ns) (*a*) 0–40°C	(*b*) 0–50% glycerol	Absolute q (20°C)	Lifetime τ (ns)
DNS-pepsin	29 ± 3*	37 ± 3	0.057	7.9
DNS-L-phenylalanine–pepsin	52 ± 4*	58 ± 5	0.09	11.0

* In 0.1M-acetate buffer, pH5.5.

at constant temperature by the addition of glycerol (Table 1, columns *b*) varied little with excitation wavelength. The molecular volumes, calculated from ρ values measured at different temperatures, were equal, suggesting that a decrease in rotational diffusion alone was produced by the increase in viscosity. The rotational relaxation times at 20°C, calculated (eqn. 2) for the anhydrous proteins approximated to spheres, are 31ns for pepsin (mol.wt. 34163; Rajagopalan, Moore & Stein, 1966) and 36ns for pepsinogen (mol.wt. 39000; Rajagopalan *et al.* 1966), assuming a value of 0.73 for the partial specific volume. In solution, the calculated ρ values depend critically on the degree of hydration, and on setting this quantity in both cases to 20% (Edelhoch, 1957) the values of ρ are 39 and 44ns respectively, so that the observed average values of 44 and 52ns are larger by a factor 1.14–1.18. If, however, prolate ellipsoids of axial ratio 2 (Vazina & Lemazhikhin, 1966; Vazina, Lednev & Lemazhikhin, 1966) are taken as plausible approximations to the actual macromolecular domains, then the observed and calculated ρ values can be reconciled. On the curve for this axial ratio in Fig. 1, $\rho/\rho_0 = 1.16$ for α in the range 55–60°. This angular range may represent the actual orientation of the emission dipoles, but is more likely to be a consequence of approximately random orientation of several similar emitters with respect to the symmetry axis of the ellipsoid. The observed relaxation time is then the harmonic mean of the principal relaxation times (Perrin, 1934; Weber, 1953*b*), so that $\rho/\rho_0 \simeq 1.18$ since $\sin^2\alpha = 2/3$; $\alpha \simeq 55°$.

Interesting differences are revealed by applying method (*c*), fluorescence quenching by iodide ions (Table 1, columns *c*). The linear Stern–Volmer plots (Fig. 2) for pepsin have equal slopes for different excitation wavelengths, showing that the emitting tryptophan groups are accessible to iodide and that tyrosine sensitization, if present, is unaffected by quenching. The absence of a temperature coefficient for the iodide quenching suggests that two opposing viscosity-dependent processes are producing compensation. The quenching process appears to be entirely collisional, probably through electron donation to the excited indole nucleus. The curved Stern–Volmer plots for pepsinogen, in contrast, show that a tryptophan moiety apparently inaccessible to iodide is present. This might result by the screening of one or two tryptophan residues by the polypeptide fragment (which contains no tryptophan) that is lost during activation of the zymogen. The relaxation times measured with excitation wavelengths absorbed by the edge of the absorption band where high values of anisotropy are found corresponded well to those obtained by method (*b*). At shorter wavelengths, however, the measured relaxation times decreased sharply. This difference in behaviour between the effects of viscosity and lifetime suggests that a time-dependent process, such as resonance-energy transfer, partly determines the polarization values at shorter wavelengths, and is suppressed by the progressive decrease in lifetime produced by iodide quenching. The slope of the reciprocal anisotropy plot, and hence the apparent relaxation time, would then be the sum of two components, the lifetime-dependent transfer depolarization and the rotational depolarization. Since the lifetime of pepsin is not altered by glycerol, only the rotational factor is measured by changing the viscosity of the medium. In effect the limiting anisotropy A_0 is a function of τ at the shorter wavelengths. The apparent absence of the transfer depolarization at longer wavelengths may indicate heterogeneity of absorption among the tryptophan groups. One residue with absorption edge displaced to longer wavelengths than the average value would be selectively excited at the edge of the band. Such a residue would function as an energy-transfer acceptor rather than a donor, so that only the rotational effects would be revealed by excitation at the absorption wavelength limit. Support for the idea of a small degree of heterogeneity among the tryptophan residues is given by the small blue shift of the emission maximum at high iodide concentrations, suggesting that the least accessible groups are, perhaps not surprisingly, situated in less polar environments than the average.

The depolarization produced by temperature (Table 1, columns *a*) gives anomalous values of relaxation time. In contrast with the effects of iodide quenching, low values are obtained for excitation at the absorption edges of the two proteins, and large values at shorter wavelengths. A detailed investigation shows that these effects are produced by thermally induced changes in the polarization spectrum. With temperature increase the limiting anisotropy at longer wavelengths increases sharply, whereas at shorter wavelengths the reverse occurs. At the same time changes in absorption and excitation spectra occur and it seems that the environments of the tryptophan residues, and hence the absorption spectra, are temperature-sensitive. Because of the complex polarization spectrum of tryptophan (Weber, 1960), A_0 is very sensitive to slight spectral shifts, and the change in A_0 apparently masks the other two depolarizing processes that must also be present.

The effects of transfer probably explain the extrapolated polarization spectra shown in Fig. 4. Tryptophan in glycerol (curve *3*) and pepsin extrapolated to zero lifetime (curve *2*) and infinite viscosity (curve *1*) have similar maximum values at the absorption limit ($\lambda > 295$nm), but at shorter

wavelengths the protein values are considerably smaller than that of free tryptophan. Moreover, curve *1* obtained at constant fluorescence lifetime shows lower values than curve *2* obtained by iodide quenching. It is tempting to ascribe the latter difference to energy transfer from tryptophan residues having absorption bands displaced to shorter wavelengths than the emitting species. Alternatively, a small amount of tyrosine sensitization could produce similar effects. Whether the large difference between curves *2* and *3* arises in the same way is debatable. Although Konev, Bobrovitch & Chernitskii (1965) have produced evidence for inter-tryptophan transfer, Weber (1960) has earlier suggested that differences in the overlap of the electronic transitions of the indole nucleus after incorporation into the protein structure are responsible. The number of transfers between randomly oriented residues necessary to produce the observed depolarization in pepsin can be calculated from the equation of Weber (1966) as being in the range 0.5–1.0. Such inter-tryptophan transfer would also increase the efficiency of energy migration to chromophoric acceptors attached to the protein surface (Badley & Teale, 1969). In the two DNS conjugates, the fluorescent moieties differ in environment, probably as a result of different modes of attachment to the macromolecule. Thus, although the emission spectra are very similar, the two conjugates differ in quantum yield and lifetime (Table 2) and response to glycerol concentration (Fig. 3). DNS-pepsin shows a relatively large intensity increase with viscosity with little increase in average lifetime, whereas the active-site-labelled DNS-L-phenylalanine–pepsin showed smaller but parallel increases of both intensity and lifetime. Apparently the environment of the DNS group in DNS-pepsin is more accessible to glycerol, perhaps as a consequence of the structural modification that inactivates the enzyme in neutral solution. Compared with DNS acid in bicarbonate solution (q 0.36, τ 13.8ns; Chen, 1966; Chen, Vurek & Alexander, 1967), the conjugates had low quantum yields relative to the observed lifetimes, suggesting complex-formation or heterogeneity of the emitting types. In both conjugates the fluorescent lifetimes and intensities decreased with temperature increase, these effects being more marked in DNS-pepsin. On taking these lifetime changes into account, the relaxation times calculated from the emission-anisotropy results differed widely between the conjugates, and in each case the value given by temperature change was smaller than that given by glycerol addition at constant temperature. Since the DNS-L-phenylalanine–pepsin could be shown to be monomeric, the high values (52 and 58ns; Table 2) obtained must originate from a specific orientation of the DNS group, since large increases in hydration or axial ratio are unlikely to accompany the coupling procedure. Taking ρ_0 to be 39ns, $\rho/\rho_0 = 55/39 = 1.61$, and, on referring to Fig. 1 for a 2:1 prolate ellipsoid, α is in the range 20–30°. If this interpretation is correct, then a specific location is indicated of the naphthalene rings near to the enzyme active site, which has been shown to bind hydrophobic groups preferentially (Schlamowitz, Shaw & Jackson, 1968). In contrast, the DNS-pepsin gives much shorter relaxation times (29–37ns; Table 2), comparable with or shorter than ρ_0 for the hydrated sphere (37ns). In glycerol, the molecular volume is the same at 3°, 19° and 35°C, so that the same rotating unit is preserved throughout this temperature range. In aqueous 0.1M buffer, and in 0.01M buffer below 20°C, a rather lower ρ value was found. In the more dilute buffer, above 20°C the relaxation time decreased abruptly to about 7ns. Thermal activation of increased freedom of independent rotation in the fluorescent unit is probable in this case, and to a smaller degree in DNS-pepsin in 0.1M buffer or in glycerol. An alternative but less probable explanation of the low ρ of DNS-pepsin is again specific orientation, normal to the symmetry axis. However, on taking the structural lability and fluorescence intensity changes into account, the former mechanism is probably correct. The relaxation times of the DNS conjugates can be compared with those reported by Frattali *et al.* (1965) for DNS-pepsinogen. Values of 70–80ns were calculated assuming τ to be 12ns, comparable with those for DNS-L-phenylalanine–pepsin. On the other hand, if the actual fluorescence lifetimes of DNS-pepsinogen and DNS-pepsin (7.9ns) are similar, as is suggested by the low quantum yield reported for the zymogen (Frattali *et al.* 1965; Figs. 3 and 4), then lower ρ values of 46–53ns are obtained, more comparable with those given by DNS-pepsin, and in good agreement with the values for native pepsinogen given in Table 1.

Taken together, these observations show that, whenever a specific orientation of a fluorescent group in an elongated molecule is possible, the observed relaxation time must be interpreted with caution. Only when random orientation may be inferred, or several equivalent groups are present, can the molecular volume be calculated. On the other hand, if the molecular domain is well characterized, then additional structural information may be furnished by relaxation-time measurements.

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