propylene glycol solution at -70° , excited with unpolarized light.

2. Phenol, cresol and tyrosine show a simple spectrum, consisting of a negative polarization region with a minimum value of -0.065 at $235\text{ m}\mu$, and a positive region with a maximum of 0.22 at $275\text{ m}\mu$ and longer wavelengths.

3. Indole, *N*-methylinole and tryptophan show no negative polarization at any wavelengths. In the region of the longest wavelength-absorption band two maxima are present at 270 and $305\text{ m}\mu$ and a minimum at $295\text{ m}\mu$. It is shown that at least two electronic transitions are required to describe these effects, one of these transitions corresponding to the spike or shoulder observed in absorption at $295\text{ m}\mu$.

4. *N*-Glycyltryptophan has a spectrum similar to the other indole derivatives but for the existence of an additional sharp polarization band with maximum at $290\text{ m}\mu$, which appears connected with the presence of a peptide bond.

5. Concentration-depolarization measurements show that the distance R at which the probability of transfer of the excitation equals the probability of emission is $17\text{ \AA}$ for phenol molecules.

6. Concentration-depolarization measurements on indole solutions show that $R = 17\text{ \AA}$ if the fluorescence is excited with light of wavelength shorter than $295\text{ m}\mu$, but that excitation with longer wavelengths is not followed by transfer with very much lower efficiency.

7. Measurements of the fluorescence spectrum in

mixtures of phenol (1M) and indole (0.01 – 0.2M) show that phenol can transfer its energy to indole with $R\ 18.5\text{ \AA}$.

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Fluorescence-Polarization Spectrum and Electronic-Energy Transfer in Proteins

By G. WEBER

Department of Biochemistry, The University, Western Bank, Sheffield 10

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The analysis of the polarization spectrum of the fluorescence of proteins is necessarily more difficult than that of the aromatic amino acids and simple derivatives described in the previous paper (Weber, 1960). The main differences arise because of the various types of Brownian molecular rotations which are possible in the protein, and also the possibility of energy transfer among the aromatic residues. The characteristic distances of transfer R for phenol-phenol, indole-indole and phenol-indole transfer calculated from concentration-depolarization and fluorescence-spectral data (Weber, 1960)

are of the order of the distance between these residues in the protein molecule. Finally, the possibility that interaction of the indole nucleus with the highly polarizable backbone of the protein may lead to changes in the relative positions of the overlapping transitions in tryptophan must be kept in mind.

EXPERIMENTAL

This paper describes the fluorescence-polarization spectrum of two classes of globular proteins.

Class A proteins containing phenylalanine and tyrosine but no tryptophan. These proteins (Teale & Weber, 1959;

F. W. J. Teale, in preparation) have a fluorescence spectrum characteristic of tyrosine with a maximum emission at 303 m μ . The quantum yield of the fluorescence is 0.03–0.05, that is one-fifth to one-seventh of that of free tyrosine (Teale & Weber, 1957).

Class B proteins, containing tryptophan as well as tyrosine and phenylalanine. These proteins show only tryptophan fluorescence with wide variations in quantum yield (0.04–0.3) and wavelength of maximum emission (320–350 m μ) (F. W. J. Teale, in preparation). No appreciable tyrosine fluorescence can be discerned in the fluorescence spectrum.

Materials. Bovine-serum albumin, bovine γ -globulin, bovine fibrinogen and lysozyme were Armour preparations. Ribonuclease, chymotrypsin, chymotrypsinogen and rabbit-muscle lactic dehydrogenase were Worthington Biochemical Corp. (N.J., U.S.A.) preparations. Ovalbumin was three-times crystallized, as described by Warner (1954). Insulin was a specimen from British Drug Houses Ltd. Human-serum albumin was a gift from Dr J. L. Oncley.

Methods. Polarizations were measured as described in the preceding paper. With protein solutions, scattering of parasitic light can falsify the results. This parasitic light is the light of wavelength longer than the cut-off of the filter used to separate the exciting from the fluorescent light. A first-order correction for the scattered parasitic light was determined by introducing into the path of the exciting beam a filter similar to that used to separate excitation from fluorescence. The light passing through both filters is to a first approximation the parasitic light that would be scattered in the absence of the excitation filter. With both filters in position the components I'_1 and $I'_\perp$ of the scattered parasitic light were measured independently. They were subtracted respectively from I_1 and $I_\perp$, the components of the fluorescence light as measured with the excitation filter removed. The polarization was calculated from the corrected components in the ordinary way Weber (1960). The intensity of parasitic light in our monochromator was practically independent of the exciting wavelength and depended exclusively on the slit width. This had to be increased as shorter wavelengths were used, particularly in the region 230–250 m μ , because of the falling output of the xenon arc in this region. The correction described was applied when $I'_1 \leq 0.2I_1$. For larger values of the scattered light the correction is not considered reliable, and this condition has determined the short-wave limit of the polarization spectra here described.

RESULTS

Class A proteins

Insulin, ribonuclease and zein were studied in 90–95% propylene glycol–water mixtures at -70° . The spectra, shown in Fig. 1, are qualitatively similar to those of phenol, cresol or tyrosine, but the absolute values of the polarization reached in both the negative and positive regions of the spectrum are smaller than in the simpler derivatives. It does not seem that these differences can be attributed to any influence other than the energy transfer among the tyrosine residues, since practi-

cally all rotational depolarization must be absent under the conditions described. The probability of transfer will be determined by the average distance between the tyrosine residues. In the absence of more detailed information, assuming for simplicity that these tyrosines are spaced in the same way as in a solution, the effective molar concentration c_e in the proteins equals $10^3 (n/V)$ where n is the number of tyrosine residues per molecule and V the anhydrous molar volume of the protein. The probability of transfer will be also determined by the characteristic distance R for inter-tyrosine transfer already computed (Weber, 1960) and the lifetime τ of the tyrosine fluorescence in the protein, as compared with the lifetime τ_0 of the tyrosine in the medium in which R has been determined. From the theory of concentration depolarization already developed (Weber, 1954),

$$\frac{1}{p} + \frac{1}{3} = \left(\frac{1}{p_\infty} + \frac{1}{3} \right) (1 + A c \tau_0), \quad (1)$$

where p is the polarization observed at concentration c , τ_0 the lifetime of the excited state and A a constant proportional to R^6 . p_∞ is the polarization in dilute solution in which no appreciable intermolecular transfer of the excitation takes place. If the assumption is made that the processes that decrease the quantum yield of the fluorescence are competitive with the emission, then $\tau/\tau_0 = q/q_0$, where q_0 and q are respectively the yields of the tyrosine fluorescence when free and in the protein. From a study of the concentration depolarization of phenol solutions (Weber, 1960) $A\tau_0 \approx 20$, and

Fig. 1. Fluorescence-polarization spectra of insulin, zein and ribonuclease in propylene glycol at -70° . The spectrum of tyrosine is included for comparison. ●, Tyrosine; ▲, zein; ×, insulin; ○, ribonuclease.

therefore the polarization p expected in the protein is given by

$$\frac{1}{p} + \frac{1}{3} = \left(\frac{1}{p_\infty} + \frac{1}{3} \right) \left(1 + 20 \frac{q}{q_0} c_e \right). \tag{2}$$

p_∞ is the polarization of the fluorescence of tyrosine in a medium of infinite viscosity, which for excitation with 275 m μ wavelength equals 0.22. As a first approximation $\frac{1}{3}$ can be neglected by comparison with $1/p$ and, replacing c_e by its value $10^3 (n/V)$,

$$p \simeq \frac{p_\infty}{1 + \frac{2q}{q_0} \frac{n}{V} 10^4}, \tag{3}$$

with the values $2q/q_0 \simeq \frac{1}{2}$ and $(n/V) \simeq 0.5$ m-mole/ml.
 $p \simeq 0.07$.

The experimental values in the three proteins studied are all greater than this (0.11–0.14), although still considerably smaller than p_∞ . A significant comparison of the observed with calculated values cannot be made since the quantum yield, orientation and relative position of the several tyrosine residues in the protein are not known.

Class B proteins

Qualitatively the polarization spectrum is that of tryptophan and glycyltryptophan. The spectra are shown in Figs. 2–10. The general features are given in paragraphs (1) to (6).

(1) The main polarization regions of tryptophan and other indole derivatives are present, with

maxima at 270 m μ and 303–308 m μ and a minimum in the intervening region in every case.

(2) An intermediate band with a maximum at 290 m μ , having a shallow minimum on the short-wave side and a sharp minimum at 295 m μ and a band width of less than 10 m μ , similar to a band observed already in *N*-glycyltryptophan, is found in most proteins.

(3) Although the bands are qualitatively similar to those of the simpler indole derivatives there is an important quantitative difference, shown in Table 1. When the ratio of the polarizations observed on excitation by 270 m μ (p_{270}) and 305 m μ

Fig. 2. Polarization spectra of human-serum albumin: ●, in buffer, pH 7.0; ×, in 8M-urea. These symbols are used in the same way in Figs. 3–9.

Fig. 3. Polarization spectra of bovine-serum albumin. See legend to Fig. 2.

Fig. 4. Polarization spectra of pepsin. See legend to Fig. 2.

Table 1. *Class B proteins*

s.d. of the quoted values is $\Delta p \pm 0.003$. 50% PG indicates measurements in 50% (v/v) propylene glycol-water mixture at -70° ; PG indicates measurements in pure propylene glycol at -70° .

Protein	Solvent	P_{270}	P_{305}	$\frac{P_{305}}{P_{270}}$
Human-serum albumin	Buffer, pH 7.0	0.079	0.172	2.18
	8 M-Urea	0.054	0.115	2.1
	50% PG	0.080	0.200	2.5
Bovine-serum albumin	Buffer, pH 7.0	0.084	0.172	2.1
	8 M-Urea	0.084	0.117	1.6
	50% PG	0.080	0.200	2.5
Ovalbumin	Buffer, pH 7.0	0.083	0.140	1.7
	8 M-Urea	0.054	0.080	1.5
Lysozyme	Buffer, pH 7.0	0.052	0.123	2.4
	8 M-Urea	0.070	0.115	1.6
	50% PG	0.070	0.210	3
Bovine γ -globulin	Buffer, pH 7.0	0.075	0.149	2
	8 M-Urea	0.070	0.105	1.55
	50% PG	0.081	0.178	2.2
Bovine fibrinogen	Buffer, pH 7.0	0.062	0.157	2.5
	8 M-Urea	0.054	0.100	1.85
	50% PG	0.045	0.135	3
Chymotrypsinogen	Buffer, pH 7.0	0.057	0.154	2.7
	8 M-Urea	0.064	0.092	1.4
	50% PG	0.048	0.154	3.2
Chymotrypsin	Buffer, pH 7.0	0.052	0.120	2.3
	8 M-Urea	0.042	0.063	1.5
	50% PG	0.054	0.170	2.9
Pepsin	Buffer, pH 7.0	0.063	0.135	2.1
	8 M-Urea	0.064	0.150	2.3
	50% PG	0.085	0.220	2.6
Rabbit-muscle lactic dehydrogenase	Buffer, pH 7.0	0.086	0.200	2.3
	8 M-Urea	0.044	0.074	1.7
Tryptophan	PG	0.145	0.210	1.45
Indole	PG	0.155	0.205	1.33
N-Glycyltryptophan	PG	0.146	0.245	1.68

Fig. 5. Polarization spectra of chymotrypsinogen.
See legend to Fig. 2.

Fig. 6. Polarization spectra of chymotrypsin.
See legend to Fig. 2.

(p_{305}) is recorded it is found that, for the simple molecules, $p_{305}/p_{270} = 1.4-1.7$, whereas for nine out of ten proteins $p_{305}/p_{270} > 2$. In ovalbumin the value is 1.7. This larger ratio in the proteins is due to a comparative decrease in the 270 $m\mu$ region. The values observed at 305 $m\mu$ approach more closely those encountered in the simple derivatives in a rigid medium (Table 1).

(4) A very small increase in polarization was found on dissolving the proteins in 60% sucrose. On the other hand, solutions of the proteins in 50% propylene glycol-water mixtures at -70° showed considerable increases in the ratio p_{305}/p_{270} .

In a series of eight proteins the ratio varied between 2.2 and 3.2 (Table 1 and Figs. 11-13). In all but one case (fibrinogen) the polarization at 305 $m\mu$ was increased, whereas p_{270} showed increases (lysozyme, pepsin), a decrease (fibrinogen) or remained the same (bovine and human albumin, γ -globulin, chymotrypsin).

(5) When the proteins were dissolved in 8M-urea the two bands at 270 and 305 $m\mu$ persisted, but

Fig. 7. Polarization spectra of rabbit-muscle lactic dehydrogenase. See legend to Fig. 2.

Fig. 8. Polarization spectrum of ovalbumin. See legend to Fig. 2.

Fig. 9. Polarization spectrum of bovine fibrinogen. See legend to Fig. 2.

Fig. 10. Polarization spectra of lysozyme: ○, in buffer, pH 7.0; ×, in 8M-urea; ●, in 50% propylene glycol-water at -70° .

they appeared usually much flatter than in the water solutions (Table 1). A decrease in the absolute values of both maxima was generally observed, but the fall in the 305 m μ maximum was much more pronounced, so that in all but one case (pepsin) the ratio p_{305}/p_{270} was much reduced, approaching the values observed in the simple indole derivatives. The effect of urea was rapid.

Fig. 11. Polarization spectra in 50% propylene glycol-water at -70° . ●, Chymotrypsin; ○, chymotrypsinogen.

Fig. 12. Polarization spectra in 50% propylene glycol-water at -70° . ●, Pepsin; ○, fibrinogen.

The polarizations were determined 30 min. after the addition of the urea. After 24 hr. at 4° the values remained the same in four proteins in which the time course was followed.

Fig. 13. Polarization spectra in 50% propylene glycol-water at -70° . ●, Bovine-serum albumin; ○, human-serum albumin.

Fig. 14. Polarization spectra of the fluorescence of proteins in buffer, pH 7.0. Proteins were dissolved in 8M-urea and kept at 4° for 24 hr. before thorough dialysis against phosphate buffer, pH 7.0. ●, Bovine-serum albumin; x, lactic dehydrogenase; ○, chymotrypsinogen.

(6) Three proteins were studied after removal of the urea. The polarization of the fluorescence was determined in the fraction that remained in solution after prolonged dialysis against neutral buffer. The proteins did not recover the polarization spectrum characteristic of the native protein, as can be seen in Fig. 14. The two main bands were less defined and the p_{305}/p_{270} ratio was much smaller than in the native proteins.

DISCUSSION

The depolarization of the fluorescence due to the Brownian rotation of the protein particle can be calculated when the rotational relaxation time of the particle and the actual lifetime of the excited state of the fluorescence τ are known (Weber, 1953). The natural lifetime of the excited state τ_e can be calculated from the integrated absorption of the electronic band of lowest frequency (e.g. Förster, 1951)

$$\frac{1}{\tau_e} \simeq 2.88 \times 10^{-9} \cdot \bar{\nu}^2 \cdot n^2 \int_0^\infty \epsilon(\nu) d\nu.$$

From the Gaussian approximation already discussed (Weber, 1960) and data for phenol and tyrosine already published (Teale & Weber, 1957)

$$\tau_e \simeq 42 \mu\text{m.sec.}$$

if the processes that decrease the quantum yield of tyrosine from unity are competitive with the emission, $\tau \simeq q\tau_e$. On this assumption the actual lifetime of phenol or tyrosine in water is $8 \mu\text{m.sec.}$ and that of insulin about $1.5 \mu\text{m.sec.}$ In the same manner calculation of the natural lifetime of indole gives $\tau_e \simeq 8 \mu\text{m.sec.}$ From the quantum yields observed in the class B proteins (F. W. J. Teale, in preparation) their actual lifetimes appear to be in the range (6) of $1-3 \mu\text{m.sec.}$ Thus the actual lifetime of the excited state of the fluorescence of proteins of either class A or B is too short to reveal changes in the relaxation time of the particles when the viscosity is increased (Weber, 1953). The polarization method may prove useful, however, in the determination of the rotational relaxation time of tyrosine and tryptophan peptides of molecular weight up to a few thousand.

The class A proteins, containing no tryptophan, show a polarization spectrum which can be interpreted unequivocally by comparison with that of tyrosine or phenol. The existence of transfer of the electronic-excitation energy among the tyrosine residues results in the proteins in smaller values of the polarization over the entire wavelength range, as compared with the polarizations observed in phenol or tyrosine solutions. Since the probability of transfer of the excited state is proportional to the

lifetime of the excitation, other things being equal, it follows that transfer of energy from the tyrosines with higher quantum yields, and therefore longer lifetime, to those with smaller quantum yield and shorter lifetime will occur more often than the converse process, and will result in a smaller quantum yield than that expected in the absence of transfer. The process of transfer may then be partly responsible for the low yield of fluorescence observed in these proteins. In the extreme case in which the probability of transfer to a group with negligible quantum yield is very high the fluorescence of the protein may be almost completely quenched, as exemplified by transfer to the non-fluorescent haem in the haem proteins (Weber & Teale, 1959).

In class B proteins the interpretation of the findings is much more uncertain. The polarization spectrum shows considerable variation among the proteins, contrasting with the small changes in the absorption spectrum. The findings in the solutions in propylene glycol-water at -70° , where rotation of the tryptophan residues between excitation and emission appears extremely unlikely, show that some influence is at work in the proteins which reduces considerably the polarization observed on excitation by $270 \text{ m}\mu$ light and has little effect on the polarization of the fluorescence excited by $305 \text{ m}\mu$. It appears tempting to suggest that energy transfer, particularly of the type observed among the indole molecules, with its dependence on the exciting wavelength, is responsible for the effect. Although this may be responsible for part of the observed effect, another possibility must be considered: the relative weights of the $G-S_1$ and $G-S_2$ transitions (Weber, 1960) in the indole nucleus will determine the relative values of the polarizations at 270 and $305 \text{ m}\mu$. In the proteins, extension of the $G-S_2$ transition towards the shorter wavelength could explain the experimental findings. The spectral changes in the $G-S_2$ transition could occur as a result of the interaction of the indole nucleus with the highly polarizable protein backbone. The effect of urea would follow the breaking of hydrogen bonds maintaining the backbone structure. The changed relations with the backbone and the increased freedom of rotation of the tryptophan residues would be sufficient to explain the observed effects. Whatever the relative importance of these two factors, namely relative weight of the $G-S_1$ and $G-S_2$ transitions and electronic-energy transfer, there is little doubt that the fluorescence-polarization spectrum is an individual and characteristic property of the proteins studied, and is sensitive to changes in the secondary structure of the protein, in a characteristic manner. Though the interpretation of the observed effects is not unequivocal, their correlation with other structural properties of the

proteins offers a possible way to the final understanding of them.

SUMMARY

1. The polarization spectrum of insulin, ribonuclease and zein is qualitatively identical with that of tyrosine or cresol, but the lower absolute values of the principal polarization indicate the existence of energy transfer among the tyrosine residues.

2. The polarization spectrum of proteins containing tryptophan is similar to that of *N*-glycyl-tryptophan but differs from it in the lower values of the polarization in the 270 m μ region. The ratio of the polarization on excitation by 305 m μ and by 270 m μ (p_{305}/p_{270} ratio), which varies from 1.4 to 1.7 in the simple indole derivatives, is found to be greater than two in nine out of ten globular proteins studied.

3. The polarization spectrum in 50 % propylene glycol-water at -70° shows an increased p_{305}/p_{270} ratio (2.3–3.0), and the polarization spectrum in 8M-urea shows this ratio decreased to the range 1.4–1.9 in eight out of ten proteins studied.

4. The changes of proteins in urea are only partially reversible. The soluble fractions obtained in three proteins had a distinctly changed polarization spectrum with a lowered p_{305}/p_{270} ratio.

5. Though energy transfer among the tryptophan residues may play a part in the observed effects, it is believed that a change in the relative intensities of the $G-S_1$ and $G-S_2$ transitions in tryptophan can explain these effects equally well.

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Amino Acid-Activating Systems from Pig Liver

BY PRISCILLA HELE AND L. R. FINCH*†

M.R.C. Experimental Radiopathology Research Unit, Hammersmith Hospital, London

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A reaction between amino acids and adenosine triphosphate catalysed by specific enzymes has been termed amino acid activation by Hoagland (1955). This is considered to consist of the formation of an enzyme-amino acyl adenylate complex with release of pyrophosphate. The amino acid-activating systems further catalyse a reaction between amino acid and 'soluble' ribonucleic acid (Hoagland, Zamecnik & Stephenson 1957) to form an amino acyl-ribonucleic acid compound which is usually assumed to be formed by the transfer of the amino acyl adenylate to 'soluble' ribonucleic acid. Evidence for these two reactions has been summarized by a number of authors (Lipmann, 1958; Zamecnik, Stephenson & Hecht, 1958; Berg & Ofengand, 1958; Novelli, 1958). It is not yet

clear whether these two reactions are catalysed by one or more enzymes, and purification of the system might help to elucidate this point. Moreover, these reactions have special interest as possible early stages in protein synthesis, and further elucidation of their function might be assisted by the availability of the purified enzymes. Pig liver was found by us to be a source of protein catalysing both reactions, and one from which the isolation and purification of these enzymes might be attempted.

In this paper we describe the isolation and simple properties of amino acid-activating preparations, which are essentially free of ribonucleic acid, including 'soluble' ribonucleic acid. This in no way impairs their ability either to catalyse amino acid-dependent exchange of pyrophosphate, or the transfer of [^{14}C]amino acids to added 'soluble' ribonucleic acid. We have compared these preparations with pH 5.0 fractions from the same liver and found them not dissimilar in their general behaviour.

* Overseas student of the Australian Commonwealth Scientific and Industrial Research Organization.

† Present address: Department of Biochemistry, University of Melbourne, Australia.