FLUORESCENT PROTEIN CONJUGATES

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I. INTRODUCTION

A. SCOPE OF THE REVIEW

The use of fluorescence techniques in chemistry and biology has become very widespread in recent years. Even if, as in this review, attention is confined to the single sector of fluorescent protein conjugates, the existing literature is most voluminous and includes a wide range of diverse applications. In what follows an emphasis will be placed upon principles rather than details.

The contents of this review will be limited to a discussion of true fluorescent conjugates, in which the fluorescent group is *chemically* linked to the protein by a primary bond. Protein–dye complexes in which the fluorescent dye is reversibly bound by non-covalent linkages will be excluded from consideration here.

In addition to being interesting objects of study in their own right, such derivatives have proven useful in investigations of protein structure and interactions and especially as highly specific histochemical stains. The opening sections will deal with the preparation and the chemical and physical properties of the conjugates themselves. This will be followed by a discussion of their application to problems of protein structure and interactions. The final section will deal with the major uses of the fluorescent antibody technique.

B. GENERAL REMARKS ON FLUORESCENCE

When, as a result of the absorption of a quantum of radiation of frequency γ_{AB}, an atom or more complicated system is raised from energy level A to energy level B, the possible reverse transitions depend upon the number of energy levels between A and B (1, 2). If there are no intermediate levels the system can return to A only by reëmission of radiation of the same frequency γ_{AB}. This is called resonance radiation. If, however, there are one or more other levels C, D, ..., between A and B, the return to B may occur by a stepwise process. As each energy gap must normally in this case be less than for the single step process the frequency of any emitted radiation ($= E/h$, where h is Planck's constant) will be less than that of the absorbed radiation. This is the basis of the empirical law of Stokes, which states that the frequency of fluorescent radiation is less than, or in the limit equal to, that of the activating ratiation (1). It is of course unnecessary that all steps of the return to the original state be by an emission of radiation. An alternative mechanism is *via* a transfer of thermal energy through collision with other molecules. This is particularly important for solutions.

An important parameter characterizing the transition between two levels by radiation emission is the excited lifetime (τ). This has been defined as the time (t) required for the intensity of fluorescence to drop from its initial value I_0 to $(1/e)I_0$ (1, 2). Thus $I_t = I_0 e^{-t/\tau}$. For most fluorescent molecules the magnitude of τ ranges from 10^{-9} to 10^{-7} second.

In the absence of all external perturbations the lifetime of the excited state would be determined by the total probability of all possible transitions to lower energy levels and would be given by $\tau_0 = 1/\Sigma_J P_{BJ}$, where τ_0 is the unperturbed lifetime of a molecule in excited state B and P_{BJ} are the probabilities of the transitions between level B and the other levels J.

If there is no exit possible from a particular intermediate level except by a "forbidden" transition of a low order of probability, then the level is termed *metastable*. Radiation-producing processes which involve the passage through a metastable state will normally be of very long excited lifetime and are referred to as phosphorescence (1, 2).

If the energy of excited systems can be dissipated as a result of collisions or other perturbations, the excited lifetime and the quantum yield of fluorescence will be reduced as a result of these competitive processes. The *quantum yield*, Q, of fluorescence may be defined as the ratio I/A, where I and A are the number of quanta of radiation emitted and absorbed, respectively, in unit time.

If the only competing non-radiative transitions from the excited state are of a *kinetic*, time-independent type (as collisional deactivation), then it may be shown that the quantum yields and excited lifetimes for two sets of conditions are related by (1, 2)

$$\text{I-(1)} \qquad Q_1/Q_2 = \tau_1/\tau_2 \qquad (\text{if } A_1 = A_2)$$

It must be emphasized, however, that this relation is by no means of universal applicability, as will be discussed subsequently.

In the case of solutions or other condensed systems the frequency of collisions is so great that radiative processes are swamped by thermal energy transfer for the vast majority of molecules. The property of fluorescence is retained only by a minority of organic molecules, almost all of which have a rigid ring structure. Whenever a molecule in solution acquires a large excess of vibrational energy, this energy is almost at once converted into thermal energy of the medium and is not restored to the initially excited molecule.

The normal process of excitation under these conditions involves a transition from the ground state to a higher vibrational level of the first excited state. The lifetime of the excited state is sufficiently long to permit ready dissipation of excess vibrational energy so that the radiative transition is from the lowest vibrational level of the excited state. The multitude of energy levels in the ground and excited states and the presence of solvent perturbations cause both the absorption and emission bands to have a very broad appearance for molecules in solution.

In addition to thermal transfer other processes competitive with respect to direct emission include *sensitized fluorescence*, in which the excited energy is transferred intact to a second molecule, which then emits it as fluorescence. The energy transfer may be by a bimolecular collision or, more interestingly, *via* a *radiationless energy transfer* which occurs without benefit of either direct molecular contact or the emission of radiation (2). Processes of this latter class have attracted widespread interest in recent years and will be discussed in detail in section IV.

The degree of polarization of fluorescent radiation is often of great importance (1, 2, 3, 4). This may be defined most generally by the equation (1, 2, 3)

$$\text{I-(2)} \qquad P' = \frac{I' - I''}{I' + I''}$$

Here I' and I'' are the maximum and the minimum intensities of the fluorescence observed through a Nicol prism which is rotated gradually about its axis. However, it is more usual to measure the apparent degree of polarization, P, which is defined by

$$\text{I-(3)} \qquad P = \frac{I_z - I_x}{I_z + I_x}$$

where I_z and I_x are the intensities of the components with electric vectors parallel to two directions, z and x, which are mutually perpendicular. The difference between P and P' disappears if the fluorescence is observed at an angle of 90° to the activating beam,

which is either unpolarized or polarized in the z-direction. In particular, if the latter direction corresponds to vertical polarization, P becomes equal to $(I_v - I_h)/(I_v + I_h)$ where I_v and I_h are the intensities of the vertically and horizontally polarized components. All the data of interest with respect to protein conjugates have been obtained for these conditions and in the subsequent discussion this meaning will be assigned to the degree of polarization, unless otherwise indicated.

II. PREPARATION OF THE CONJUGATES

A. THE NATURAL ULTRAVIOLET FLUORESCENCE OF PROTEINS

All proteins which contain one or more tryptophan or tyrosine groups show a definite fluorescence spectrum in the near ultraviolet (5, 6). The third fluorescent amino acid, phenylalanine, does not appear to contribute to the ultraviolet fluorescence of proteins. Because of the widespread occurrence of the first two amino acids the property of ultraviolet fluorescence can be regarded as almost universally present for proteins.

The quantum yield and spectral distribution of the natural fluorescence of proteins are dependent upon their molecular organization, as will be discussed in subsequent sections.

B. TYPES OF SYNTHETIC CONJUGATE

In general the fluorescent protein conjugates of greatest interest and usefulness are those which leave the secondary and tertiary structure of the protein itself essentially intact. It follows that the coupling process should proceed to completion under mild conditions and should not involve any group of the protein essential to its structural integrity. These, together with the third obvious requirement of a highly stable covalent linkage, serve to reduce sharply the number of possibilities.

The protein side chains which are sufficiently reactive to be potential sites for conjugation under mild conditions include those terminating in imidazole, aliphatic hydroxyl, phenolic, carboxyl, ϵ-amino, and sulfhydryl groups. Although information as to the site of conjugation is incomplete in many cases, it is probable that the vast majority of the conjugates prepared to date have involved linkage to sites of the latter two classes only.

The most popular methods of conjugation have involved the conversion of the fluorescent dye to an isocyanate, isothiocyanate, or sulfonyl chloride and its subsequent combination with the acceptor groups of the protein *via* a heterogeneous reaction. In the case of anthracene the alternative procedure exists of coupling to free –SH groups after conversion to the mercuric chloride. Derivatives of all the above types have the advantage of combining rapidly under mild conditions to form highly stable conjugates, usually with a minimal alteration of the fluorescence properties of the free dye.

Only a few of the large number of known fluorescent molecules have been used for the preparation of protein conjugates. Conjugates have been prepared from the isocyanates or isothiocyanates of fluorescein (7), rhodamine B (8), 2,2′,4-trihydroxy-4′-aminoazobenzene (9), anthracene (10, 11), 4-dimethylamino-stilbene (12), 2′-methyl-4-dimethylamino-stilbene (12); 10-methyl-1,2-benzanthracene (11), 1,2,5,6-dibenzanthracene (11), 3,4-benzpyrene (11), 1,2-benzanthracene (11, 13), and 3-phenylcoumarin (14); the sulfonyl chlorides of 1-dimethylaminonaphthalene (15, 16), anthracene (16), 3-hydroxypyrene (14), rhodamine B (17), fluorescein (17), and acridine orange (17); and the mercuric chloride of anthracene (18).

C. SITES OF COMBINATION WITH THE PROTEIN

There is little direct information available as to the sites of attachment of the fluorescent reagents in common use. However, it is possible to make reasonable conclusions as to the loci of conjugation on the basis of results obtained with analogous compounds.

Phenyl isocyanate has long been known to interact readily with proteins (19). The reaction is accompanied by a decrease in available amino nitrogen. It follows that the ϵ- and α-amino groups are among the protein sites which interact with the isocyanate reagents, forming in this instance phenylureido derivatives.

Accessible sulfhydryl groups likewise react rapidly with phenyl isocyanate and its derivatives. Fraenkel-Conrat and Olcott have reported that the –SH groups of native ovalbumin display even greater reactivity with *m*-chlorophenyl isocyanate than do the amino groups (20).

The evidence as to reaction with tyrosine is inconclusive. In any event, in view of the correspondence between the number of *p*-bromophenyl isocyanate groups introduced into serum albumin and the number of amino groups which simultaneously disappear, it is safe to conclude that the susceptibility of the phenolic hydroxyl of tyrosine to attack by reagents of this class is slight in comparison with that of the amino and sulfhydryl groups (21).

It is clear that the amino and sulfhydryl groups are the most likely sites of combination for the fluorescent isocyanates. However, this conclusion must remain tentative pending a more direct confirmation.

The evidence in the case of the fluorescent sulfonyl chlorides is likewise of an indirect nature. Thus 2-naphthalenesulfonyl chloride and benzenesulfonyl chloride have been widely used as protein acetylating

agents (22). In this case the ϵ- and α-amino groups and the sulfhydryl groups have been conclusively identified as the loci of reaction. There is no reason to doubt that this specificity extends to the fluorescent sulfonyl chlorides as well. Amide sulfonate derivatives of 1-dimethylaminonaphthalene-5-sulfonyl chloride with glycine and polylysine have in fact been prepared and identified (15, 23).

Massey and Hartley have studied the reaction of 1-dimethylamino-naphthalene-5-sulfonyl chloride (DNS) with a series of amino acids (23). In harmony with expectations arising from experience with other sulfonyl chlorides, the presence of an available amino or sulfhydryl group invariably permitted coupling. However, they also found evidence for a reaction under certain conditions with the imidazole ring of histidine to form a somewhat less stable derivative. These authors also have attributed the unusual properties of the DNS conjugate of α-chymotrypsin formed at low degrees of labelling to a preferential attack upon an imidazole group which is an element of the enzymatically active site. This is at present the sole proposal for the possible attachment of DNS to a protein site other than an amino or sulfhydryl group. Even in this case the evidence for the occurrence of this process in the protein is indirect and by no means conclusive.

In the case of anthracene mercuric chloride the identification of the acceptor site as the sulfhydryl group is fairly conclusive from the stoichiometry of the reaction, the combination of the reagent with the $-SH$ group of cysteine, and the known behavior of other organic mercurials (18).

D. PREPARATION OF REAGENTS

All of the reagents mentioned earlier are relatively easy to synthesize and most of them are, in fact, commercially available. 1-Dimethylaminonaphthalene-5-sulfonyl chloride is prepared from the corresponding sulfonate. This, in turn, is prepared easily by methylation of the cheaply available 1-aminonaphthalene-5-sulfonic acid. Weber has reported that the method of Fussganger is satisfactory (15).

The sulfonate, in the form of a sodium salt, is readily converted to the sulfonyl chloride by grinding with PCl_5 in a weight ratio of 1.5 to 1 (15). The heat of reaction usually fuses the mixture, which is poured into water at 0°. The yellow (or orange) sulfonyl chloride is dissolved in a minimal volume of acetone and then reprecipitated by pouring into four volumes of water. After centrifugation the reagent may be dried in a vacuum desiccator and then stored at room temperature.

Uehleke has described the preparation of a sulfonic acid derivative of fluorescein *via* the combination of 4-sulfonic-phthalic anhydride and resorcinol (17). The same author also has prepared sulfonated derivatives of acridine orange by treating the dye with concentrated sulfuric acid at 80°. The product is somewhat heterogeneous and difficult to purify (17).

Chadwick, McEntegart, and Nairn have prepared and coupled the sulfonyl chloride of rhodamine B (24).

Both fluorescein isocyanate and isothiocyanate can be prepared by the action of phosgene or thiophosgene upon fluorescein amine. The preparation, in purified form, of the isomers of fluorescein amine has been described by Coons and Kaplan (25). In brief, nitrofluorescein is obtained by heating a 1:1 mixture of nitrophthalic acid and resorcinol for 12 hours at 200°. After washing with water and drying, the crude nitrofluorescein is refluxed with a four-fold excess of acetic anhydride for 2 hours. The resultant crude nitrofluorescein diacetate is washed with ethanol and recrystallized from acetic anhydride. At this stage the nitrofluorescein diacetate is a mixture of isomers. By fractional precipitation from saturated alcoholic sodium hydroxide, the individual isomers can be separated.

The nitrofluorescein is reduced to the corresponding fluorescein amine by hydrogenation of a suspension of nitrofluorescein and Raney nickel (2:1.5) in ethanol. After centrifugal removal of nickel the ethanol solution of amine is diluted with an equal volume of water. The fluorescein amine crystallizes out upon standing.

Conversion of the amine to isocyanate is accomplished by dropwise addition of a 1% solution in acetone to three volumes of acetone saturated with phosgene. The isocyanate may be recovered in solid form upon evaporation of solvent. If conjugation is to be carried out at once the isocyanate may be dissolved in a 2:1 acetone–dioxane mixture for direct addition to protein.

Analogously, fluorescein isothiocyanate may be prepared by the addition of the amine to thiophosgene in acetone (26, 27). After standing overnight the resultant precipitate is collected by filtration, washed with petroleum naphtha, and dried over calcium chloride.

The preparation of the isocyanate of rhodamine B, as described by Silverstein, is entirely analogous to that of fluorescein isocyanate (28). The nitro derivative of rhodamine B is prepared by the condensation of two equivalents of *m*-diethylaminophenol with one of 4-nitrophthalic anhydride. The nitro group is converted to an amino group by catalytic reduction. Subsequent treatment with phosgene yields the isocyanate.

A fluorescent conjugate of a quite different kind has been prepared by Dowdle and Hansen (9). A non-fluorescent compound, 2,2′4-trihydroxy-4′-aminoazobenzene was converted to the isothiocyanate by treatment with thiophosgene and coupled to γ-globulin. Upon chelation with aluminum the conjugate acquired a brilliant yellow fluorescence.

Creech and Peck have described the synthesis of the isocyanates of 2′-methyl-4-dimethylaminostilbene and 4 dimethylaminostilbene (12). In both cases the corresponding amino derivatives were converted to isocyanates by the addition of phosgene in toluene solution. The parent amino derivatives were prepared by reduction of the corresponding nitro compounds. The isocyanates of a number of polycyclic aromatic hydrocarbons have been prepared by entirely analogous means (11, 13).

In general any dye which can be prepared as an amino derivative can be converted to an isocyanate or isothiocyanate. For most purposes the use of a mixture of isomers probably is adequate.

Williams and Foster have prepared the mercuric chloride derivative of anthracene by a simple process involving the heating of anthracene with mercuric acetate in a propionic acid solution (18). Upon completion of the reaction, conversion to the chloride is made by addition of ethanolic calcium chloride.

E. CONDITIONS OF COUPLING

A complicating factor in the use of reagents of the types described earlier is their insolubility in water. Thus the coupling reaction must be of a heterogeneous nature. The fluorescent reagent may be present as a suspension or as an adsorbate bound by some inert material of high surface area.

In the case of 1-dimethylaminonaphthalene-5-sulfonyl chloride (DNS) the most widely used procedure has been to add the sulfonyl chloride in the form of an aqueous colloidal suspension to the protein under weakly alkaline conditions (0.1 *M* $CO_3^=$, pH 8–9) at 0–3° (15, 16). The colloidal suspension is formed by adding an acetone solution of DNS to a five-fold excess of water. Usually the total weight of reagent added is of the order of 1–2% of the weight of protein.

No systematic studies exist as to the kinetics of the reaction or as to the influence of pH and temperature. The time required for clearing of the suspension appears to vary somewhat from protein to protein. In most cases the reaction is complete after 10 hours at 0–3°.

The acetone solution of DNS may, of course, be added directly to the protein solution, but with the risk of exposing the protein to a local high concentration of acetone.

Rinderknecht (29) has developed an alternative technique of labelling based upon the adsorption of the fluorescent reagent from dry chloroform by diatomaceous earth (Celite, Super-cel, *etc.*). Labelling is carried out by the direct addition of dry DNS-containing Celite to the protein solution. It is claimed that, in addition to avoiding exposure of the protein to organic solvent, this method permits a greatly enhanced rate of coupling.

A variant of this technique has been described by Goldman and Carver (30). These workers have absorbed the reagent from solution with filter paper. The dried filter paper then is added to the protein solution. It is claimed that this method is rapid and convenient.

Fluorescein isocyanate, although largely superseded at present by reagents of greater stability and convenience, was the original reagent of Coons and was used for most of the earlier fluorescent antibody work.

The poor stability of this reagent under ordinary conditions makes it imperative either to couple it to the protein at once or else take special measures for its preservation. As in the case of the other agents, a weakly alkaline solution (1% protein, 0.1–0.5 *M* carbonate, pH 8–9) appears to be favorable for reaction. Most workers have used procedures similar to the original one of Coons and Kaplan (25), who recommended the direct addition of 5% by weight of isocyanate dissolved in 2:1 acetone–dioxane.

However, the literature is full of complaints that this procedure results in the inactivation of a major fraction of antibody. The techniques of Rinderknecht and of Goldman and Carver are probably to be preferred with respect to both milder conditions of coupling and enhanced stability of the reagent. According to the latter authors fluorescein isocyanate can be stored in the absorbed form for long periods without perceptible decomposition, provided that the filter paper is thoroughly dry.

The above statements apply equally well to fluorescein isothiocyanate. However, the much higher stability of this reagent renders the whole problem of preservation much less acute. In addition to the techniques already described, it has been reported that good results were obtained by the direct addition of solid reagent (5% of total weight of protein) to a 1% protein solution in 0.15 *M* NaCl, 0.5 *M* $CO_3^=$, pH 9 (31). It is claimed that inactivation of antisera is minimal under these conditions.

There appears to be no important difference in behavior of the other fluorescent isocyanates or isothiocyanates. Thus conjugates have been prepared by similar procedures from the corresponding derivatives of anthracene and rhodamine B.

Creech and Peck have described the preparation of serum albumin conjugates with the isocyanates of 4-dimethylaminostilbene and 2′-methyl-4-dimethylaminostilbene (12). A dioxane solution of reagent was added to a stirred and buffered solution of albumin (1 *M* $CO_3^=$, pH 10, 0°).

Williams and Foster have prepared anthracene conjugates of bovine mercaptalbumin *via* the reaction of anthracene mercuric chloride with the available sulfhydryl group of the protein (18). The extreme insolubility of the reagent made it necessary to add it as a

stirred suspension in water. The reaction is not profoundly sensitive to pH and temperature. A neutral pH and 0–3° appear to be satisfactory, permitting completion of the reaction within 24 hours (18).

For most applications it is important to remove all reagent which is not combined chemically with the protein. Excess reagent in suspension may be removed by centrifugation or filtration through a Millipore or equivalent filter. However, the reagent may be slightly soluble in water or (as in the case of DNS) may be converted to a soluble form by hydrolysis. Moreover, uncoupled reagent may be bound strongly by the protein. This is the case especially for serum albumin.

The methods employed include prolonged dialysis, precipitation of the protein with ammonium sulfate or with ethanol, and passage of the solution through an ion-exchanger, as Dowex-2. All combinations of these have proven successful in individual cases. It has been reported that extraction with ethyl acetate is an effective way to remove excess reagent from fluorescein-conjugated antisera (32).

III. Properties of the Conjugates

A. Modification of the structure and properties of the protein

It is obviously impossible to generalize as to the degree of alteration of the secondary and tertiary structure of the labelled protein. Clearly such variable factors as the degree of conjugation, the lability of the protein, and the importance of the ϵ-amino or sulfhydryl groups in maintaining its structural integrity will be crucial. There exist a number of studies which appear to indicate that the protein fine structure is usually not grossly altered by limited degrees of conjugation. Thus, as is well known, a wide variety of labelled antibodies are known to retain their immunological activity (7, 8, 17). The partial inactivation which has often been reported to occur is almost certainly a result of denaturation caused by faulty labelling technique, in particular the exposure of the protein to a local excess of organic solvent. The molecular kinetic properties of such proteins as serum albumin (10, 15), ovalbumin (15), insulin (33), and thyroglobulin (34) are essentially unchanged by low degrees of conjugation with DNS (<about 3 DNS residues per 100,000 molecular weight unit). Soybean trypsin inhibitor has been shown to retain inhibitory activity in the labelled state (35).

A preliminary study of fluorescein conjugates of bovine serum albumin has failed to reveal any important effect of conjugation upon the sedimentation coefficient or intrinsic viscosity of the protein (36). There was however an appreciable acid shift of the isoelectric point, as would be anticipated if the ϵ-amino groups were the principal sites of conjugation (36).

The rather cursory existing data likewise appear to indicate that fluorescein labelling has no major influence upon the behavior of bovine albumin *in vivo*. The disappearance rates of albumin conjugates from the circulation of rats are similar to those of isotopically labelled albumin and slower than that of fluorescein itself (36).

The rate of absorption from skin of the albumin conjugates was found to be very slow, in harmony with expectations if no dissociation occurs. Diffusion through capillary walls was likewise very slow and comparable to the behavior of unlabelled plasma albumin (36). Moreover, it was also reported that fluorescein conjugation did not confer antigenicity upon homologous plasma albumin (36).

It must be acknowledged that in no case has a truly *systematic* study been made of the dependence of structure and properties upon the extent of conjugation. In particular no effort has been made to detect any *subtle* changes which might not be found by the above rather insensitive criteria.

B. Absorption spectra

The usual sites of attachment of the commonly used fluorescent reagents are polar terminal groups of saturated aliphatic side chains. No very pronounced modification of the electronic state of the fluorescent residue is to be expected under these circumstances. In general the absorption spectra of conjugates of the above class show only minor deviations from those of the free dye.

The spectra of DNS conjugates of bovine serum albumin, ovalbumin, and polylysine are rather similar to that of the glycine conjugate and to each other (15) (Fig. 1). The absorption maximum for ovalbumin

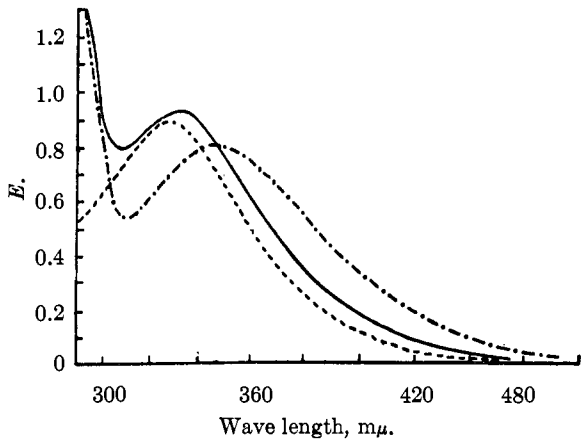

FIG. 1.—Absorption spectra of several DNS conjugates (15): —— 0.65% bovine serum albumin (2.3 moles DNS/mole protein); -·- 0.48% ovalbumin (2 moles DNS/mole protein); - - - 0.5% polylysine.

(344 mμ) is displaced noticeably to the red in comparison with those of serum albumin (332 mμ) or

polylysine (329 mμ) (15). Conjugates of this type have only a single absorption band in the near ultraviolet. The presence of only a single electronic transition is of importance in studies of fluorescence polarization and results in the degree of polarization being independent of the wave length of excitation.

Weber has reported that an appreciable change in the absorption spectrum of DNS conjugates of ovalbumin is produced by thermal denaturation at 100° (15). A definite blue shift occurs in the position of the maximum (Fig. 2). In view of these results and those

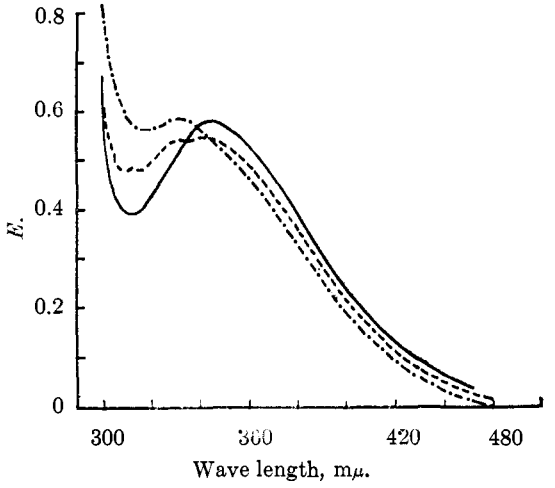

FIG. 2.—Effect of thermal denaturation upon absorption spectrum of an ovalbumin DNS conjugate (15): —— undenatured ovalbumin; - - - 1 minute at 100°; -·-·- 5 minutes at 100°.

obtained with the anthracene conjugates to be discussed presently, it is likely that further investigation will reveal a general dependence of the absorption spectra of the conjugates upon the physical state of the protein.

Hartley and Massey have found that important differences exist between the DNS conjugates of α-chymotrypsin, chymotrypsinogen, and the diisopropyl fluorophosphate (DIP) derivative of α-chymotrypsin (23). For conjugates with about 0.5 DNS group per mole the position of the absorption maximum was at 340 mμ for α-chymotrypsin, at 333 mμ for DIP-chymotrypsin, and at 328 mμ for chymotrypsinogen.

The absorption spectra for α-chymotrypsin conjugates also showed some dependence upon the degree of labelling. A progressive blue shift occurred with increasing extents of conjugation.

Hartley and Massey have found the initial conjugate of DNS and α-chymotrypsin (<1 DNS group per molecule) to have unusual properties which they attributed to a preferential combination of the label with an element of the active site itself (23). If this view is correct, it is not surprising that modifying or blocking the enzymatic site should result in a change in the DNS absorption spectrum. The α-chymotrypsin conjugate will be discussed further in a later section.

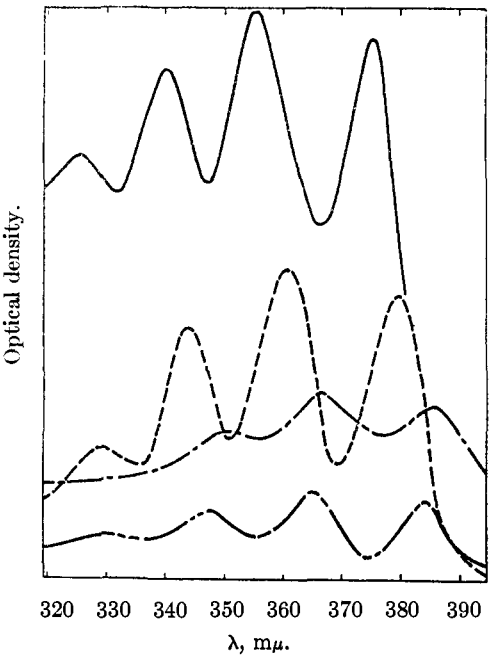

FIG. 3.—Relative absorption spectra of anthracene and its conjugates (18): —— anthracene in cyclohexane; - - - anthracene—Hg in cyclohexane; -·- Hg-anthracene–bovine albumin conjugate in 0.02 *M* NaCl; -··- cysteine–Hg-anthracene in 0.02 *M* NaCl.

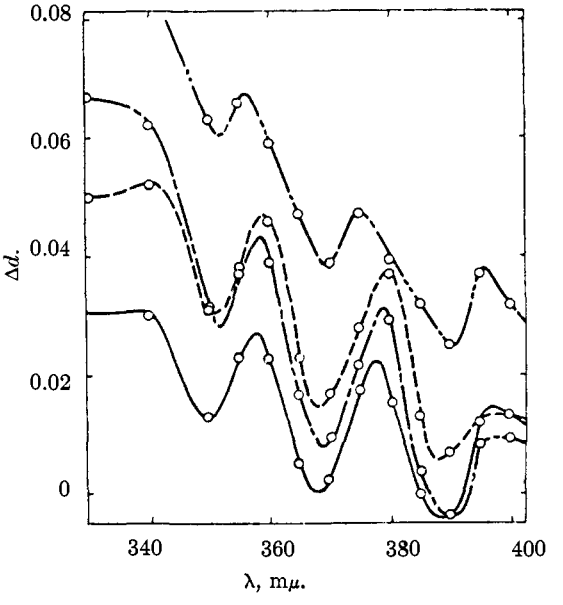

FIG. 4.—Difference spectra of Hg-anthracene–bovine albumin conjugate in 0.02 *M* NaCl (18); the pH of the reference solution is 2: —— pH 4.18; - - pH 5.53; -·- pH 7.25; -··- pH 10.6.

The absorption spectrum of the conjugate of anthracene mercuric chloride with mercaptalbumin is qualitatively similar to that of the corresponding conjugate with cysteine (Fig. 3). However, Williams and Foster (18) have shown that a definite perturbation is present which depends upon the over-all state of the protein (Fig. 4). At neutral pH the presence of the protein

appears to produce a definite red shift in all three near ultraviolet bands (18).

The absorption spectrum was appreciably pH-dependent. Thus the absorbancy at 355–360 mμ decreased at pH's acid to 5. Serum albumin is known to undergo a structural change below pH 4, which is reflected by a molecular inflation at low ionic strengths (10). However, a large fraction of the acid spectral change is completed by pH 4 (18).

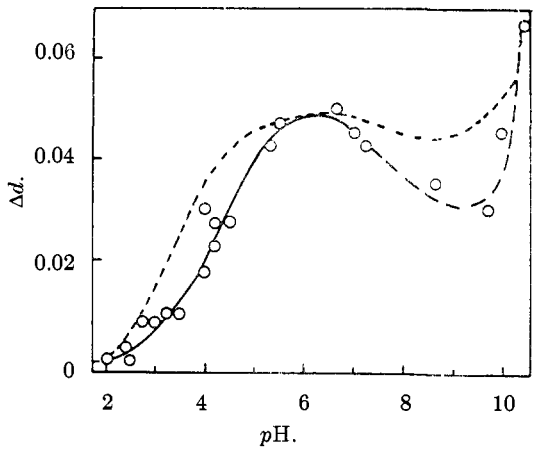

FIG. 5.—pH profile of difference spectrum (18) for anthracene–mercaptalbumin conjugate at 360 mμ (lower curve) and for bovine albumin at 287 mμ (upper curve, divided by 20).

The absorbancy at 355–360 mμ passes through a maximum at about pH 6 (Fig. 5). After a minor decrease between pH 7 and pH 9, a sharp and time-dependent increase occurs at about pH 10.

The correlation of these spectral effects with the known conformational changes of serum albumin is imperfect. It is possible that vicinal charge effects may also be a significant factor.

Williams and Foster have tentatively attributed the spectral perturbation of the coupled anthracene to the enfolding of this chromophore in a non-polar environment (18). This would of course require that the albumin structure possess sufficient deformability to permit the entry of this group into its interior. It is difficult to judge the plausibility of this model at the present time.

Creech and Peck have examined the ultraviolet absorption spectra of conjugates of bovine serum albumin with isocyanates of 4-dimethylaminostilbene and 2′-methyl-4-dimethylaminostilbene (12). There were no obvious *major* differences in appearance between the spectra of the protein conjugates and those of conjugates with ε-aminocaproic acid. These authors have, in fact, utilized ultraviolet absorbancy as a means of determining the extent of conjugation.

Emmart has compared the absorption spectrum of fluorescein itself with that of a conjugate of fluorescein isocyanate with bovine serum albumin (37). While a quantitative comparison was not made, no important change in the positions of the peaks at 284, 325, and 495 mμ occurred, at least at pH 7.6 where the fluorescein is fully ionized in both cases (Fig. 6).

The binding of a proton by fluorescein at pH's below 6 is accompanied by a shift in position of the primary band from 495 to 430 mμ and by a total quenching of green fluorescence. There are no data available on the acid absorption spectra of the conjugates.

It is clear from the limited data presented above that the influence of the protein upon the absorption spectrum of the attached fluorescent label is in general quite appreciable. The absorbancy of the conjugate is thus responsive to any alteration in the molecular organization of the protein.

However, as in the case of the ultraviolet absorption spectra of unlabelled proteins, it is often difficult to differentiate between the results of a structural transition and any trivial effects induced by a change in the state of ionization, possibly *via* a vicinal charge effect. Moreover, a comprehensive theory of the dependence of the properties of the label upon the structure of the protein carrier is still lacking. This question will arise again in connection with the H^+ ion titration and the fluorescence properties of the conjugates.

The positions of the absorption and emission peaks of the principal fluorescent labels are given in Table I.

TABLE I

ABSORPTION AND EMISSION PEAKS FOR SEVERAL FLUORESCENT LABELS

Label	Absorption maxima, mμ	Emission maxima, mμ	References
DNS	340,	500	15, 40
Fluorescein	250, 495, 325, 285	550	1, 37
Anthracene	350, 365, 385	400	1, 18, 40
Rhodamine B	250, 300, 350, 420, 550[a]	605[a]	1
1,2,5,6-Dibenz-anthracene	280, 289, 300, 327, 342 357, 377, 399	390, 415, 450[a]	1, 11

[a] Data refer to dye itself in uncombined form.

C. HYDROGEN ION TITRATION

Many of the conjugate dyes discussed earlier can bind a proton. The ionization is usually accompanied by a significant change in both the absorption and emission spectra. It is thus very easy to obtain a titration curve for this group alone, analogous to the spectral titration curves often made for tyrosine in proteins. For example, both fluorescein and DNS undergo major spectral shifts and fluorescence quenching upon binding a proton.

A more than superficial discussion of the hydrogen ion titration curves of proteins is beyond the scope of this review. However, it is well known that a central problem in interpreting data of this kind is to account for the often major displacements of the pK's of the ionizable groups from those found for the same groups in the free amino acids. The most popular explanation

has been in terms of hydrogen bonding of the ionizable group. Scheraga and Laskowski have developed ingenious variants of this model, which have been shown to be capable of accounting for many of the existing data (38).

It would clearly be advantageous to obtain titration data for a group which is unlikely to be involved in any hydrogen bonding and which can be resolved completely from other ionizable groups. Such a possibility is offered by DNS conjugates of proteins (39). As Klotz and Fiess have pointed out (39), the dimethylamino group contributes its pair of electrons to resonance with the ring and hence cannot be a good acceptor of an H-bond. Thus any change in its titration properties accompanying conjugation to a protein must arise from some other cause.

Klotz and Fiess have found that the pK of the $(CH_3)_2$-NH^+-group is 1.67 when attached to bovine serum albumin and 4.0 when attached to glycine (39). The difference is much too large to arise from any electrostatic factor and, if hydrogen bonding is absent, must reflect some more general property conferred by conjugation to protein.

In 8 M urea the pK of the dimethylamino group returns to 3.3. This suggests that the integrity of the albumin internal structure may be essential for the effect, possibly as a consequence of an envelopment of the DNS group by nonpolar regions of the intact protein. This model would be similar to that proposed by Williams and Foster to account for the spectral perturbation of anthracene when coupled to albumin (18).

However, Klotz and Fiess have preferred to interpret their results in terms of a postulated ice-like character of the hydrate water in the vicinity of the non-polar portions of the protein, including the DNS group. Such an environment would tend to stabilize the uncharged form of the dimethylamino group, as the creation of a charged group in place of the uncharged one would require some breakdown of the ice lattice.

It is clear that the choice of the correct explanation must be left open at present. In any event, information of this kind is sufficient to suggest that some revision may be necessary of current ideas as to the origin of abnormal behavior in protein titration curves.

D. FLUORESCENCE SPECTRA

The limited available information suggests that in most instances the presence of the protein is without any major influence upon the spectral distribution of emitted radiation, at least for DNS and fluorescein conjugates. The *intensity* of fluorescence, however, can be very dependent upon the state of the protein (40).

The emission spectra of DNS conjugates (40) are broad, with a maximum usually at 500–520 mμ (Fig. 7). There appears to be little variation in position with either degree of conjugation or the nature of the protein. The emission band of fluorescein conjugates is likewise broad, with a maximum in the range 520–550 mμ (Fig. 6) (1, 37).

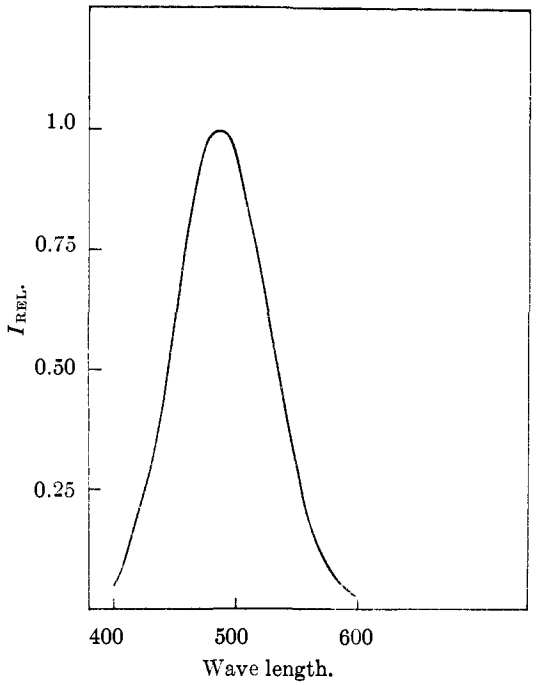

FIG. 6.—Emission spectrum for a DNS conjugate of bovine serum albumin in H_2O, pH 7, activated at 340 mμ (40). There are 0.5 DNS groups per mole.

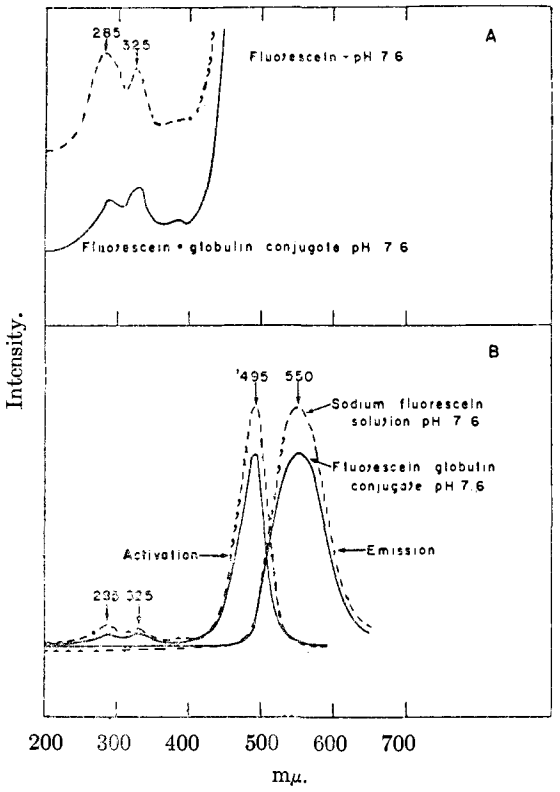

FIG. 7.—Absorption and emission spectra for a fluorescein conjugate of γ-globulin (37).

While no quantitative information as to quantum yields exists it is clear from the few available studies that the intensity of fluorescence is, in general, sensitive to external conditions and appears to reflect, to some degree, the molecular organization of the protein. Thus, in the case of DNS conjugates of bovine serum albumin (40), a dramatically sharp drop in fluorescence occurs at about pH 11.5 (Fig. 8). This corresponds to the alkaline zone of pronounced molecular disorganization, as indicated by molecular kinetic and fluorescence polarization studies (15). The intensity is essentially constant and independent of pH in the zones 5–7 and 9–11. Minor changes are observed between pH's 7 and 9. These are variable in magnitude and may be either positive or negative depending upon the extent of conjugation (40). While the significance of these is unclear, it is of interest that there is some evidence for a minor structural transition in this pH zone (41).

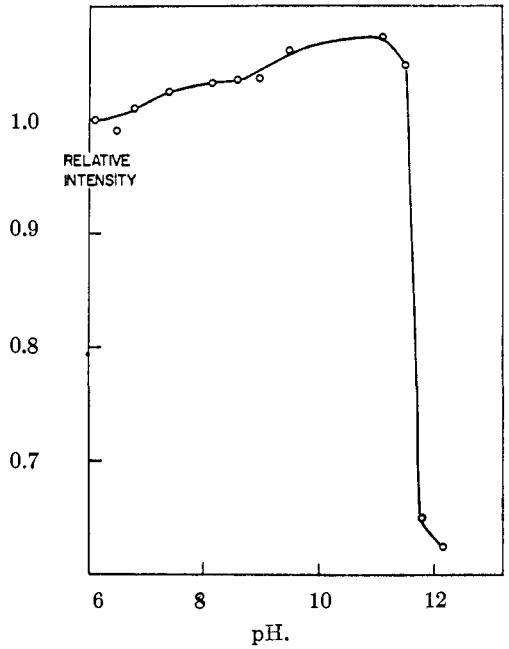

FIG. 8.—pH profile of relative fluorescence intensity for the DNS albumin conjugate of Fig. 6 in H_2O. The wave lengths of activation and emission are 340 and 520 mμ, respectively (40).

In the case of fluorescein conjugates of serum albumin (40) a zone of rapid increase of intensity with pH between pH 6 and 8.5 is followed by a flat region between pH's 8.5 and 11.5 (Fig. 9). At the latter pH a sharp drop in emission intensity occurs, which parallels that observed for the naphthalene conjugates. Below pH 5 the issue is obscured by the onset of titration of the fluorescein group itself.

The intensity of fluorescence of the mercuric-anthracene conjugate of mercaptalbumin is relatively invariant to pH, although minor variations are observed in the regions of extensive structure change (40).

The intensity of fluorescence of the conjugates may also depend upon their state of association. Thus Dandliker and Feigen have reported that appreciable changes in the fluorescence intensity of fluorescein conjugates of ovalbumin accompany their combination with rabbit anti-ovalbumin antibody (42). Similarly, the formation of electrostatic complexes with oppositely charged serum albumin (40) results in an enhancement in the fluorescence intensity of DNS conjugates of lysozyme (Fig. 10). The presence of a sufficiently high level of electrolyte abolishes the interaction (43) and renders the fluorescence intensity independent of the concentration of added albumin.

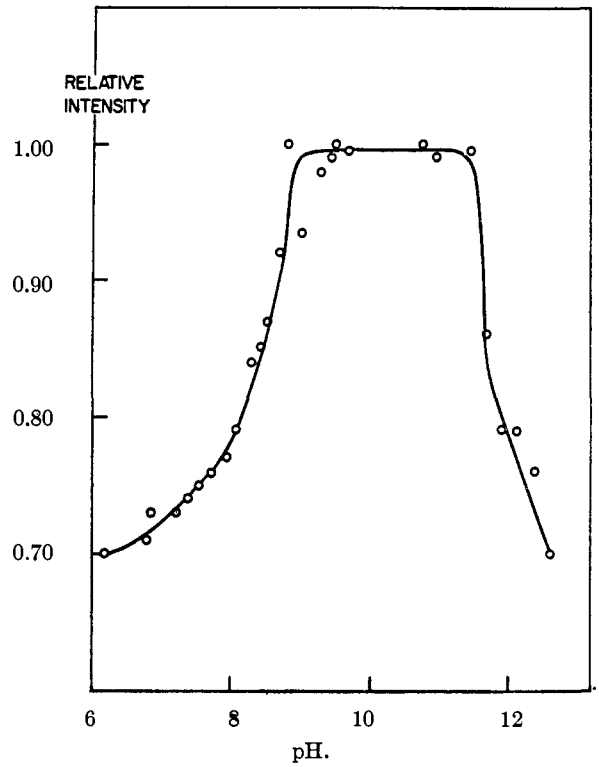

FIG. 9.—pH profile of relative fluorescence intensity for a fluorescein conjugate (0.04 group/mole) of bovine serum albumin in water (40). The wave lengths of activation and emission are 490 and 550 mμ, respectively.

The dependence of the fluorescence intensity of the conjugates upon the conformational state of the attached protein is paralleled by the behavior of the natural ultraviolet fluorescence of proteins (44).

The dependence of the intensity of fluorescence, either natural or acquired, upon the molecular organization of the protein is only beginning to be explored. It can be expected to become a technique of great utility, especially in view of its ready adaptability to kinetic measurements.

The excited lifetimes of the fluorescent conjugates will be discussed in a later section. In most cases they are close to those of the free dye.

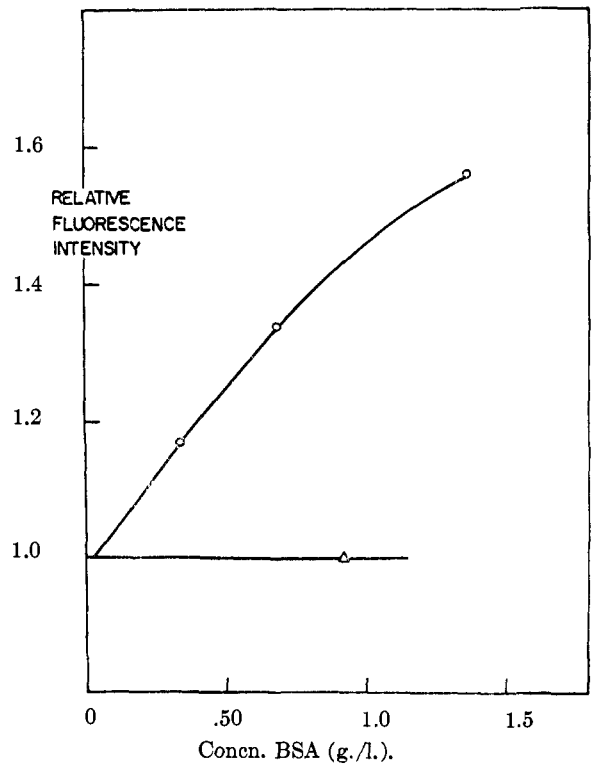

FIG. 10.—Enhancement of fluorescence of a DNS conjugate of lysozyme (0.9 g./l.) as a result of electrostatic complexing with bovine serum albumin at pH 7.8 (40): ⊙ 0.001 *M* tris; △ 0.001 *M* tris, 1 *M* KCl.

E. ENZYME INHIBITION

The DNS conjugate of α-chymotrypsin appears to be a special case. Hartley and Massey have found that, for low degrees of labelling, the enzyme is almost stoichiometrically inhibited (23). The presence of a competitive inhibitor of the enzyme, β-phenylpropionate, protects the activity during reaction.

Moreover, DNS conjugates of α-chymotrypsin will no longer react with organophosphorus inhibitors, as diethyl *p*-nitrophenyl phosphate. The obvious implication of these results is that some group of the enzymatically active site is the locus of conjugation. This hypothesis is reinforced by the capability of DNS conjugates of chymotrypsinogen to be activated to chymotrypsin of normal enzymatic properties (23).

The chymotrypsin conjugate is unusual in several respects, including the orange color of its fluorescence and its low stability to acid or thermal hydrolysis. A comparison with a series of amino acid conjugates showed that only the conjugates formed with the imidazole ring of histidine had properties approximating those of the chymotrypsin derivative.

On this basis Hartley and Massey have proposed that preferential reaction occurs with an imidazole ring which is an essential part of the active site (23). The enhanced activity of this imidazole presumably is conferred by its special environment. Further labelling is to more conventional sites, as the ε-amino groups, and is accompanied by a gradual transition to the usual properties of DNS conjugates.

On the whole, the above evidence is rather compelling as to the preferential attack of DNS upon some element of the active site. Further work will be required to establish conclusively whether the imidazole ring is indeed the true point of attachment. If this is the case, the further problem remains of accounting for its unusual reactivity in this case.

IV. NON-RADIATIVE ENERGY TRANSFER IN PROTEIN CONJUGATES

A. GENERAL REMARKS

The term "radiationless energy transfer" is used to designate a class of processes in which the energy of an excited fluorescent group is lost *via* transfer to an acceptor group, *without any emission of radiation by the primary group or any collisional contact of the two groups* (45). The latter conditions serve to differentiate this mechanism from such trivial processes as the reabsorption of *emitted* radiation and the transfer of excitation energy by collision or chemical complexing. Radiationless energy transfer is competitive with respect to direct emission by the primary group and among its consequences is a quenching of the fluorescence of the primary group (2, 45).

Since the early work of Cario and Franck, examples of sensitized fluorescence, both in the gas phase and in solution, have multiplied (46). The energy transfer invariably occurs from a sensitizer absorbing at a shorter wave length to an acceptor absorbing at a longer (46). A transfer in the reverse direction is normally energetically impossible.

The relative broadness of absorption and emission bands in solution and the high frequency of molecular collisions render it essential to exclude trivial mechanisms with particular care in this case. The exclusion of a collisional mechanism is done most readily by examining the dependence of sensitized fluorescence upon solvent viscosity (45). An increase in viscosity reduces the frequency of collisions and hence any collisional transfer, but has no direct influence upon non-radiative transfer.

Radiationless transfer may be distinguished from simple reabsorption by the secondary group of the emitted fluorescence of the primary group by several criteria. Transfer generally involves a decrease in sensitizer excited lifetime, which is unchanged by reabsorption (45). Transfer is generally independent of the geometry of the system, in contrast to a reabsorption mechanism which depends upon the volume of the solution (45).

The third possible trivial mechanism, transfer by molecular complexing, is usually readily distinguished by changes in the absorption spectrum of the sensitizer.

The efficiency of radiationless transfer tends to be remarkably high in view of the large distances—as high as 100 Å.—which often are involved and the shortness of the sensitizer lifetimes, which are usually of the order of 10^{-8} second. It was early realized that such a process is comprehensible only if some kind of resonance condition is fulfilled. While a classical treatment was made by J. Perrin at an early date, an adequate quantum mechanical derivation was only achieved in recent times by Forster (2). Only the molecular case for solutions will be considered here.

If the energy drop for a possible radiative deactivation for the sensitizer corresponds precisely to that for a possible absorption transition for an acceptor molecule in the vicinity, then it can be shown by a quantum mechanical argument that there is a finite probability of a simultaneous occurrence of both processes, resulting in a net transfer of energy from the sensitizing to the accepting group (45). Because of the requirement of correspondence of energy changes, this kind of process is sometimes referred to as a resonance transfer.

Despite the confusing formal similarity of this condition to that for reabsorption of fluorescence, it must again be emphasized that the two mechanisms are totally different as the transfer occurs *before* the emission of fluorescence.

The correspondence requirement is not very restrictive for solutions, as the multiplicity of closely spaced levels ensures that this condition will be met, provided that some degree of overlap occurs between the sensitizer emission spectrum and the acceptor absorption spectrum.

As the transfer process depends upon the mutual coupling of the electronic systems of both molecules, the efficiency of transfer naturally falls off with increasing separation of the sensitizer and acceptor groups. The interaction energy is of a dipole–dipole character and varies as the inverse third power of the separation. The probability of transfer is proportional to the square of the interaction energy and hence to the inverse sixth power of the distance.

The detailed derivation of Forster leads to an expression of the type:

$$k = \text{rate constant for transfer}$$

$$k = KJ/\tau_s R^6$$

where K is a collection of constants, τ_s the intrinsic lifetime of the sensitizer, and R the separation of sensitizer and acceptor groups. J is an overlap integral, defined by

$$J = \int_0^\infty f(\nu)\epsilon(\nu)\,\frac{d\nu}{\nu^4}$$

where f and $\epsilon(\nu)$ are the normalized spectral distributions of emission and absorption, respectively, as a function of the frequency, ν.

Alternatively, one may define a new parameter, R_0, as the separation for which transfer and spontaneous deactivation are of equal probability. In terms of R_0

$$k = (R_0/R)^6/\tau_s$$

The inverse sixth power dependence upon separation renders the phenomenon very sensitive to the mutual position of the interacting groups.

In general the equation of Forster must be regarded as only a first approximation at best. In certain instances it fails entirely, including the case where transfer occurs before the attainment of thermal equilibrium. In addition, when R is very small, interactions of a type other than dipole–dipole may become important.

B. RADIATIONLESS ENERGY TRANSFER IN PROTEIN CONJUGATES

Although most of the experimental work upon energy transfer has dealt with transfer between different molecules, there exists also the possibility of transfer between different electronic systems of the same molecule, as in the case of aromatic complexes of certain rare earths. More pertinently, it would be expected that exchange could occur between a fluorescent group and an acceptor group attached to the same protein molecule, provided that sufficient overlap of their emission and absorption bands exists.

An obvious possibility is exchange between tryptophan and an acceptor group, such as fluorescein or DNS. In either case extensive overlap of emission and absorption bands is present. Thus it is not surprising to find that the apparent activation spectrum of a DNS conjugate of serum albumin has a double maximum (40). The lower wave length peak (λ_{max} = 290 mμ) cannot be due to the DNS itself and must arise *via* transfer from the tryptophans.

The potential interest of such intramolecular transfer arises from its profound dependence upon the separation of primary and acceptor groups. One would thus expect it to be particularly sensitive to any structural change of the protein. In effect the influence of any structural transition upon the sensitized fluorescence of DNS conjugates will represent the cumulative result of any change in either group, plus the influence of any change in their separation. One example is the observation that the sensitized fluorescence of DNS conjugates of bovine serum albumin shows more pronounced variation with pH in the zone pH 7–8.5 than does the emission of either group alone (Fig. 11). At pH's above 10, in the region of tryptophan quenching, the transfer fluorescence also decreases.

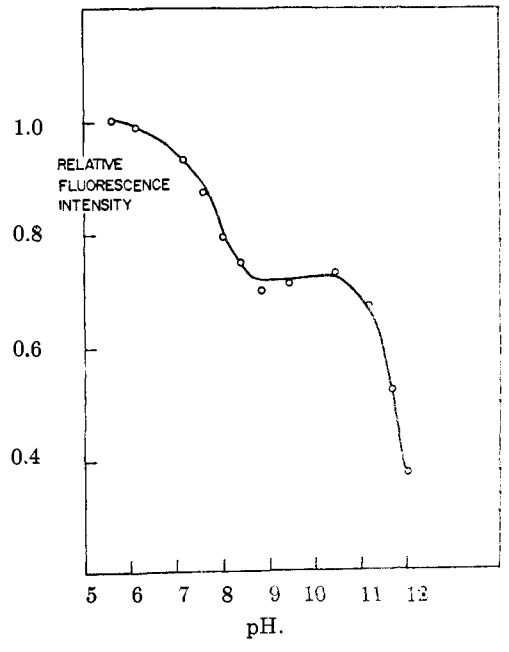

Fig. 11.—pH profile of relative intensity of sensitized fluorescence of the DNS conjugate of bovine serum albumin of figure (6). The wave lengths of absorption and of emission are 290 and 520 mμ, respectively (40).

C. ENERGY TRANSFER IN HEME PROTEINS

The heme-containing proteins, including hemoglobin, myoglobin, peroxidase, and catalase, possess very efficient built-in acceptors of the emitted radiation of DNS conjugates, as well as of tryptophan. In both cases a high degree of spectral overlap is present. As the heme groups are not fluorescent, exchange is manifested only by a quenching of the primary groups.

Weber and Teale have made a detailed study of the fluorescence of native and conjugated heme proteins (42). The approach utilized was to compare the fluorescence efficiencies of the intact and heme-free proteins. These were compared with computed curves giving the degree of quenching through transfer as a function of the ratio R_0/D, where R_0 is that separation of primary and acceptor groups for which emission and transfer are of equal probability and D is the diameter of the protein, approximated by a sphere.

In general, for n_1 equal fluorescent centers and n_2 equal quenching centers, Weber and Teale write for the ratio of intensities in the presence (F) and absence (F_0) of quenchers

$$\frac{F}{F_0} = \sum_{i=1}^{i=n_1} \frac{1}{1 + \sum_{j=1}^{j=n_2} (R_0/r_{ij})^6}$$

where r_{ij} is the separation of the ith fluorescent group and the jth acceptor.

To make numerical calculations some assumption must be made as to the distribution of primary groups. If the distribution of fluorescent groups is sufficiently continuous to permit the replacement of summation by integration, we have if only one quencher is present

$$\frac{F}{F_0} = 2\int_0^1 \frac{1}{1 + R_0^6/z^6D^6} F(z)dz$$

where $F(z)$ = fraction of fluorescent centers at distance z from the quenching center ($z = r/D$).

Analogous equations hold for two or more quenchers, if some plausible model is assumed for their positions. Numerical evaluation of these equations, upon the assumption of the random distribution of fluorescent centers, gives F/F_0 as a function of R_0/D.

A comparison of the experimental ratio F/F_0, which is the ratio of intensities in the presence and absence of hemes, with the computed curve permits an estimate of R_0/D and hence of R_0 if the equivalent diameter of the protein is known. Since quenching of the tryptophan fluorescence was virtually quantitative except in the case of peroxidase, only the data for the DNS conjugates could be analyzed.

The values of R_0 obtained in this way were compared with those computed from Forster's equation. The discrepancy in R_0, which amounts to 40% or more, appears to be outside of any systematic errors involved in its more direct determination. Weber and Teale attribute this to the approximate character of Forster's theory (47). However, the data indicate a proportionality between transfer probability and overlap integral, in agreement with the theory.

In the case of the four-heme proteins, hemoglobin and catalase, it was found that the observed dependence of quenching upon the degree of labelling was inconsistent with a model which placed all four hemes at one place. The location of the hemes in two oppositely situated pairs gave better agreement with observation.

It is clear from this work that radiationless transfer to the heme groups is quite efficient and, in the case of tryptophan emission, provides a potential means of photochemical activation. The biological importance of this remains to be assessed.

V. FLUORESCENCE POLARIZATION OF PROTEIN CONJUGATES IN SOLUTION

A. GENERAL REMARKS

The polarization phenomena of fluorescent molecules can be formally accounted for by imagining them to consist of two linear oscillators of absorption and of emission which are clamped together at a fixed angle, α (48–52). For an assembly of such systems, the degree of polarization of fluorescence at 90° to the incident beam is completely determined by the average value of the angle between the direction of the absorption oscillator at the moment of absorption of a quantum of radiation and the direction of the fluorescence oscillator

at the moment of emission. In the absence of Brownian rotation, as in a highly viscous medium, this angle will equal α and the polarization will have its limiting value (3, 4, 48, 49).

If, however, the systems have some freedom of rotation, then the emission oscillator will be swept through a finite angle during the lifetime of the excited state. Obviously, the average value of this angle will depend upon the excited lifetime and the time constant of the rotation. In the limit of very long lifetime or rapid rotation the average angle between the two oscillators will approach that for *random orientation* ($\cos^{-1} 1/\sqrt{3}$) (48). In this case the theory predicts a zero value for the polarization. In intermediate cases polarizations lying between zero and the value for immobilized molecules will be observed.

The parameters which most explicitly characterize the Brownian rotation of a dissolved particle are the rotational relaxation times about its principal axes, which are three in number if the particle can be approximated as an ellipsoid (48, 49).

The rotational diffusion of a spherical particle can be described by a single relaxation time. If each member of an assembly of such particles is imagined to have a particular radial direction which is distinguishable, then the relaxation time of the spheres may be defined in terms of the motion of these radii. If all particles are further supposed to have the same orientation at zero time, then after a finite time interval each radius will, as a result of Brownian rotation, make an angle ϕ with the original direction. The time required for the average value of cos ϕ to attain a value of $1/e$ is the relaxation time, ρ_0, of the spheres.

If the particle is ellipsoidal in shape then three relaxation times, ρ_1, ρ_2, and ρ_3, are required to describe the rotation. These correspond to rotation about each of the three characteristic axes of the ellipsoid. If it is an ellipsoid of revolution, then two of these will be equal.

In the limiting spherical case, $\rho_1 = \rho_2 = \rho_3 = \rho_0$. In this case ρ_0 has been shown to equal $3\eta V/R'T$, where η is the solvent viscosity; V, the molar volume; R', the gas constant; and T, the absolute temperature.

The molar volume, V, here refers to that of the actual kinetic unit, including any bound solvent.

For an ellipsoidal molecule one can also define a mean relaxation time, ρ_h, which is equal to the harmonic mean of the three relaxation times about the principal axes, $(3/\rho_h = 1/\rho_1 + 1/\rho_2 + 1/\rho_3)$.

Any deviation from spherical symmetry always increases ρ_h. For a rigid ellipsoid of revolution, which is impermeable to solvent, the ratio ρ_h/ρ_0 is a function of axial ratio alone (48).

It probably is unnecessary to point out that no real protein molecule can be expected to conform exactly to the idealized model described above. The best that can be expected is that the apparent value of ρ_h suffices to describe the behavior of the effective molecular domain. This problem, of course, reoccurs for all the hydrodynamic methods. The observed polarization of a system of fluorescent conjugates can normally be described in terms of ρ_h, τ, and the mutual orientation of the axes of absorption and emission and the characteristic axes of the attached particle, approximated as an ellipsoid.

The basic theory for the polarization of fluorescence of molecules in solution was developed in essentially complete form by Perrin (3, 50, 51, 52). More recently, Weber has developed an alternative approach which leads to essentially the same conclusions (48, 49).

Only the results of Perrin's theory will be cited in this section (50, 51, 52). The basic assumptions of his derivation are threefold: (a) The solvent may be regarded as a continuum. (b) The actual molecular domain of the macromolecule may be approximated by an ellipsoid. (c) The ellipsoids are completely rigid and possess no internal degree of rotational freedom.

We consider the degree of polarization of the fluorescent radiation in a direction 90° to the incident beam, which will be assumed to be unpolarized. In this case Perrin's relationships take the form, for unpolarized incident light

$$V\text{-}(1) \qquad \frac{1}{P} + \frac{1}{3} = \frac{10\,(A + 2B)\,(1 + 12\theta\tau + 36\sigma^2\tau^2)}{3\left\{G + \Sigma[2\,G\theta_i + F_i(\theta_j - \theta_k)]\tau - 9\sum \frac{D_i(\theta_j - \theta_k)^2\tau^2}{1 + 3(\theta_i + \theta)\tau}\right\}}$$

For vertically polarized light, $1/P + 1/3$ is replaced by $1/P - 1/3$ (48, 49). Here P is the degree of polarization; $A = \Sigma A_i$; $B = \Sigma B_i$; $D = \Sigma D_i$; $G = A - B + 3D$; $F_i = (A_j + 2B_j - 3D_j) - (A_k + 2B_k - 3D_k)$; $A_i = \alpha_i^2 f_i^2$; $2B_i = \alpha_j^2 f_k^2 + \alpha_k^2 f_j^2$; $D_i = \alpha_j \alpha_k f_j f_k$; α_i, f_i = direction cosines (with respect to ith axis of ellipsoid) of absorption and emission oscillators, respectively; $\theta = 1/3\ \Sigma\theta_i$; $\sigma^2 = 1/3\ \Sigma\theta_i\,\theta_j$; θ_i = rotary diffusion coefficient of ellipsoid about ith axis. Since $A + 2B = \Sigma\alpha_i^2\ \Sigma f_i^2 = 1$, equation (1) becomes:

$$V\text{-}(2) \qquad \frac{1}{P} + \frac{1}{3} = \frac{10(1 + 12\theta\tau + 36\sigma^2\tau^2)}{3\left\{G + 2G\tau\Sigma\theta_i + \Sigma F_i(\theta_j - \theta_k)\tau - 9\sum \frac{D_i(\theta_j - \theta_k)^2\tau^2}{1 + 3(\theta_i + \theta)\tau}\right\}}$$

Upon expanding in series form and introducing a new parameter P_0, the limiting value of P when $\theta = 0$, equation V-(2) takes the form (16)

$$V\text{-}(3) \quad R = \frac{1/P + 1/3}{1/P_0 + 1/3} = 1 + 2\tau\Sigma\theta_i + \frac{\tau}{G}\Sigma F_i(\theta_j - \theta_k) + \ldots$$

The higher terms are of negligible magnitude for virtu-

ally all cases of interest. The second term is dependent solely upon the molecular parameters of the particle and is, in particular, independent of the orientation of the oscillators of absorption and emission with respect to the axes of the particle.

The third term of equation V-(3) is not, in general, of negligible magnitude and moreover does depend upon the mutual orientation of the fluorescent group and the particle. Since independent information as to the latter is normally lacking, it will usually be possible to utilize equation V-(3) to yield molecular parameters only when the third term is equal to zero. Fortunately, this is by no means a remote possibility.

It is easy to show that the third term of eq. V-(3) is zero if any of the following hold (16):

(a) The particle is spherically symmetrical ($\theta_1 = \theta_2 = \theta_3$)

(b) The axes of emission and absorption are randomly oriented so that $\bar{F}_1 = \bar{F}_2 = \bar{F}_3 = 0$. This case is very likely to be realized for a protein conjugate, where the number of possible sites of attachment is very large

(c) The axes of absorption and emission coincide and are either randomly oriented or else uniformly oriented so that $\alpha_1^2 = 1/3$, provided that the particle is an ellipsoid of revolution ($\theta_2 = \theta_3$)

Case (b) is, in practice, so likely to be true for fluorescent protein conjugates that equation (2) may with considerable confidence be rewritten as V-(4) (since $\rho_i = 1/(\theta_j + \theta_k)$

$$V\text{-}(4) \quad R = 1 + (3\tau/\rho_h)$$

$$1/\rho_h = 1/3\,(1/\rho_1 + 1/\rho_2 + 1/\rho_3).$$

If the particle has spherical symmetry then $\rho_1 = \rho_2 = \rho_3 = 3\eta V/R'T$, where V is the molecular volume. In this case equation V-(4) has the form

$$R = 1 + (R'T\tau/\eta V)$$

In practice this linearity in T/η generally persists for non-spherical particles. Therefore, a linear extrapolation of $1/P + 1/3$ *versus* T/η to $T/\eta = 0$ is usually the most convenient way of obtaining P_0.

B. TECHNIQUE OF FLUORESCENCE POLARIZATION

If τ, the excited lifetime, is known it is possible to compute ρ_h for a labelled protein from values of polarization as a function of T/η. Thus some kind of apparatus for measuring polarization at different temperatures must be available.

The devices described in the literature are divided among two categories. The first and simplest of these is the direct measurement of horizontally and vertically polarized components with a photomultiplier tube and a galvanometer. In practice a light scattering photometer equipped with a Polaroid or Nicol analyzer often has been used (16, 53). The only other modification necessary is the introduction of a cell holder equipped with coils for the circulation of water from a constant temperature bath.

The precision of this method depends upon the stability of the light source and photocell power supply and upon the scale of the galvanometer. Since the measured polarization involves the difference of two numbers the precision tends to decline for small polarizations. With an instrument of very high stability, polarizations have been measured to within 0.002 by this method. A distinct advantage of this technique is the ease with which background fluorescence can be corrected for.

However, most investigators have preferred to use more sophisticated optical techniques. Weber, in his earlier investigations, utilized an Arago compensator in conjunction with a Savart polariscope (15). The compensator consisted of four glass plates which could be rotated about a vertical axis. By the preferential reflection of one component, the degree of polarization of the beam transmitted by the compensator could be altered by rotating it about its axis. At a particular critical angle, whose value depends upon the initial degree of polarization, the transmitted beam has zero polarization. After calibration with a source of variable known polarization the unknown polarization can be obtained from the angle of rotation of the compensator required to abolish polarization. The Savart polariscope, consisting of a Savart plate and a Nicol prism, serves to render the presence of polarization visible by the appearance of interference fringes, which disappear when compensation is complete.

This method has yielded good results in the hands of Weber and others. However, the use of fringe disappearance as a criterion cannot but introduce an intrinsic subjective element into the measurement. In the author's experience, several independent observers often have made widely varying estimates of the endpoint for the same system. Moreover, the presence of any background fluorescence is difficult to correct for in this technique.

Harrington, Johnson, and Ottewill (54) have modified the apparatus of Weber by introducing a quartz biplate and a second compensator between the first compensator and the Savart plate (Fig. 12). The biplate has the effect of producing a divided field. By rotating the second compensator a slight polarization is introduced which results in the appearance of fringes in the two halves of the field. If compensation by the first compensator is complete the two sets of fringes will be matched as to both position and intensity. However, the presence of a slight amount of residual polarization in the beam emerging from the first compensator causes the two sets of fringes to show important differences in intensity. The additional optical elements thus serve

to sharpen the endpoint obtained with the polariscope of Weber.

The polariscope of Harrington, Johnson, and Ottewill undoubtedly represents an improvement over the original version of Weber. However, in the author's experience it still suffers from the usual disadvantages of visual instruments, including a tendency to produce severe eyestrain. For polarizations of the order of those usually observed for protein conjugates (0.05–0.25) it possesses no crucial advantage in precision over the ordinary photoelectric method described earlier. However, for very small polarizations (<0.05) it possesses a definite advantage.

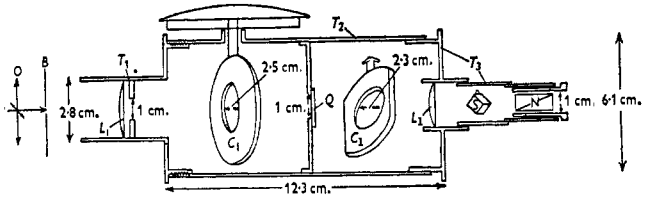

FIG. 12.—The polariscope of Harrington, Johnson, and Ottewill (54): T_1, T_2, T_3 are tubular brass sections; L_1 and L_2 are lenses; C_1 and C_2 are compensators; S is the Savart plate; N is a Nicol prism; Q is a quartz biplate.

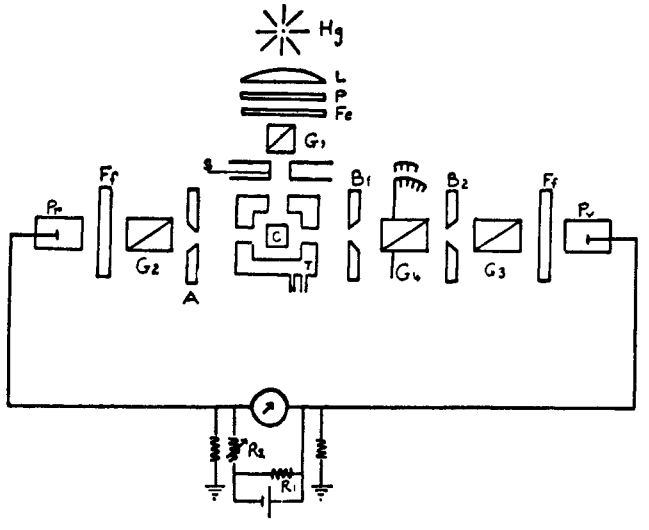

FIG. 13.—The photoelectric polarization photometer of Weber (55): G_1, G_2, G_3, G_4 are Glan–Thomson prisms; F_e, F_f are complementary filters; A, B_1, B_2 are apertures; P_v and P_h are photomultiplier tubes; T is a cell-holder.

Weber subsequently has developed an elaborate photoelectric instrument which differs from the simpler photoelectric devices described above in that an invariant reference signal from one of two photomultipliers is compared with a signal from the second photomultiplier arising from the vertically or horizontally polarized components of the emergent fluorescent beam (Fig. 13). Essentially, the two outputs are balanced for the smaller of the two polarized components by a set of variable slits. By rotation of the polarizer the output of the second photomultiplier is then altered to that corresponding to the other direction of polarization. The outputs are then rebalanced by rotation of one (G_4) of two analyzers in series before the second photocell, thereby varying the intensity of light striking it. A spot galvanometer serves as a null point indicator. By the use of the known relationship between the angle of rotation of the two analyzers and the reduction of intensity, the ratio of the two polarized components may be computed (55). The plane of polarization of the second analyzer (G_3) is kept fixed. The plane of the polarizer (G_1) is instead rotated by 90° for the two balancings.

The precision claimed for the photoelectric polariscope of Weber (±0.0005) much exceeds those reported for any of the other techniques. It clearly should have distinct advantages provided that due care is taken to eliminate the numerous potential systematic errors arising from the complexity of its optical system.

The measurement of the other parameter needed to compute rotational relaxation times, the excited lifetime, is a somewhat more formidable undertaking. Several techniques have been employed successfully. An essential element in any technique is the production of a modulated light beam whose frequency is comparable in order of magnitude to the reciprocal of the lifetime to be measured.

For excited lifetimes of the order of magnitude usually encountered for fluorescent materials (10^{-9} to 10^{-7} second) the frequencies of modulation required are much too high to be produced by a rotating disc or mechanical shutter. The shutter must have essentially zero inertia. The two most commonly employed methods have used a Kerr cell between crossed Nicol prisms or else a piezoelectric quartz crystal, driven by a voltage varying with high frequency (1). In the former case the light beam is transmitted only by virtue of the induced birefringence which appears and disappears in phase with the applied voltage. In the latter case the crystal acts as a diffraction grating when the field is on and is isotropic when the field is off. If the unmodulated zero order spectrum, which corresponds to the light transmitted when the field is off, is intercepted by a suitable stop, the remaining higher order spectra are modulated at twice the frequency of the applied field.

Once a modulated beam has been produced the problem reduces to the determination of the phase lag introduced by the finite lifetime (1). The earlier devices made use of either two electric shutters in series or else a single quartz crystal used as a double shutter. If the pair of shutters were in phase, then, when the time required to traverse the optical path between them was equal to one-half the reciprocal of their frequency, no light would be transmitted. The intensity of the transmitted beam was measured by a photocell. In practice

TABLE II

ROTATIONAL RELAXATION TIMES[a]

Protein	Mol. wt. (× 10⁴)	Label	Moles label/Mole protein	Solvent, *M*	pH	τ (×10⁸)	ρ_h^∞ (×10⁷)	ρ_h/ρ_0	Ref.
BSA	6.8	DNS	2.7	0.02 PO_4^-	6.8	1.14	1.5	2.3	16
BSA	6.8	DNS	2.3	0.06 PO_4^-	7.1	(1.4)[b]	1.44	2.2	15
BSA	6.8	AS	2.6	0.02 PO_4^-	7.5	1.60	1.3	2.0	16
BSA	6.8	FI	0.08	0.02 PO_4^-	6.8	0.49	1.5	2.3	16
EA	4.5	DNS	2.0	0.06 PO_4^-	7.0	(1.4)[b]	0.88	2.1	15
EA	4.5	DNS	0.45	0.02 PO_4^-	6.7	1.27	0.90	2.2	16
EA	4.5	FI	0.03	0.02 PO_4^-	7.1	0.52	0.86	2.1	16
L	1.5	DNS	0.56	0.02 PO_4^-	6.8	1.22	0.28	2.0	16
L	1.5	FI	0.14	0.02 PO_4^-	7.5	0.45	0.26	1.9	16
STI	2.0	DNS	1.1	0.1 OAc^-	4.1	(1.4)[b]	0.38	2.0	35
GA	7.1	DNS	..	0.001 Na Versenate	7.5	(1.4)[b]	1.6	2.4	66

[a] The symbols used have these meanings: BSA, bovine serum albumin; EA, egg albumin; L, lysozyme; STI, soy bean trypsin inhibitor; GA, G-actin; DNS, 1-dimethylaminonaphthalene-5-sulfonyl chloride; FI, fluorescein isocyanate; AS, anthracene sulfonyl chloride. [b] Assumed value.

the length of the light path first was varied until a minimal transmitted intensity was attained with the beam deflected by a diffusely reflecting surface. Then the reflecting surface was replaced by the fluorescent material and the path length again varied until minimal transmission was attained. The excited lifetime could be computed from the difference between the two lengths.

More recent variants of the basic technique have determined the phase lag directly. In the apparatus of Steiner and McAlister the modulated beam passes successively through two square cells, each adjacent to a photomultiplier. The output of the first cell is fed through a phase shift unit consisting of a multiswitch arrangement permitting known lengths of transmission line to be thrown in or out of the circuit. Phase shift can be computed from the length of line L added to or subtracted from the circuit by the relation

$$V\text{-}(5) \quad \phi = 2\pi f \epsilon^{1/2} L/c$$

where f = frequency of modulation; ϵ = dielectric constant of line dielectric; and c = velocity of light *in vacuo*.

The transmission lines terminate in a mixer circuit which couples the mixed signal from the two photocells into the input of a tuned receiver. The receiver serves as a very sensitive and selective output detector.

The actual measurement consists of varying phase until a minimum combined signal is obtained with Ludox in both cells. Then the fluorescent material is placed in the second cell and the phase readjusted until a new null is attained. The difference in phase between the two nulls, as computed from equation V-(5) is related to the excited lifetime by $\phi = 2\pi f\tau$.

The Steiner–McAlister apparatus is based upon an earlier instrument developed by Ravilious, Farrar, and Liebson (56). Bailey and Rollefson have used an electrical phase shift unit in place of a variable transmission line length (57).

C. VALUES OF EXCITED LIFETIME

Determinations have been made by Steiner and McAlister of the excited lifetime of a series of DNS and fluorescein conjugates of serum albumin, ovalbumin, and lysozyme (16). The values obtained are cited in Table II. For the examples studied there appears to be little variation in τ with activation wave length or from protein to protein. In the case of anthracene conjugates the values cited vary from protein to protein and also differ greatly from most of the literature values for anthracene itself. However, the variance of the latter is so large that the correct value of τ for anthracene itself must be left open for the present.

In general a value for τ of 1.2×10^{-8} probably can be assumed safely for an uncharacterized DNS conjugate. However, as was mentioned earlier, there may be an exception to this in the case of α-chymotrypsin (58). Massey, Harrington, and Hartley have found an apparent dependence of ρ_h/τ upon the degree of labelling, which they attributed to an actual dependence of τ upon the extent of conjugation. The effect was disconcertingly large, the apparent lifetime decreasing from 1×10^{-8} at very low degrees of labelling to 0.5×10^{-8} for more than three DNS groups per molecule.

However, α-chymotrypsin polymerizes extensively under the conditions (0.01 *M* $PO_4^=$, pH 7.9) utilized by these authors. Thus the limiting value of ρ_h/τ at zero concentration could be obtained only by a very steep extrapolation. The presence of curvature in the plots of ρ_h/τ *versus* concentration probably renders the values of τ cited essentially lower limits. Moreover, the calculation required rather arbitrary assumptions about the shape and axial ratio of the chymotrypsin monomer. A further potential source of error is the presence of internal degrees of rotational freedom.

The above ambiguities aside, it is by no means implausible that the lifetime could vary with extent of labelling in this case, in view of the preferential attach-

ment of the first coupled DNS to a site involved in the active center and the unusual characteristics of the resultant conjugate (23). However, more direct lifetime measurements probably will be required to settle the matter.

D. ROTATIONAL RELAXATION TIMES FOR INTACT PROTEINS

Values have been published for the apparent mean rotational relaxation times of a large number of proteins. In the few cases where more than one determination exists for the same protein, the agreement appears to be acceptable. In the cases of bovine serum albumin, ovalbumin, and lysozyme similar values have been obtained with fluorescein and DNS conjugates (16). The existing values are collected and cited in Table II.

In order to eliminate the possibility of the various artifacts discussed in the theoretical section, it is necessary, in principle, to show that the apparent value of ρ_h is independent of both the extent of conjugation and the lifetime of the fluorescent label. Only the three proteins mentioned above have been characterized with this degree of thoroughness. Even in these cases it is questionable whether the available data are precise enough to establish completely the fulfillment of these conditions. Pending the application of these criteria, it is necessary to retain some reservations about the other relaxation times cited. However, it is the opinion of the authors that the errors involved are unlikely to be large.

The problem of relating ρ_h unambiguously to the over-all size and shape of the molecular domain in other than a purely formal way is still essentially unsolved. The ratio ρ_h/ρ_0 invariably is greater than unity, except in those cases where internal rotation is likely to be present. The values of ρ_h/ρ_0 cited in Table II are much too large to be accounted for by any reasonable degree of hydration and can only be attributed to deviation of the protein shape from spherical symmetry.

Unfortunately, it is not at present possible to determine ρ_h by an alternative method for comparison purposes. The method of dielectric dispersion, which was long believed to yield relaxation times directly, is now the subject of considerable controversy as to interpretation.

Some concepts of the problems encountered in this area may be gained by considering the case of bovine serum albumin, whose size and shape parameters probably have been investigated more intensively than those of any other protein. Results from several laboratories agree in assigning a value of ρ_h close to 1.3×10^{-7} (10, 15, 16). If a hydration of 0.15 (g. H_2O/g. protein) is assumed, the prolate ellipsoidal model requires an axial ratio of 4:1 (48, 52).

Krause and O'Konski have succeeded recently in refining the electrical birefringence technique so that it can accommodate proteins of relaxation time as short as 10^{-7} (59). Its application to bovine serum albumin at neutral pH gave a relaxation time of 2×10^{-7} for the long axis. This is in remarkably good agreement with the value of 1.7×10^{-7} obtained by Edsall and Foster using streaming birefringence in 88.5% glycerol solution (60).

The very fact that birefringence can be observed at all is virtually conclusive in ruling out a spherical shape, as proposed by Champagne (61). In principle the observed value of ρ_1, the relaxation time of the long axis would be consistent with either a prolate or oblate ellipsoidal shape. However, a comparison of values of ρ_h computed for the two models using Perrin's equations showed that the prolate shape gave much better agreement with the fluorescence polarization value of ρ_h (52). The shape found to be most consistent with their data was a prolate ellipsoid of axial ratio 6:1 (assuming a hydration equal to 0.3).

The preceding discussion serves to bring out the power of the combined methods of electrical birefringence and fluorescence polarization when applied to this problem. It is the belief of the authors that this kind of approach will find much wider employment in the future.

This discussion would be incomplete without mentioning that the existing low angle X-ray studies are in disagreement with the above models and with each other (62, 63). Of the various widely differing models proposed on this basis, the asymmetric prism of Low bears the closest resemblance to those arising from fluorescence polarization and electrical birefringence.

The hydrodynamic and low angle X-ray data for other proteins are not comparable in completeness with those for serum albumin. Thus, it is not worth while to make a detailed comparison of the fluorescence polarization results with those obtained from the older techniques. While the tabulated values of ρ_h/ρ_0 provide a useful empirical index of the deviations of the shape of the protein from the limiting case of an unhydrated sphere, it is not possible to assign a high order of accuracy to the axial ratios computed therefrom.

E. STRUCTURAL TRANSITIONS

It has been recognized since the earliest studies of the subject that the apparent rotational relaxation time, as computed by Perrin's equation, is sensitive to the presence of internal degrees of rotational freedom and will, in general, correspond to that of the labelled macromolecule as a unit only if the latter is completely rigid. Conversely, any decrease in apparent relaxation time can be used as a direct index of the deviation of the protein from the limiting stage of complete rigidity. Fluorescence polarization can thus be a sensitive indicator of any molecular event which is reflected by a gain in flexibility.

The first example of this kind of application was the observation by Weber that the relaxation time of serum albumin decreased sharply at pH's below 4 and above 11 (15). Indeed, although the original interpretation was in terms of a molecular dissociation, this was actually the earliest demonstration of the now well-studied acid structural transition of serum albumin. The pH-profile of polarization parallels closely those of optical rotation and viscosity. While some controversy continues to exist as to the detailed mechanism of this process, agreement is general that it is reflected by a change in conformation, a loss in rigidity, and an ionic strength-dependent inflation of the molecular domain.

The original observations of Weber subsequently were confirmed and extended by Harrington, Ottewill, and Johnson, who interpreted the fall in ρ_h below pH 4 as arising from a "rotational dissociation" into discrete, freely rotating sub-units (10). However, there is no reason to prefer this model to one involving a wide spectrum of relaxation times.

Weber has failed to find any appreciable change in the polarization of ovalbumin conjugates between pH 2 and 12 (15). This is in harmony with the absence of any evidence for a structural change of this protein within these limits. Similarly, no appreciable dependence of the polarization of lysozyme DNS conjugates upon pH has been observed between the pH limits 3 and 11. This is again consistent with conclusions reached by other methods, which have failed to detect any structural transition in this range (43).

Weber has observed a major drop in the value of ρ_h for serum albumin in the presence of high concentrations of urea. However, the thermal denaturation of ovalbumin or serum albumin resulted in an increase of ρ_h (15). The dominant factor in these cases is clearly aggregation.

Steiner and Edelhoch have made a detailed study of thyroglobulin conjugates over a wide range of denaturing conditions (34). Depending upon the nature and concentration of added reagent, almost any level of molecular organization can be attained, from the essentially completely rigid state of the intact molecule to a state approaching that of a random coil.

The molecular events which have been detected by fluorescence polarization and other methods are of two kinds. The first of these is a fragmentation into half molecules, which can be further split under more drastic conditions. However, the additional splitting is only found under conditions where processes of the second kind are occurring (34). These latter correspond to varying degrees of disruption of the internal fine structure of the molecule and occur at extremes of pH and at high levels of urea, guanidine, or detergent.

The relaxation time was found to fall continuously at pH's above about 10. However, much of the drop was accounted for by the molecular splitting although a value of ρ_h/ρ_0 close to 0.83 indicated some degree of loss of rigidity at pH 12.2.

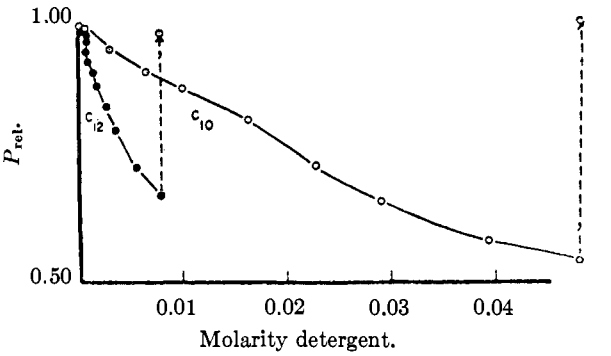

FIG. 14.—The variation of relative polarization with detergent concentration for a DNS conjugate of thyroglobulin (6.1 g./l.) in 0.01 *M* KNO_3, pH 7.1, 25.1° (34): ⊙ Na decyl sulfate; ● Na dodecyl sulfate. The vertical dashed lines indicate the reversal occurring upon thirtyfold dilution with 0.01 *M* KNO_3.

The action of urea and detergent was very different from the above (Fig. 14). In the presence of high levels of urea or sodium dodecyl sulfate (SDS) the fall in ρ_h at neutral pH is much greater than expected for the observed change in molecular weight, so that the ratio ρ_h/ρ_0 drops as low as 0.1. Clearly most of the internal rigidity of the molecule has been lost under these conditions.

That all molecular organization has not vanished is indicated by the profound further drop of ρ_h which occurs at alkaline pH in 8 *M* urea. Indeed, at pH 12 in 8 *M* urea, the value of ρ_h is so low (1.4×10^{-8}) as to suggest that the thyroglobulin molecule is approaching the limiting state of a random coil.

An interesting aspect of these findings is the surprising capacity of thyroglobulin to recover from major distortions of its structure, at least by the criterion of internal rigidity. Thus, the SDS and urea profiles of polarization at neutral pH were essentially reversible. However, the combined action of urea and alkaline pH was incompletely reversible. While the fluorescence polarization results for this system were basically in accord with those obtained by viscosity and optical rotation, fluorescence polarization appears in general to be somewhat more sensitive than the other two criteria and responds to levels of molecular disorganization insufficient to permit any gross inflation of the molecular domain or any important loss in helical content.

A similar study recently has been made of rabbit and bovine γ-globulin (64). Here also there is a continuous gradation of molecular state from the highly organized to the near-random coil. However, the process is not complicated by the occurrence of molecular scission.

A drop in ρ_h occurs at pH's outside the limits 4–9. The alkaline transition is somewhat more pronounced than the acid. The order of occurrence of the various processes is as follows. The earliest change observed is a

loss in molecular rigidity, indicated by a drop in ρ_h, which begins at about pH 9.0. At about pH 10.0 some inflation of the molecular domain, as reflected by an increase in viscosity, begins to be observed. Finally, at pH 11 a major and incompletely reversible change in conformation occurs which is accompanied by a change in optical rotation and a loss in solubility.

The presence of urea, SDS, or the cationic detergent trimethyldodecylammonium chloride (TDAC) produces a major drop in ρ_h. The combined action of urea or TDAC and alkaline pH results in the loss of almost all rigidity. Thus, in 9 *M* urea at pH 12, the value of ρ_h/ρ_0 is as low as 0.04.

Massey has examined the polarization of fluorescence for DNS conjugates of the enzyme fumarase (65). The value of ρ_h^{20} was found to undergo dramatic changes with temperature and in the presence of severe inhibitors of enzymatic activity. Thus in the presence of substrate in 0.06 *M* phosphate, pH 6.35, the apparent value of ρ_h^{20} decreases sharply at about 18°. The fall in ρ_h^{20} parallels a change in the activation energy of the enzyme-catalyzed reaction.

The addition of the potent enzyme inhibitors thiocyanate or transaconitate reduces ρ_h^{20} to about half its value for the intact molecule. In the case of thiocyanate, but not of transaconitate, this drop is accompanied by a molecular splitting. The action of transaconitate is thus apparently confined to the disruption of internal structure.

The limited number of available examples is sufficient to show the sensitivity and power of this approach. Indeed, it may well be that fluorescence polarization will find its area of greatest utility as a structural probe.

F. PROTEIN ASSOCIATIONS

Another area where the fluorescence polarization technique offers a high potentiality is that of protein associations. A change in the state of association of a fluorescent protein conjugate may be reflected by changes in emitted intensity, in polarization, or both. Either effect may, in principle, provide a means of following the association process.

In general, an increase in the size of the kinetic unit to which a fluorescent label is attached will result in an increase in the measured rotational relaxation time. As a qualitative means of detecting association, this approach has several advantages, including the capacity of observing a single component of a complex mixture.

For an association of two different proteins, one of which is labelled, it is often possible to relate the observed polarization to the fraction of the labelled reactant which is uncombined (43). The starting point is the equation of Weber for the polarization of a mixture of molecules of different size carrying the same fluorescent oscillator (eqn. 8, ref. 49)

$$\frac{1/P + 1/3}{1/P_0 + 1/3} = R = \frac{1}{\Sigma x_i/(1 + (3\tau/\rho_i))}$$

where x_i is the fraction of the total emitted intensity which arises from species i. The parameter x_i is equal to the product of the mole fraction of the labelled component present in species i and a factor accounting for any change in fluorescence intensity accompanying combination.

Upon inversion and rearrangement one ultimately obtains: $X_f = R_f(\bar{R}_c - R)/R(\bar{R}_c - R_f)$, where X_f is the fraction of the intensity arising from the labelled component which is uncombined and R_f, $\bar{R}_c$ are the values of R for the *free* labelled species and the average value of R for the complex species, respectively.

In general $\bar{R}_c$ will be independent of composition and hence of X_f only if both components are monofunctional or else if the unlabelled component has a relaxation time very long in comparison with τ, so that $\bar{R}_c = 1$. If the labelled component is monofunctional and of relaxation time short in comparison with that of the unlabelled, it may be a legitimate approximation to regard $\bar{R}_c$ as effectively constant.

The parameter X_f may be converted into y_f, the mole fraction of free reactant, by the relation $y_f/(1 - y_f) = x_f w/(1 - x_f)$, where w is the ratio of the fluorescent intensities in the combined and free states.

If the labelled component is monofunctional, it is possible to analyze the data for y_f as a function of composition and total concentration by one of several straightforward procedures to obtain values of the consecutive association constants (43).

The sole example at present of the quantitative application of fluorescence polarization to the problem of computing consecutive equilibrium constants is a study of the electrostatic interaction of lysozyme with bovine serum albumin and with deoxyribonucleic acid (DNA). Interaction with albumin was found only when the two proteins were oppositely charged, in the pH zone bounded by the isoionic points of serum albumin (5.3) and lysozyme (9.5). At sufficiently high levels of electrolyte the interaction was abolished and the polarization of labelled lysozyme was independent of the presence of albumin over the entire pH range (43).

Interaction of lysozyme with DNA was much stronger for comparable conditions. In this case there are no data obtained by another technique available for comparison.

Dandliker and Feigen have described briefly the use of fluorescence polarization in studying the association of labelled ovalbumin with its rabbit antibody (42). While the expected increase of polarization in the presence of antibody occurred, no quantitative analysis of the interaction is available as yet.

Most applications of fluorescence polarization to this kind of problem have been as a purely qualitative index of association. Massey, Harrington, and Hartley ex-

amined the polarization of DNS-conjugates of α-chymotrypsin in 0.01 *M* phosphate, pH 7.9 (58). It was found that the increase in ρ_h with concentration was much too large to be attributed solely to the dimerization found by Schwert and Kaufman at more acid pH and higher ionic strength. Sedimentation measurements subsequently verified that, under the conditions of these experiments, the self-association of α-chymotrypsin takes the form of a monomer–polymer equilibrium in which the aggregates of low degree of polymerization, as the dimer and trimer, are present in very low concentrations, if at all.

It is of interest that Massey, Harrington, and Hartley also have found ρ_h to be concentration-dependent in the case of chymotrypsinogen under the same conditions, revealing an association process hitherto unrecognized.

Steiner and McAlister have examined the self-association of insulin under a wide range of conditions (33). The value of ρ_h was found to decrease at extremes of pH, as would be expected from earlier studies. Again in harmony with earlier work, ρ_h was found to increase with ionic strength at constant pH and protein concentration.

The presence of urea, ethanol, or acetic acid in high concentration results in a major drop in the limiting value of ρ_h at low protein concentration. This probably reflects primarily the splitting of the 12,000 units into 6,000 units under these conditions. In addition, part of the drop in ρ_h may arise from a disruption of internal structure by these reagents.

Fluorescence polarization also has been used to follow the interaction of trypsin with soy bean inhibitor (34). The extent of interaction was found to decline sharply between pH 4.5 and 3.0. Dissociation of the complex species was favored by the addition of ethanol or by an increase in ionic strength. To the extent that these results overlap those of other studies, they are in basic agreement with them.

The same cannot be said of the work of Tsao upon the polymerization of actin (66). In brief, the apparent relaxation time of F-actin was found to be much too low to be consistent with a completely rigid molecule of the order of molecular weight ($>10^6$) found for F-actin by other techniques. It corresponded, in fact, to the value expected for a dimer of the monomer, G-actin (mol. wt. 6×10^4). Tsao therefore concluded that the polymerization did not proceed beyond the dimer. The light scattering, birefringence, viscosity, and sedimentation results upon F-actin were believed to be artifacts, arising from some form of intermolecular interaction.

Actually, as has been pointed out by Weber (48), it is possible to bring these results into register with the more conventional model for F-actin simply by postulating a minor degree of departure from complete rigidity. The existence of some free rotation between the G-actin sub-units could easily account for the low relaxation times observed.

Somewhat more difficult to explain is the observation of Tsao that ρ_h for F-actin *decreases* upon the addition of myosin and the formation of the actomyosin complex. In the presence of ATP the relaxation time of F-actin is restored. The interpretation of these findings must remain open for the present.

VI. The Use of Fluorescent Protein Conjugates as Histochemical Stains

A. Immunological Staining

Fluorescent protein conjugates have found perhaps their most widespread and fruitful biological application as highly sensitive and specific histological stains (67). This is an immuno-histochemical technique which originates from the finding that fluorescent antibodies, if carefully prepared, retain to a major extent both their activity and their specificity.

There is usually little difficulty in obtaining antibodies to a given microörganism or cellular component. The injection of a foreign antigen into a rabbit or other animal usually is followed, after an interval of several weeks, by the appearance of antibodies to the injected material in the γ-globulin fraction of the plasma. The entire γ-globulin fraction, including the antibodies, can be treated with a suitable fluorochrome in the usual manner and thereby converted to the fluorescent conjugate. It is usually unnecessary to make a preliminary separation of the antibody from the inert protein. The most useful fluorescent labels are those for which the activation and emission bands are widely separated, in particular those possessing activation bands in the ultraviolet. Fluorescein and rhodamine B conjugates have been used for most investigations thus far.

The fluorescent antibody conjugate thereby produced provides an intense stain whose specificity is limited only by the immunological specificity of the system. When illuminated by a suitable ultraviolet source, the loci of antibody attachment appear as bright areas in a dark background. In this manner the fluorescent antibody preparation can be used as a specific visual indicator for the antigen, either as a cellular constituent *in vivo* or in the form of a suspension or solution *in vitro*.

Needless to say, this method does not lack its own characteristic difficulties and drawbacks. The antigen may fail to induce an adequate production of antibodies. If the antigen carries impurities which are also antigenic, a variety of antibodies will be produced. This may largely vitiate the specificity of the staining technique.

The identification of a particular tissue antigen may be carried out in lyophilized or frozen sections, in smears, or in tissue culture preparations. In the case of insoluble

antigens the preparation may be stained directly with antibody. If the antigen is soluble a preliminary fixation generally is required with an agent which leaves its antigenicity intact.

There are two general variations of the fluorescent antibody staining technique, as applied to whole tissue. The first of these is the direct application of a labelled antibody preparation which is specific for the antigen to be identified (67). An alternative procedure involves two consecutive interactions (68). The antigen is first treated with nonfluorescent antibody, with subsequent application of a fluorescent preparation of antibodies directed against γ-globulin itself. These combine with the fixed antibody which is itself a γ-globulin. This latter variant has been called the double layer or "sandwich" method. It has the advantage of requiring only a single preparation of fluorescent antibody.

B. APPLICATION OF STAIN

The fluorescent conjugate is applied directly to the specimen, such as a tissue section, as a drop. The time required for reaction is variable and may be as long as an hour or more. Excess antibody then is removed by washing with buffered physiological saline, pH 7.0. After the use of a nonfluorescent glycerol mountant, the material is suitable for direct observation by fluorescence microscopy.

Activation of fluorescence normally is *via* one of the ultraviolet bands of the label. Ultraviolet activation eliminates the visual background of scattered or reflected light. A very strong ultraviolet source is of course required. A high pressure mercury arc has been most frequently used. An ultraviolet filter, cutting off radiation of wave length above 4000 Å., is used in conjunction with the arc source. Under favorable conditions the sensitivity of this technique is quite comparable to that obtainable with the use of radioactive isotope tracers. It has been shown that antigen in concentrations as low as 10 μg./ml. can be detected.

C. THE DISTRIBUTION OF NORMAL CELLULAR COMPONENTS

Fluorescent antibodies have found extensive application in the location of particular homologous proteins. The findings to date have been of vital importance in several sectors.

Gitling, Landing, and Whipple have used this technique to identify a series of plasma proteins in human tissue sections (69). These included serum albumin, γ-globulin, β-lipoprotein, and fibrinogen. The distribution of each of the antigens proved to be quite wide. Serum albumin and γ-globulin were identified in many cells, especially connective tissues, lymphatics, and blood vessels; β-lipoprotein was found in the nuclei of all cell types. Fibrinogen occurred mainly in the lymphatic and vascular channels, connective tissue, and the interstitial spaces.

Lacy has employed fluorescent antibodies to beef insulin to identify this protein, or its precursor, in the β granules of the islets of Langerhans of several species (70). The β cells of the rat, dog, cat, rabbit, and chicken were all stained with antibodies to insulin, suggesting that the endogenous insulins of these species are immunologically similar to beef insulin. On the other hand, the β cells of the guinea pig did not cross-react, indicating that a difference exists in this case.

Specific staining with labelled antibodies was used by Marshall to study the distribution of several enzymes and enzyme precursors in the acinar cells of bovine pancreas (71). Antibodies to chymotrypsinogen, procarboxypeptidase, deoxyribonuclease, and ribonuclease were produced and converted into their fluorescein derivatives.

Thin sections of beef pancreas were prepared, fixed, and stained with labelled antibody. The staining pattern of each of the antibodies was sharply specific. No evidence was found for any specialization among the acinar cells with respect to zymogen synthesis and storage.

The localization of an auto-antigen in the thyroid gland has been accomplished by White, using a fluorescent antibody technique (72). It is known that the sera of patients with Hashimoto's disease contain an antibody reactive in the precipitin test with a saline extract of normal thyroid or with purified thyroglobulin. By staining with labelled antibodies obtained from the sera of patients with Hashimoto's disease, White was able to identify the auto-antigen, presumably thyroglobulin, in tissue sections of the thyroid. The antigen was present in most of the thyroid follicles in all cases and in the hypertrophic follicular epithelial cells from toxic thyroids.

Immuno-histochemical methods have the potentiality of detecting an immunological similarity of different cellular elements which contain a common precursor. Went and Mazia have found evidence by this method for the occurrence of such a precursor in the mitotic apparatus of sea urchin eggs (73).

The enzyme elastase has been located in porcine pancreas by Moon and McIvor (74). Rabbit antibodies to porcine elastase were prepared and shown to be specific. An indirect staining procedure was used. Pancreas sections were fixed and treated successively with unlabelled anti-elastase and fluorescein-conjugated anti-γ-globulin. The results were very clear-cut and indicated that elastase occurred only in the acinar tissue of porcine pancreas. No elastase was present in the islets of Langerhans.

A fluorescent antibody approach has led to the demonstration of a common connective tissue antigen in the basement membrane, reticulum, sarcolemma,

and neurolemma of several organs of the rat (75). A parallel study succeeded in localizing the kidney antigen responsible for the artificial disease ("nephrotoxic nephritis" (76). A subsequent extension of this work, using a double layer staining technique, demonstrated the fixation of the antibody in the kidney glomeruli (77).

Another example is furnished by a study of the cytological distribution of the lipopolysaccharide Forssman antigen of several species (78).

Rappaport has prepared fluorescent derivatives of skin-sensitizing human antibodies and used them in studies of the antigen–antibody interaction at the challenged skin site (79).

As final examples of the use of fluorescent antibodies to detect specific normal cellular components, we might cite the location within the pituitary of adrenocorticotropic hormone (80) and Raben human growth hormone (81).

D. STRUCTURE OF THE MYOFIBRIL

The elegant studies by Holtzer, Marshall, and others upon the architecture of the myofibril deserve special consideration as perhaps the most successful and interesting of the many recent efforts to bridge the gap between molecular biology and cytology (82, 83).

The objective of these studies was the localization within the sarcomere of chicken muscle of the principal protein antigens. Rabbit antibodies to the three major protein components of the myofibril-actin, myosin, and tropomyosin—were isolated and conjugated with fluorescein isocyanate. Staining and observation were carried out in the usual manner.

The basic results can be quickly summarized. Antibodies to myosin stain the A-band region of the sarcomere, in harmony with earlier evidence for the localization of myosin in this band. Some variation in staining pattern was noted, particularly for the center of the A band.

Antibodies to actin stain the I-band and the M-line, but leave the greater part of the A-band unstained. Anti-tropomyosin antibody stains only the I-band weakly.

A major complicating factor in structural studies of this kind is the possibility of artifacts arising from antigenic impurities in the protein preparations. If antibodies are produced to these, the specificity of the fluorescent antibody preparation can be severely blurred. The ordinary unbound antigens can be recognized easily from their behavior in gel diffusion. These do not appear to be a problem in the present case. A more severe difficulty is presented by the bound antigens, which can be very difficult to recognize.

Considerable effort has been devoted to identifying the known myosin fragments within the myofibril. Antibodies were prepared to the L- and H-meromyosins. The staining pattern of glycerol-extracted chicken muscle fibril showed that the L-antigens appeared to be concentrated in the lateral parts of the A-band region, while the H-antigens were predominantly at or near the M-line in the center of the A-band. While the H- and L-patterns are roughly complementary, there is some degree of overlap.

It is not easy to bring these results into register with what is known of the dimensions of the myosin molecule in solution. To do so requires the assumption that the myosin molecule *in situ* is either dissociated into fragments or else greatly extended. More work will be needed to remove the anomaly.

Holtzer subsequently attempted to extend the fluorescent antibody studies to living muscle fibers (82). However, preliminary results appear to indicate that only damaged fibers can admit antibody.

Labelled anti-myosin antibodies have been used to analyze the development of trunk myoblasts in the chick embroyo. Specific staining by antimyosin occurred only in differentiating myoblasts. Cross-striated myofibrils appeared first in stage 16 to 17 embryos (84).

E. LOCALIZATION OF INJECTED ANTIGEN AND SITES OF ANTIBODY SYNTHESIS

The earliest application of the fluorescent antibody technique to the problem of detecting the areas of concentration of foreign antigen was the pioneering investigation of Coons, Creech, Jones, and Berliner upon the distribution of pneumococcal antigen in tissues (7). This work subsequently was refined and extended by Kaplan, Coons, and Deane, who examined the distribution in mice of types II and III pneumococcal polysaccharide (85). This proved to be unexpectedly widespread. The most constant and heavy concentrations of the antigens were in the cells of the reticuloendothelial system, the capillary endothelium, and fibroblasts throughout the body. Other sites included the hepatic cells, cardiac and smooth muscle cells, lymphocytes, and various steroid-forming cells. The persistence of antigen was found to be quite long—of the order of several months. Quite similar behavior was found for the capsular polysaccharide of the Friedlander bacillus, type B (86).

In contrast, the type-specific polysaccharide of *Streptococcus pyogenes* showed very slight retention by the mouse, being excreted rapidly in the urine (87).

Fluorescent antibodies also have been used to locate injected protein antigens in mouse tissue (88). Ovalbumin, bovine serum albumin, and human γ-globulin have thereby been found in the reticulo-endothelial system, connective tissue, vascular endothelium, spleen lymphocytes, and epithelium of kidney tubules.

By ingenious adaptations of the fluorescent antibody technique, White has been able to confirm the con-

clusion that single cells from lymph nodes, when stimulated simultaneously with two antigens, form antibody to one or the other, but never to both (89).

One approach used was to expose the previously stimulated cells to a mixture of both antigens and subsequently to the labelled antibody to one antigen. After the distribution of fluorescent cells had been photographed, the fluorescent antibody was bleached photochemically and the process repeated, using the labelled antibody to the second antigen.

The second approach was to treat the stimulated cells with a mixture of antigens and then with both antibodies simultaneously. The two antibodies were conjugated with different labels, including fluorescein, DNS, and rhodamine B.

Both methods were in substantial agreement in establishing the selective response of individual cells.

Ortega and Mellors have made a study of the cellular sites of formation of γ-globulin in lymphatic tissues (90). A direct staining technique was used. The findings indicated that γ-globulin is synthesized in the germinal centers of lymphatic nodules and in the cytoplasm of mature and immature plasma cells—both those with and without Russell bodies.

The cells found to form γ-globulin appear to be identical with those which synthesize specific antibody. Thus it is likely that, if a normal, non-immune γ-globulin exists, it originates in the same cells which produce antibody.

F. THE LOCATION OF INFECTIOUS AGENTS IN ORGANS OR TISSUES

Fluorescent antibodies offer by far the most powerful and rapid approach to the identification of a viral agent directly in the attacked organism. The technique does not differ basically from that used to locate other antigens.

Thus Noyes and Mellors have succeeded in detecting by this method the viral antigens of the Shope papilloma virus in papillomas of the wild rabbit (91). In the wild rabbit papillomas the Shope viral antigens occurred solely in the nuclei of differentiating cells of the keratohyaline layers and in the keratinized layers. No antigens were detected in the proliferating cells. The authors attributed this surprising result to the existence of the virus in early, non-antigenic form.

Boyer, Denny and Ginsberg have studied the infection of HeLa cells by adenovirus (92). The host cell nuclei were shown to be the principal sites of adenovirus synthesis. The occurrence of type-specific adenovirus antigen was noted in the characteristic intranuclear inclusions.

Similar methods have been used to determine the distribution of viral antigens in frozen sections of wing tumors produced in chickens by initiating doses of Rous sarcoma virus (93). It was estimated that a quantity of virus approaching one tumor-producing dose was detectable by these means.

Watson has used specific staining to follow the fate of mumps virus in the embryonated egg (94). The multiplication of virus was limited to cells which came into contact with infected fluid. Following intraamniotic inoculation into eight-day embryos, these included cells lining the amniotic membrane and the epidermal and pharyngeal epithelium.

Coffin, Coons, and Cabasso have made a histological study of the progress of infectious canine hepatitis (95). The intranuclear inclusions characteristic of this disease were found to contain high concentrations of viral antigen. The multiplication of virus began on the nuclear membrane and spread to the interior of the nucleus with the gradual formation of larger granules.

The influenza virus infection of the chick embryo also has been investigated (96). The major sites of multiplication of PR 8 and Lee B strains of influenza virus in chick embryos injected by the amniotic route were in cells lining the amnion and in the epidermal and pharyngeal epithelium.

The specific influenza viral antigens have been detected in infected ferrets by Liu, using fluorescein-labelled antibodies (97). This technique also has been used by Liu for rapid identification of influenza infection by direct staining of nasal washings (98).

Rhodamine B-labelled antibodies to influenza A were used to show the presence of virus in the brain tissue of mice in the early stages of infection by Fraser, Nairn, McEntegart, and Chadwick (99). Injection of both neurotropic and non-neurotropic strains resulted in the early appearance of specific staining in the membranes and parenchyma of young mice. In adult mice only the membranes were affected in the early stages of infection. In the later stages fluorescent staining was more generalized. While the morphological lesions detected by conventional methods were stained intensely by the fluorescent antibody, the correspondence was not perfect.

Carski has used immunological fluorescence staining to observe antigen localization during the progress of foamy degeneration of monkey kidney tissue cultures (100).

Burgdorfer and Lackman were able to identify a large number of Colorado tick fever virus isolates in suckling mice injected with suspensions of *Dermacentor andersoni* (101). Sections of quick-frozen brain and heart tissues were stained directly with labelled antibody. Parallel control tests indicated that the staining was specific.

Goldwasser and Kissling have utilized a fluorescent antibody technique to identify rabies in brain smears of infected mice (102). Evidence for the viral origin of Negri bodies was obtained.

Staining was found in both the cytoplasm and nu-

cleus of infected ciliated epithelial cells covering the nasal turbinates.

Purified horse rabies antibody has been prepared and conjugated with fluorescein isocyanate by Etchebarne, Bernal, and Leyton (103). The staining of brain smears from rabies-infected dogs and mice permitted the prompt identification of the virus. The speed offered by the fluorescent antibody technique is of particular value in the case of this disease, where early information is crucial.

G. IDENTIFICATION OF MICROÖRGANISMS IN CULTURES

The use of immunological techniques to identify bacterial cultures has been a standard procedure for many years. The introduction of fluorescent antibodies has permitted the attainment of much greater sensitivity as well as enhanced ease and rapidity of measurement.

As an example of the superiority of the fluorescent antibody method, as applied to this problem, let us consider its use in the grouping of streptococci (104, 105, 106). The conventional methods for grouping by the precipitin test require the cultivation of large quantities of organisms in pure culture for the preparation of an extract. The time required to accomplish this, starting from a throat smear, is from two to four days. A total of three to five days is thus required for identification by the older procedure. By the use of the fluorescent antibody technique this may be shortened to one or two hours.

Moody, Ellis, and Updike have tested strains consisting of stock cultures of groups A, B, C, D, F, and G, all of which previously had been grouped by the precipitin test (104). The globulin fractions of the antisera to each group were isolated and labelled with fluorescein isocyanate.

Smears for testing were made from saline suspensions of broth culture sediment or surface growth of slants. Controls consisted of unstained smears and those stained with labelled normal rabbit globulin.

The observed specificity was high, but not complete. Thus all strains of group A gave a strong reaction with antibodies to group A. About two-thirds of the strains of group C showed reaction with anti-A globulin. Some strains of group G gave a very weak reaction. The remaining groups (B, D, F) gave no reaction. Groups B, D, and F were stained only by their homologous conjugates.

The potential danger of mistaken identification of groups A, C, and G arising from the cross-reactions shown by these groups could be eliminated by adsorption of each antibody preparation with cells of those heterologous groups for which cross-reaction occurred. This procedure completely removed the cross-reaction but did not affect the homologous reaction. In this manner complete specificity could be obtained.

The sensitivity of the technique was such as to permit identification by staining of smears made directly from throat swabs in many cases. Alternatively, smears could be made from six-hour broth cultures inoculated with swabs or from sediment obtained from these.

The identification procedures which have been developed for other microörganisms are in general analogous to those described above. Carter has applied the indirect staining technique to this problem, using a single labelled anti-globulin antibody preparation (107). This variant of the basic procedure avoids the necessity of preparing and storing a large number of conjugates.

Goldman has used a fluorescent antibody technique to stain *Toxoplasmii gondii* organisms in smears of peritoneal exudate (108). The evidence indicated clearly that the staining was due to an antigen-antibody reaction at the cellular level. A variant of this approach was described, which was based upon inhibition of specific staining with fluorescent antibody.

Goldwasser and Shepard employed an indirect staining method to identify antibodies to murine and epidemic typhus rickettsiae in human sera (109). The immunological response of individuals exposed to both was found to be erratic.

Beale and Kacser have examined the fluorescent staining of *Paramecium aurelia* preparations which had been immobilized by placing the organisms in fluorescent antisera (110). The areas of intense staining included the entire surface of the organisms and the clumped tips of the cilia. No fluorescence was seen in the nuclei or cytoplasm, but the food vacuoles were brightly stained.

Finkelstein and Labrec have developed a fluorescent antibody method which permits the rapid recognition and serological identification of small numbers of cholera vibrios (*Vibrio comma*) in human stool suspensions (111). By utilizing selective enrichment it was possible to minimize the non-specific reactions encountered in the direct examination of such suspensions.

Dacres and Groth have devised a fluorescent technique for the recognition of *Erysipelothrix insidiosa*, the etiological agent of swine erysipelas (112). A rigorous system of controls excluded the possibility of any non-specific staining.

Cultures of *Shigella flexneri* have been grouped and typed by Labrec, Formal, and Schneider, using a fluorescent technique (113). By using adsorbed antisera, cross-reactions could be avoided.

The pathogenic yeast *Histoplasma capsulatum*, whose identification often has proved troublesome, has been stained selectively with labelled antibody by Gordon (114).

An indirect staining procedure has been used by Vogel and Padula to detect antibodies to pathogenic

fungi in sera from patients with systemic mycoses (115). The technique consisted of the addition of human sera to a dried smear of the organism tested for, then the addition of conjugated anti-γ-globulin antibodies. Despite some complications arising from the presence of some intrinsic fluorescence of the fungi themselves, the method was entirely successful.

Labelled antibodies to isolates of rumen bacteria have been used to demonstrate their presence *in situ* in rumen contents. Hobson and Mann have found that the occurrence of a specific organism in rumen contents, as detected serologically, paralleled observations upon isolations of the same organism from this source (116).

The detection and identification of coagulase-positive staphylococci have been accomplished by Carter (117). Unadsorbed fluorescent antibodies to types I and II of *Staphylococcus aureus* stained all coagulase-positive cultures of staphylococci, but did not interact with coagulase-negative cultures.

For clinical purposes, when the time factor may be crucial, it is obviously advantageous to be able to identify a microörganism directly in the collected specimen. The possibility has been explored by Thomason, Cherry, and Edwards in the case of Salmonella in faecal smears (118). Cross-reactivity proved to be a serious problem, which in this case could not be resolved completely.

Wolochow has successfully applied the fluorescent technique to the identification of *Pasteurella pestis* (119). In this case DNS conjugates of antibody were substituted for the usual fluorescein conjugates.

Goldman has described the preparation of labelled antibodies to *Endamoeba histolytica* (120). Unadsorbed preparations showed a strong cross-reaction with *Endamoeba coli*. After adsorption by intact *E. coli* organisms the cross-reaction was abolished, without affecting appreciably the staining of *E. histolytica*.

As a final illustration of the power of the fluorescent antibody method as applied to this problem, we may cite the work of Moody, Goldman, and Thomason, who selectively stained 33 strains of *Malleomyces pseudomallei* and three strains of *Malleomyces mallei* (121). It was possible to detect these organisms in concentrations as low as 220 cells/ml. (122).

In addition to the bacteria which have been identified in cultures, fluorescent antibody methods also have been used to demonstrate the presence of virus in tissue cultures. Examples include vaccinia (123), measles (124), Egypt 101 (125), psittacosis (126), and poliomyelitis (127).

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