Fluorescence of solutions: A review

R. T. WILLIAMS AND J. W. BRIDGES

*From the Department of Biochemistry, St. Mary's Hospital
Medical School, London*

The use of fluorescence as an analytical technique in clinical biochemistry and pathology is in the process of developing and it is therefore important that those who use it should understand the underlying principles governing the phenomenon of fluorescence and its application, so that this elegant and sensitive technique can be used to its greatest advantage. Fluorescence has been studied by physicists and photochemists for over a century but the results of these studies have penetrated but slowly into biology and medicine, partly due to difficulties of communication and partly because the compounds, temperatures, and solvents used were of little interest from the biological point of view.

Present-day research in medicine and biochemistry requires many highly sensitive analytical techniques and workers in these fields frequently have to develop their own physical instruments to meet the needs of their researches. This type of development has occurred in the field of fluorescence, for the first commercial spectrofluorimeter (Aminco-Bowman spectrophotofluorometer; see section on fluorimeters) was based upon a model devised by a medically qualified scientist, Dr. R. L. Bowman (Bowman, Caulfield, and Udenfriend, 1955). With this instrument it is possible to measure not only visible but also ultraviolet fluorescence, and to record the wavelengths of maximum fluorescence and of maximum excitation or activation. Some vitamins and drugs have been assayed fluorimetrically for many years (Bowen and Wokes, 1953) but this earlier work was done with filter fluorimeters which are less sensitive and less selective than the modern spectrofluorimeters (or fluorescence spectrometers) and usually only measure visible fluorescence.

Fluorescence is a highly sensitive analytical tool which can be used to measure concentrations as low as 10^{-8} to 10^{-10} g./ml. (0.01 to 0.0001 μ g./ml.) whereas few substances can be estimated colorimetrically below 10^{-7} g./ml. (0.1 μ g./ml.). Since the use of fluorescence in analysis is in its infancy one can expect improvements in instrumentation such as better detectors of fluorescence and better light

sources. There is no doubt that fluorescence analysis will become even more sensitive and more accurate than it is at present.

THE MECHANISM OF FLUORESCENCE

In this section it is proposed to give a simplified account of the origin of fluorescence and to give some guidance as to what sort of molecules one could expect to fluoresce. Fluorescence is essentially an electronic phenomenon and is primarily concerned with light of wavelengths in the region of 200 to 800 m μ . Some compounds when illuminated with light of this region only absorb specific wavelengths of this light and the wavelengths absorbed are characteristic of the particular compound being examined. The extent to which the light of these wavelengths is absorbed constitutes the absorption spectrum of the compound. As a consequence of the absorption of light some of the molecules of the compound become excited because certain electrons in the molecule are raised to a higher energy level. This is shown diagrammatically in Figure 1. The energy levels¹ are represented in Fig. 1 by horizontal lines and the directions of the energy transitions by vertical or inclined arrows. On absorption of light, the molecule is raised from the ground state G to the excited state E as indicated by the vertical arrow A. The molecule returns to the ground state, emitting some of its absorbed energy as fluorescence, as indicated by the arrow marked F. An electronic transition due to light absorption is almost instantaneous (10^{-15} second), whereas the lifetime of the excited state is about 10^{-8} second, and therefore, the whole process of light absorption and fluorescence emission takes place in about 10^{-8} second. Now some of the absorbed energy is lost partly by collisions with other molecules and partly by other means, so that less energy is emitted as fluorescence than was absorbed from the exciting light. According to the quantum theory, light is absorbed in discrete units called quanta and the energy (E) of a quantum is related to vibrational frequency (ν), thus:—

¹To simplify the picture only the lowest vibrational level of each energy level is shown.

FIG. 1. Diagram relating energy levels, absorption, fluorescence, and phosphorescence.

$E = h\nu$, where h = Planck's constant. Frequency is related to wavelength (λ) according to the expression, $\nu = \frac{c}{\lambda}$, where c is the velocity of light.

From these two equations, $E = \frac{hc}{\lambda}$, and, since h and c are constants, E varies inversely as λ . As mentioned above, the energy emitted as fluorescence is less than the light energy absorbed and therefore from the last equation the wavelength of fluorescence is longer than that of the absorbed light.

The mechanism of phosphorescence is also illustrated in Figure 1. This phenomenon is distinguished from fluorescence by the much longer life time of the excited state (which may be up to several seconds). To understand the reason for this difference one must consider the spin of the electrons of a molecule. Electrons in most molecules are found in even numbers and are paired. In each pair, the two electrons spin about their own axes in opposite directions (anti-parallel spins, see below) and, for reasons which need not be considered here, such molecules are said to have a 'singlet' electronic level. When the molecule is raised to the excited state two things could happen: (1) the electrons may remain 'singlet' and the molecule can then return to the ground state with the emission of fluorescence (path F), or, (2) one electron, by some internal energy transition, may have its spin reversed (parallel spins, see below) and the molecule is then said to have a 'triplet' electronic level:—

In Fig. 1, this change is indicated by the top inclined arrow t and the 'triplet' excited state is indicated by the horizontal line T . Such a molecule is regarded to be in a metastable state. The life time of the triplet excited state is longer than that of the singlet excited state, for the molecule has to return

to the ground singlet state by what is known as a 'forbidden transition' which has a low probability. During this return there is emission of phosphorescence as indicated by the inclined arrow P . In the transition from the singlet excited state to the triplet state there is a loss of energy, for the energy level T is lower than E . Therefore, there is less energy emitted in the transition from T to G , so that the wavelength of the fluorescence that would have been produced by the same excitation. Fluorescence is thus an emission from a singlet excited state (electron spin paired) whereas phosphorescence is emission from a triplet excited state (electrons unpaired). Phosphorescence persists longer than fluorescence and this persistence is prolonged and the intensity enhanced by low temperatures. The biological applications of phosphorescence are as yet limited.

FLUORESCENT COMPOUNDS An examination of the literature shows that not all organic compounds are fluorescent. However, those which show fluorescence are usually aromatic or contain conjugated double bonds (*i.e.*, alternating single and double bonds between atoms). Since fluorescence is an electronic phenomenon, it might be expected that those molecules containing electrons which undergo energy transitions readily would be capable of fluorescing. Such electrons are (*a*) the so-called ' π ', 'mobile', or 'delocalized', electrons and (*b*) the 'lone pair' electrons². The nature of π -electrons may be explained briefly as follows. The application of quantum mechanics to molecular theory has shown that substances containing two or more conjugated double bonds (*i.e.*, $-\text{C}=\text{C}.\text{C}=\text{C}-$, etc.) have a certain number of electrons possessing greater mobility than the other electrons (*i.e.*, σ electrons) of the molecule. One of these π -electrons is derived from each carbon atom associated with the double bond, and these electrons form a cloud which circulates the molecule (Fig. 2). Thus benzene, which has six

²The 'lone pair' is a pair of electrons not directly concerned in chemical bonding and they are found particularly associated with N, O, P, and S atoms in compounds. They may interact with π -electrons to increase an electron cloud, but usually on excitation they yield phosphorescence rather than fluorescence (Kasha, 1960).

carbon atoms and the equivalent of three conjugated double bonds, has six of these electrons whose orbits form a cloud above and below the molecule. None of these electrons has any special relation to one particular carbon atom, but all six electrons are equally related to all six carbon atoms. The other electrons of the molecule, termed σ electrons, are localized and tend to be located along the line joining the nuclei of the two participating atoms. The freedom of the π -electrons, however, can be influenced by substituent groups and, in heterocyclic systems, by heteroatoms, *i.e.*, atoms other than carbon, such as O, N, and S, so that the π -electrons become relatively more or less delocalized under their influence.

FIG. 2. Diagram showing the π -electrons of conjugated double bonds.

(a) Formal way of showing conjugated double bonds

(b) The electrons of the bonds

(c) Representation of the π -electrons; the electrons between the C atoms are the σ electrons.

If a compound contains π -electrons there is a good possibility that it will fluoresce, and if a substituent, which increases the freedom of these electrons, is added to the compound, then the substituted compound is likely to be more fluorescent than the unsubstituted parent compound. On the other hand, if the substituent tends to localize the π -electrons, there will be a diminution or abolition of fluorescence. Let us take the simple examples of cyclohexane, benzene, and vitamin A. Cyclohexane contains no conjugated double bonds and is non-fluorescent. Benzene is an aromatic compound and is weakly fluorescent. Vitamin A is not aromatic, but contains five conjugated double bonds and is therefore fluorescent (see below):—

Cyclohexane, non-fluorescent no absorption peak

Benzene absorption peak, 254 $m\mu$; excitation max. 269 $m\mu$ (observed); fluorescence max. 291 $m\mu$ (Bridges and Williams, 1963)

Vitamin A absorption 325 $m\mu$; excitation 327 $m\mu$ (corrected); fluorescence 510 $m\mu$ (Hagins and Jennings, 1959)

It will be noted that the excitation maximum of benzene has a longer wavelength than the absorption peak, whereas the excitation of vitamin A is fairly close to the absorption peak. Theoretically the absorption and excitation peaks should coincide, and the differences that occur are due to instrumental errors largely as a result of the variation in energy output with wavelength of the light source used for excitation (see p. 16 and Fig. 11). These errors can be corrected if necessary and when these corrections are made the excitation maximum is found to be close to the absorption peak.

The effects of substituents upon fluorescence can be illustrated with benzene, aniline, and nitrobenzene. In dilute solutions in water, aniline is 40 to 50 times more fluorescent than benzene, whereas nitrobenzene is non-fluorescent (Bridges and Williams, 1962).

The NH_2 group in aniline tends to activate the benzene ring and thus increases the freedom of the π -electrons. The NO_2 group in nitrobenzene, on the other hand, deactivates the ring by tending to withdraw the π -electrons from the ring thus reducing their freedom by increasing their localization. Now the NH_2 group is one of the classical *ortho-para*-directing groups and the NO_2 group is one of the classical *meta*-directing groups, so that one might conclude that mono-substituted benzenes containing *ortho-para*-directing groups are fluorescent whereas those containing *meta*-directing groups are non-fluorescent. This conclusion is partly, but not wholly, true, as can be seen from Table I.

The data quoted in Table I were obtained in this laboratory (Bridges and Williams, 1962; 1963) with the Aminco-Bowman spectrofluorimeter and it is important to indicate how much of the compounds quoted were needed to produce fluorescence. Benzene, ethylbenzene, and chlorobenzene are weakly fluorescent and with the first two at least 20 to 25 $\mu g./ml.$ was needed to detect fluorescence, whilst with chlorobenzene at least 500 $\mu g./ml.$ was needed. With aniline and phenol, fluorescence could be detected down to concentrations of 0.01 $\mu g./ml.$ The main points about this table are that aromatic compounds containing NH_2 , OH , F , OCH_3 , $NHCH_3$, and $N(CH_3)_2$ groups are likely to be fluorescent, whilst those containing Cl , Br , I ,

TABLE I
FLUORESCENCE OF MONOSUBSTITUTED BENZENES¹ (BRIDGES AND WILLIAMS, 1963)

Compound	C_6H_5R			
	Substituent R	Excitation (λ_{max} , m μ)	Fluorescence (λ_{max} , m μ)	Relative Intensity ²
<i>Ortho-para-directing</i>				
Benzene	H	269	291	1
Aniline	NH ₂	290	345	46
Monomethylaniline	NHCH ₃	290	360	—
Dimethylaniline	N(CH ₃) ₂	297	363	114
Acetanilide	NHCOCH ₃	—	None	0
Fluorobenzene	F	265	285	13
Chlorobenzene	Cl	281	294	0.02
Bromobenzene	Br	—	None	0
Iodobenzene	I	—	None	0
Toluene	CH ₃	273	292	3.8
Ethylbenzene	C ₂ H ₅	275	292	1.2
Phenol	OH	282	330	112
Anisole	OCH ₃	279	302	92
Phenoxide ion	O ⁻	—	None	0
<i>Meta-directing</i>				
Benzoic acid	COOH	—	None	0
Benzoate ion	COO ⁻	—	None	0
Nitrobenzene	NO ₂	—	None	0
Benzene sulphonic acid	SO ₃ H	—	None	0
Benzenesulphonamide	SO ₂ NH ₂	—	None	0
Benzaldehyde	CHO	—	None	0
Benzenearsonic acid	AsO ₃ H ₂	—	None	0
Benzonitrile	CN	287	294	45

¹The wavelengths given here are instrumental values (Aminco-Bowman spectrofluorimeter) and the solutions were either in water or in water containing ethanol.

²Relative intensities are calculated from concentration, molecular weight, and photomultiplier readings of the fluorimeter and are therefore practical values.

NHCOCH₃ and most of the *meta*-directing groups, except CN, are likely to be weakly fluorescent or non-fluorescent. The quenching effect (see also p. 14) of bromo and iodo substituents upon fluorescence is well illustrated with the highly fluorescent dye, fluorescein. The quantum efficiency³ of fluorescence of fluorescein is 70%, that of its tetrabromo derivative, eosin, is 15%, and that of its tetra-iodo derivative, erythrosine, is 3% (Bowen and Wokes, 1953).

So far, the effect of single substituents upon the fluorescence of benzene has been examined, and now consideration must be given to the effect of more than one substituent. At present, there is little systematic data pertaining to the fluorescence of disubstituted benzenes. However, some information has been obtained in this laboratory (Bridges and Williams, 1962) which suggests that the fluorescence of these molecules depends upon the resultant of the combined action of both substituents upon the π -electrons. The results are complicated, for some *meta*-directing groups, which normally abolish the fluorescence of benzene, when combined

with *ortho-para*-directing groups which normally increase the fluorescence of benzene, may produce more or less fluorescence than the *ortho-para*-directing groups alone, as shown in the examples given in Table II.

It will be noted that benzenesulphonamide, which contains the weak *meta*-directing sulphonamide group, is non-fluorescent, but when combined with an amino group as in sulphanilamide (*p*-aminobenzenesulphonamide), the resulting compound is five times as fluorescent as aniline. On the other hand, the weak *ortho-para*-directing chloro group in chlorobenzene suppresses the fluorescence of benzene to a very small value, and in combination with an amino group in *p*-chloroaniline, the fluorescence of aniline is also reduced. Nitrobenzene is non-fluorescent, and, if the strongly *meta*-directing nitro group is combined with an amino group as in *p*-nitroaniline, the effect of the nitro group is sufficient to make *p*-nitroaniline non-fluorescent. The effect of more than one substituent on fluorescence is thus a resultant effect upon the mobility or freedom of the π -electrons. Weak *meta*-directing groups, such as SO₃H and SO₂NH₂, can, in combination with strong *ortho-para*-directing groups (NH₂, OH), often increase fluorescence by increasing the freedom (perhaps one could say chaos) of the π -electrons. A strong *meta*-directing group (except

³The quantum efficiency of fluorescence is a measure of the number of excited molecules returning to the ground state by emission of fluorescence along path F (Fig. 1). If all the excited molecules fluoresced, the quantum efficiency would be 100% but such a value has never been observed. In Table I, no compound has a quantum efficiency of more than 10%.

TABLE II
FLUORESCENCE OF SOME DISUBSTITUTED BENZENES COMPARED WITH
RELATED MONOSUBSTITUTED BENZENES (BRIDGES AND WILLIAMS, 1962)

Compound	R	R ¹	Excitation	Fluorescence	Relative Intensity ¹
Aniline	NH ₂	H	290	350	46
Benzenesulphonic amide	H	SO ₂ NH ₂	—	None	0
Sulphanilamide	NH ₂	SO ₂ NH ₂	275	350	220
Chlorobenzene	H	Cl	281	294	0.02
<i>p</i> -Chloroaniline	NH ₂	Cl	300	360	32
Nitrobenzene	H	NO ₂	—	None	0
<i>p</i> -Nitroaniline	NH ₂	NO ₂	—	None	0

¹Benzene = 1

CN)⁴ will usually tend to diminish or suppress fluorescence. A good example is the nitro group, for most simple nitro derivatives are non-fluorescent. Two examples are shown below. Diphenyloxazole (PPO) (Ott, Hayes, Hansbury, and Kerr, 1957) is fluorescent but its nitro derivative is not.

2, 5-Diphenyloxazole
(PPO) fluorescent (flu: 365
and 381 mμ in toluene)

2-Phenyl-5 (p-nitrophenyl)
oxazole non-fluorescent

(Diphenyloxazole derivatives are used for scintillation counting which depends on the fluorescence of the scintillator when excited by radioactivity. The scintillator POPOP is 2,2'-*p*-phenylenebis-(5-phenyloxazole) which has a fluorescence efficiency approaching 100%.)

2,2'-*p*-phenylenebis-(5-phenyloxazole);
POPOP (flu: 420 and 440 mμ in toluene)

Nevertheless, if there is sufficient conjugation in the molecule to overcome the effect of the nitro group, a molecule containing a nitro group can be fluorescent, as in the case of 5-dimethylamino-4'-nitrostilbene below (Lippert, Lüder, and Moll, 1959).

Fluorescent (λ max. ca. 740 mμ in isobutanol)

Again benzoic acid, which contains the *m*-directing COOH group, is non-fluorescent, but in combination with the OH group, as in the hydroxy-

⁴The CN group has a triple bond, *i.e.*, C ≡ N, and the electrons of this bond can interact with the π-electron cloud of the benzene ring to produce the conditions for fluorescence.

benzoic acids or the NH₂ group as in the amino-benzoic acids, is does not abolish fluorescence and in some cases, *e.g.* *o*-hydroxybenzoic (salicylic) acid and *o*-aminobenzoic (anthranilic) acid, the fluorescence is greater than that of either phenol or aniline.

In polycyclic aromatic systems, the number of π-electrons available is greater than in benzene and therefore these compounds and their derivatives are usually much more fluorescent than benzene and its derivatives. Naphthalene, anthracene, and biphenyl derivatives, for example, are much more fluorescent than the corresponding fluorescent benzene derivatives.

Naphthalene (flu: ca.
323, 340 mμ in
ethanol; Parker and
Rees, 1962)

Anthracene (flu: ca.
372, 400, 425 mμ in
ethanol; Parker and
Rees, 1962)

Biphenyl (flu: 318
mμ in ethanol;
Bridges, Creaven,
and Williams, 1963)

The fluorescence of heterocyclic systems depends upon the nature of the hetero atom and upon substituents. The hetero atoms or groups usually encountered are -N=, >NH, -O-, and -S-. Doubly bound nitrogen, *i.e.*, -N=, in a heterocyclic system tends to deactivate the ring by drawing the π-electrons towards it. Such rings are said to be π-deficient. Heterocyclic systems containing doubly bound nitrogen tend to be non-fluorescent unless there are substituents present which counteract the effect. Thus pyridine is non-fluorescent (Kerr, Hayes, and Ott, 1957), whereas 3-hydroxypyridine is fluorescent (Bridges, Davies, and Williams, 1963), because of the effect of the electron-donating OH group.

Pyridine non-fluorescent

3-Hydroxypyridine fluorescent
(see p. 20)

When $>NH$, $-O-$, and $-S-$ occur in the heterocyclic system, there is a tendency for them to contribute to the π -electron system. Such rings are called π -excessive and the order of contribution is $-NH->-O->-S-$. There is, therefore, a tendency for compounds containing such ring systems to be fluorescent, *i.e.*, those containing the pyrrol, furan, and thiophene rings, although the fundamental ring compounds themselves may not be fluorescent or only weakly fluorescent. When the heterocyclic

Pyrrol

Furan

Thiophene

system has more than one type of hetero atom, then fluorescence will depend upon the resultant of the effect of the two hetero atoms on the π -electrons, and on the effect of any substituents. Thus thiazole and isothiazole derivatives tend to be non-fluorescent because the effect of the $-N=$ upon the π -electrons is greater than $-S-$, but oxazole and isoxazole derivatives could be expected to fluoresce (Ott *et al.*, 1957) because the effect of $-O-$ is greater than $-N=$. However, the substituents in heterocyclic systems play a most important role in the fluorescence of such compounds (see pp. 373 and 374).

Thiazole

Isothiazole

Oxazole

Isoxazole

CHEMICALLY INDUCED FLUORESCENCE Organic compounds which are fluorescent as such are said to possess 'native' fluorescence. It is often possible by simple chemical means to convert non-fluorescent compounds into fluorescent ones and thereby allow their detection and estimation by fluorescence. This type of fluorescence is referred to as 'chemically induced' fluorescence. Thus the adrenal cortical steroid, cortisol, is non-fluorescent; in fact, an examination of its structure shows that it has an insufficient number of conjugated double bonds for fluorescence. However, if it is dissolved in concentrated sulphuric acid in the presence of ethanol, it is converted into intensely fluorescent products,

Cortisol
non-
fluorescent

and, although the nature of these products⁵ is unknown, their fluorescence can be used for the assay of cortisol in minute quantities (Mattingly, 1962). Chemically induced fluorescence can also be used in other ways. Sometimes the 'native' fluorescence of a compound is too weak for it to be useful quantitatively, but by a simple chemical reaction the compound may be converted into a highly fluorescent product which can be readily assayed fluorimetrically. Thus the tetracycline antibiotics, which probably have a relatively weak native fluorescence, can be converted by calcium ions and a barbiturate (usually barbitol) into highly fluorescent complexes (Kohn, 1961), the suggested structure of one of which is shown below.

(Exc. 405 m μ ; flu;
530 m μ)

Closely related substances often have native fluorescences so similar to each other that it is impossible to estimate one of a group of such substances in the presence of the others. However, it is frequently possible to convert one of these compounds into another fluorescent species, so that it can be estimated in the presence of its congeners. Thus adrenaline and noradrenaline show a native fluorescence which is common to most simple catechols, including catechol itself. Both adrenaline and noradrenaline, however, can be oxidized at pH 6.5 to 3,5,6-trihydroxyindoles (adrenolutine and noradrenolutine) which are highly fluorescent at alkaline pH values. The estimation of adrenaline in the presence of noradrenaline can be achieved by oxidizing at pH 3.5, for at this pH adrenaline is converted into a trihydroxyindole whereas noradrenaline is not (Udenfriend, 1962).

Adrenaline
Native fluorescence at
330 m μ Adrenolutine formed by
oxidation of adrenaline:
fluorescence 520 m μ in
alkali

⁵It is believed that strong sulphuric acid has the effect of introducing double bonds into the cortisol molecule.

Changes in the *pH* of a solution will sometimes allow the differentiation of two substances with similar native fluorescence characteristics. Thus both phenol and anisole fluoresce at 300 to 310 *mμ* at neutral *pH*, but at *pH* 12 phenol is converted into the non-fluorescent phenoxide ion whereas anisole remains unchanged (Rosen and Williams, 1961):—

FACTORS AFFECTING FLUORESCENCE INTENSITY

The intensity of fluorescence of a compound is affected by a number of factors, the most important of which are the following: (a) instrumental factors, (b) concentration, (c) solvent, (d) *pH*, (e) temperature, and (f) stability of the compound in light. Instrumental factors are discussed in the section on fluorimeters (see p. 385).

CONCENTRATION (see also quenching p. 383) The intensity of fluorescence is proportional to the concentration of the fluorescent compound only in highly dilute solutions and therefore the concentration of the compound to be assayed is a very important consideration in quantitative work. In most fluorimeters, the fluorescence emitted from the cell holding the solution is measured at right angles to the path of the exciting light (see p. 385 and Fig. 10). The fluorescence emitted has therefore to pass through the solution to the detector and during this passage some of it is re-absorbed by other molecules of the compound under examination. The higher the concentration of the compound, the greater is the proportion of the emitted fluorescence re-absorbed. Therefore, linearity between fluorescence intensity and concentration can only be expected at high dilutions where the number of molecules present is small enough to make the extent of re-absorption unimportant compared with the amount of fluorescence emitted. However, the effect of concentration is dependent to some extent upon instrumental factors such as slit widths, intensity of the exciting light, and whether the instrument is one of the common type which detects fluorescence at right angles to the exciting light or one of the more specialized instruments which detects fluorescence at the same face as that being excited. Slit-width and light intensity should be kept constant during any fluorimetric assay. The

FIG. 3. Concentration quenching of the fluorescence of phenol in water. The large graph covers the concentration range 0-400 $\mu\text{g./ml.}$, the inset 0-12 $\mu\text{g./ml.}$ (excitation, $\lambda_{\text{max.}}$ 295 $m\mu$; fluorescence, $\lambda_{\text{max.}}$ 330 $m\mu$; *pH* 6.5; temp. 22°C.; Aminco-Bowman spectrofluorimeter).

following approximate concentration ranges in $\mu\text{g./ml.}$ over which fluorescence is proportional to concentration have been found by us for certain compounds, using an Aminco-Bowman spectrofluorimeter:—phenol, 0.01-8.0; sulphanilamide, 0.05-5.0; salicylic acid, 0.01-2.0; aniline, 0.01-6.0; pyridoxine, 0.02-5.0; and 3-hydroxypyridine, 0.25-5.0 (Bridges and Williams, 1963). These ranges, of course, depend upon the inherent fluorescence intensity of the compound, for a compound with a weak fluorescence could be expected to show a linear fluorescence intensity/concentration range at a higher concentration than an intensely fluorescent compound. Figure 3 shows the effect of concentration on the fluorescence intensity of phenol. This is a typical example of the effect of concentration.

EFFECT OF SOLVENT Intensity and wavelength of fluorescence can be affected by change of solvent but the effect is often unpredictable. In discussing the solvent effect it will be convenient to consider three aspects, namely, purity of the solvent, non-aqueous solvents, and aqueous solvents.

Since fluorescence is a highly sensitive technique it is important that the solvents used should themselves be non-fluorescent and free from fluorescent impurities. These solvents may be used either for extracting the desired material or for the actual fluorescence measurement. Apart from water,

solvents used in fluorescence work may include the simple alcohols from methanol to butanol, ether, benzene, ethylene dichloride, hexane, heptane, etc. All solvents should be checked that they do not contain any undesirable fluorescence, and otherwise rigorously purified. Details of the purification of solvents can be obtained from standard texts (*e.g.*, Udenfriend, 1962). There are other sources of fluorescent impurities apart from solvents and particular attention should be paid to detergents used to clean glassware. These detergents may themselves be fluorescent. On the other hand, there are some cleansing agents which quench fluorescence and these should be avoided or carefully removed. Chromic acid, for example, absorbs ultraviolet light (see p. 383) and it is preferable to clean cuvettes in nitric acid rather than in chromic acid. Some solvents also absorb specific wavelengths of light *e.g.*, benzene, and should be avoided for fluorescence involving the regions of their absorption. Apart from solvents, the preparation of solutions for fluorescence assay often involves materials such as adsorbents, *e.g.*, alumina and buffer chemicals, and obviously the possibility of the addition of fluorescent impurities or quenching agents from these materials to the solution to be assayed must be borne in mind.

Non-aqueous solvents are not frequently used as media for fluorescent substances in biological work, but there is no reason why they should not be used. Fluorescence often varies with solvent and several reasons have been put forward to explain this (Van Duuren, 1963), such as the dielectric constant of the solvent, the association of solvent and solute by hydrogen bonding, quenching by solvent molecules, and ionization. Indole, for example, shows the same maximum excitation of 285 m μ in five solvents, but the wavelength of maximum fluorescence is 297 in cyclohexane, 305 in benzene, 310 in dioxan, 330 in ethanol, and 350 m μ in water. The fluorescence wavelength thus increases with the dielectric constant of the solvent, due to an effect on the π -electrons. At 5 $\mu\text{g./ml.}$, indole-3-acetic acid is not fluorescent in cyclohexane or benzene, but fluoresces at 325 in dioxan, 340 in ethanol, and 360 m μ in water. Chlorophyll shows very little fluorescence in rigorously dried non-polar solvents, but the addition of a polar solvent (methanol, ethanol, or water) causes a remarkable enhancement of fluorescence probably due to hydrogen bonding between chlorophyll and the polar solvent (Livingston, Watson, and McArdle, 1949). Pyridoxine fluoresces at 335 m μ in dioxan and 400 m μ in water and this change is due to ionization. In dioxan, pyridoxine occurs in the uncharged form whilst in water as the dipolar ion (Bridges *et al.*, 1963):—

Pyridoxine (molecular). Exc. 296 m μ ; flu: 335 m μ in dioxan

Pyridoxine (dipolar ion). Exc. 332 m μ ; flu: 400 m μ in water

Another interesting example of ionization is 2-hydroxybiphenyl which fluoresces at 348 m μ in ethanol and at 415 m μ in water although the excitation (295 m μ) is the same in both solvents (Bridges, Creaven, Davies, and Williams, 1963). In this case, the difference in fluorescent wavelengths is due to excited state ionization in water but not in ethanol (see p. 380).

A good illustration of the effect of solvent upon fluorescence is shown in Table III, where the intensity of fluorescence of solutions of sulphanilamide (2 $\mu\text{g./ml.}$) in various solvents is shown. It will be noted that sulphanilamide does not fluoresce in two ketones, in two highly chlorinated methanes, in the nitro compound, nitromethane, and *p*-xylene. The fluorescence of sulphanilamide is most intense in the lower alcohols and least in the aromatic hydrocarbons (Bridges, 1963).

TABLE III
INTENSITY OF FLUORESCENCE OF SULPHANILAMIDE
IN VARIOUS SOLVENTS¹ (BRIDGES, 1963)

Solvent	Relative Intensity	Solvent	Relative Intensity
Water	100	1,2-Dichloroethane	69
Methanol	72	<i>n</i> -Hexane	42
Ethanol	107	Benzene	17
Propanol	89	Toluene	9
Isopropanol	94	<i>p</i> -Xylene	0
<i>n</i> -Butanol	69	Light petroleum (b.p. 60-80°)	24
Isobutanol	7	Ether	78
<i>n</i> -Pentanol	48	Ethyl acetate	33
Acetone	0	Formamide	70
Butan-2-one	0	Dimethylformamide	44
Chloroform	0	Dioxan	24
Carbon tetrachloride	0	Nitromethane	0

¹Measured in an Aminco-Bowman spectrofluorimeter. The excitation wavelengths varied slightly with the solvent from 270 m μ in isobutanol to 305 m μ in toluene. The fluorescence occurred at 350 m μ in every case. The fluorescence intensity in water is taken as 100. Concentration of sulphanilamide, 2 $\mu\text{g./ml.}$

RAMAN SPECTRA OF SOLVENTS When very small amounts of a fluorescent substance in solution have to be measured, there is the possibility that the measurement may be interfered with by the so-called Raman scattering of the solvent. When this occurs it is advisable to change the solvent. Raman scattering is a phenomenon which is common to gases, liquids, and solids, for when monochromatic light falls on a substance, some of it is scattered by the molecules of that substance. If the spectrum of the scattered light, which is called a

Raman spectrum, is examined, it is found to contain wavelengths of light which are characteristic of the substance illuminated and there is a constant relationship between the wavelengths of the Raman lines and the wavelength of the incident light. In fact, Raman spectra are useful in determining molecular structure (Cleveland, 1955). However, Raman spectra, unlike fluorescence spectra, have no absolute excitation and emission wavelengths and can arise from any wavelength of incident light. If a Raman line of the solvent happens to coincide with the fluorescence maximum of the compound being estimated, it could interfere considerably with the estimation and the solvent should be changed. However, Raman scattering is usually weak and may interfere only at high dilutions of the fluorescent substance. According to Parker (1959), the Raman spectrum of a solvent can provide a most useful means of checking the day-to-day sensitivity of a spectrofluorimeter.

BUFFERS In biological work, fluorescence is frequently measured in aqueous buffer solutions. It is therefore important to know whether the constituents of the buffer affect the fluorescence. Table IV shows the effect of various buffers upon the fluorescence of sulphanilamide at 2 $\mu\text{g./ml.}$ and it can be seen that buffers containing 0.1M phosphate, borate, citrate, and phthalate and 0.05M tris-(hydroxymethyl)aminomethane (Tris) have an effect on fluorescence intensity although not a very marked one (Bridges, 1963). It has been reported by Cowgill (1963) that 0.05M Tris buffer (pH 7.5) had no quenching effect upon the fluorescence of indole or phenol. Phosphate, however, quenched both indole and phenol fluorescence, and in the quenching of phenol, the di-anion, HPO_4^{2-} , was more effective

than the mono-anion, H_2PO_4^- , whilst in the quenching of indole the mono-anion was more effective than the di-anion. Both acetic acid and sodium acetate are weak quenchers of tyrosine fluorescence but, in the case of the fluorescence of tryptophan, acetic acid but not sodium acetate is a quencher. Commercial buffer solutions contain constituents which are not always disclosed by the manufacturers. These constituents could have a fluorescence of their own or they could be quenching agents. Buffers therefore should always be checked for their effects on fluorescence. In the case of phosphate buffers, an increase of phosphate concentration frequently leads to a diminution in fluorescence intensity.

pH EFFECTS Many biologically important compounds are capable of undergoing ionization and the ionic forms of a compound often have different fluorescence characteristics from the unionized form. The effect of pH upon the fluorescence of a compound is thus of considerable importance, and a knowledge of the changes in fluorescence brought about by pH changes in the medium can be valuable from several aspects. Thus a compound may be fluorescent only over a short range of pH, as in the case of sulphapyridine, which is practically non-fluorescent above pH 4 and is maximally fluorescent at pH 0.5 to 1.0 (Bridges, 1963). Again, a compound may be fluorescent over a considerable range of pH, but over a certain section of that range it may be much more fluorescent than over the rest. Thus salicylic acid shows some fluorescence from pH 0 to 14, but over the range pH 4 to 12, it is 100 times as fluorescent as at pH 1 (Rosen and Williams, 1961). pH can also be used to distinguish compounds of similar structure as in the case of tryptamine and 5-hydroxytryptamine (5HT). At pH 2 to 10, both these compounds fluoresce at about 350 to 360 $\text{m}\mu$, but at pH approximately -0.5 (about 3N acid) 5-hydroxytryptamine fluoresces at 540 $\text{m}\mu$, whereas tryptamine is non-fluorescent (Bridges and Williams, 1963).

pH-Fluorescence changes are also the basis of the so-called fluorescent indicators. In this case the fluorescence is usually visible, and the appearance of visible fluorescence is useful as an indicator in the titration of cloudy or coloured solutions where normal indicators, which show pH changes by alteration in colour, are of little use. A good example of such an indicator is 2-naphthol which, when viewed under ultraviolet light, changes from colourless to blue at pH 6.8. At low pH values, 2-naphthol emits the invisible ultraviolet fluorescence of unionized naphthol, but above pH 6.8 it emits the blue fluorescence of the naphtholate anion.

TABLE IV
EFFECT OF BUFFER COMPOSITION ON THE
FLUORESCENCE OF SULPHANILAMIDE¹ (BRIDGES, 1963)

Solvent	pH of Solution	Fluorescence Intensity ²
Water ³	4.3	27
Water ³	8.1	27
0.1M-Citrate- Na_2HPO_4 buffer	4.0	25
	5.2	26
	6.0	24
	7.1	23
	8.0	22
0.1M- K_2HPO_4 - KH_2PO_4 buffer	7.0	21.5
0.05-M-Tris-HCl buffer	7.0	26.5
Burroughs & Wellcome buffers:		
0.1M-phthalate	4.0	25
0.1M-phosphate	6.99	24
0.1M-borate	9.15	23

¹2 $\mu\text{g./ml.}$

²Photomultiplier reading on the Aminco-Bowman spectrofluorimeter with excitation at 273 $\text{m}\mu$ and fluorescence at 350 $\text{m}\mu$.

³Glass-distilled water to which a drop of dilute HCl or NaOH was added.

pH-Fluorescence changes can also be used for the determination of the approximate pKa of acids and bases in minute amounts. These can be determined from pH/intensity curves and sometimes from pH/excitation or fluorescence wavelength diagrams, provided that there is a gradual change in wavelength from unionized to ionized forms.

The points mentioned above in this section can best be illustrated by actual pH-fluorescence curves. Figure 4 shows the pH-fluorescence intensity curve of aniline. Below pH 2, aniline is non-fluorescent and at this pH it occurs as the anilinium cation. Above pH 2, the fluorescence intensity rises and the curve follows exactly the ionization curve of the cation to molecular aniline (pKa 4.5). At about pH 7.5 to 8.0 the intensity reaches a maximum and remains constant to about pH 11 to 12 and then falls to a small value at higher pHs. The constant intensity is due to molecular aniline, but the fall at about pH 12 may be due to the formation of an aniline anion. The formation of this anion is suggested by the fact that monomethylaniline, which has a replaceable hydrogen atom, behaves exactly like aniline, but dimethylaniline, which does not have such a hydrogen, does not lose its fluorescence at high pH values (Williams, 1959). Indole also loses its characteristic fluorescence at high pH values (e.g., 5*N*-NaOH) for a similar reason, but *N*-methylindole is still fluorescent at these pH values since it has no replaceable hydrogen (White, 1959).

Figure 5 shows the pH/intensity curves for indole, dimethyltryptamine, and 5-hydroxydimethyltryptamine. Between pH 3 and 11, these three compounds fluoresce in the same region of 340 to

FIG. 4. The effect of pH upon the fluorescence intensity of aniline in aqueous solution (concentration 5 $\mu\text{g./ml.}$). (The pKa of aniline is 4.5 and the ionization fits the fluorescence intensity curve exactly; excitation, λ_{max} . 290 $m\mu$; fluorescence, λ_{max} . 348 $m\mu$; Aminco-Bowman spectrofluorimeter.)

360 $m\mu$. However, at low pH values, i.e., from pH-1 to 1, 5-hydroxytryptamine alone shows a fluorescence at 540 $m\mu$, whereas at high pH values, i.e., 14 to 15 (5*N*-NaOH), only dimethyltryptamine shows any appreciable fluorescence and this occurs at 415 $m\mu$ (see Table V). Figure 6 shows the pH/intensity curve for 5-hydroxyindole-3-acetic acid. This compound (see Table V) shows the typical indole fluorescence between pH -0.2 to 13, but in strong acid it shows a fluorescence at 545 $m\mu$ characteristic of 5-hydroxyindoles and in half-normal and normal alkali it shows a fluorescence at 455 $m\mu$. In strong alkali (5-10*N*) it is non-fluorescent (Bridges and Williams, 1963).

EXCITED STATE IONIZATION The fluorescence of a compound capable of ionizing depends upon the pH of the solution, for the fluorescence of the ionic form is frequently different from that of the

FIG. 5. The effect of pH upon the fluorescence intensity of indole (— — —), dimethyltryptamine (— · — · —), and 5-hydroxydimethyltryptamine or bufotenine (—). Concentration in each case, 1 $\mu\text{g./ml.}$ (Aminco-Bowman spectrofluorimeter.)

TABLE V
FLUORESCENCE OF SOME INDOLES¹
(BRIDGES AND WILLIAMS, 1963)

Compound	Excitation (mμ)	Fluorescence (mμ)	pH Range
Indole	—	None	—1 to 0
	285	355	0 to 13·7
Dimethyltryptamine	285	355-400 (weak)	14 to 15
	—	None	—1 to 0
	290	365	0·4 to 13·0
	295	415	14·0 to 14·7
5-Hydroxydimethyltryptamine	300	358	15
	304	540	—0·7 to 1·4
	304	342	—0·3 to 12
	—	None	12·5 to 15
5-Hydroxyindole-3-acetic acid	300	545	—1·0 to 1·0
	300-305	340-350	—0·2 to 13·0
	362	455	13·4 to 14·0
	—	None	14·4 to 15

¹Concentration 0·8 μg./ml.

FIG. 6. The effect of pH upon the fluorescence intensity of 5-hydroxyindole-3-acetic acid (1 μg./ml.). The scale for the 545 mμ fluorescence has been multiplied by 3 (Aminco-Bowman spectrofluorimeter.)

non-ionized or molecular form. The process of excitation and fluorescence of a compound MH, which is capable of ionizing, can be represented as follows:

For the ion M^- , the process is:

Some compounds, however, when excited, undergo ionization, so that excitation of the ionized form gives rise to fluorescence corresponding to the ionized form. The compound has thus undergone excited state ionization and the process can be represented as follows:—

In practice, this means that some compounds when excited yield the fluorescence of the corresponding ion at pH values at which, under normal circumstances, the ion does not exist. In fact, the excited molecule has become a much stronger acid than the unexcited or ground molecule. This phenomenon also occurs with bases and the excited base becomes much weaker than the unexcited base. Several examples of this phenomenon are known and it is probably more widespread than hitherto suspected. In our laboratory we have been able to use this phenomenon to estimate fluorimetrically the two isomers, 2- and 4-hydroxybiphenyl, produced enzymically from biphenyl by liver microsomes. 4-Hydroxybiphenyl (pKa 9·5) fluoresces at 340 mμ (excitation, 288 mμ) from pH —1·0 to 9·2, and at 401 mμ (excitation, 311 mμ) from pH 10 to 14. The change of fluorescence with pH is what would be expected, since the change occurs at pH 9 to 10 where 4-hydroxybiphenyl undergoes ionization. With 2-hydroxybiphenyl (pKa 10·0), however, the fluorescence changes at pH 1 whereas the excitation changes at pH 9·5 to 10·5. From pH —1 to 1, 2-hydroxybiphenyl fluoresces at 348 mμ (excitation, 295 mμ); from pH 1 to 9·5 it fluoresces at 415 mμ (excitation, 295 mμ); and from pH 9·5 to 14 it fluoresces at 415 mμ but the excitation is now at 320 mμ. These changes are shown in Figure 7. From the fluorescence changes it can be calculated that the pKa of excited 2-hydroxybiphenyl is about 1·5, so that in the excited state it is a hundred million times stronger as an acid than in the unexcited state. It can be seen that at pH 6, the 4-isomer fluoresces at 340 mμ whereas the 2-isomer fluoresces at 415 mμ and therefore one can be estimated in the presence of the other.

We have found several compounds to exhibit this phenomenon and a list is given in Table VI. The effect of pH is further discussed in the section on fluorescent molecules of biological interest.

TEMPERATURE Fluorescence intensity tends to in-

FIG. 7. The effect of pH upon the maximum excitation and fluorescence wavelengths of 2-hydroxybiphenyl (4.2 $\mu\text{g./ml.}$) ●—● excitation, ○—○ fluorescence. The pK_a of 2-hydroxybiphenyl is 10.0; the excited pK_a is about 1.5 (Aminco-Bowman spectrofluorimeter.)

FIG. 8. The effect of temperature on the fluorescence intensity of phenol (○—○) at 2 $\mu\text{g./ml.}$ and pH 6.8 (fluorescence $\lambda_{\text{max.}}$ 330 $m\mu$) and of *p*-anisidine (●—●) at 2 $\mu\text{g./ml.}$ and pH 9.5 (fluorescence $\lambda_{\text{max.}}$ 350 $m\mu$) (Aminco-Bowman spectrofluorimeter.)

TABLE VI

SOME COMPOUNDS SHOWING EXCITED STATE IONIZATION

2-Hydroxybiphenyl	1- and 2-Naphthol ¹
3-Hydroxypyridine	1- and 2-Naphthylamine ¹
Pyridoxine	<i>p</i> -Toluidine
Pyridoxal	<i>p</i> -Anisidine
Pyridoxal phosphate	2,2'-Dihydroxybiphenyl
Pyridoxamine	

¹See Förster (1950) and Hercules and Rogers (1959).

crease to a maximum with fall in temperature and to decrease to zero at high temperatures. The effect of temperature upon fluorescence is not fully understood but one can assume that it is connected with the movement of molecules in the medium. When the temperature rises, the motion of the molecules increases and there is a greater tendency for collisions. This would result in the loss of some of the energy which might have been radiated as fluorescence. For most practical purposes temperature changes within a few degrees are unimportant, but one must be aware of them because there are some compounds whose fluorescence intensity is particularly sensitive to temperature changes. Most fluorimeters have no means of controlling the temperature of the cell compartment, which can change several degrees as a result of the heat emitted by the light source. With most compounds a change of 1°C. may cause an intensity change of about 1%. However, compounds are known which exhibit a

change of fluorescence intensity of 5% per degree. These include aqueous solutions of indole-3-acetic acid, tryptophan (Udenfriend, 1962), *p*-anisidine, and *p*-toluidine (Bridges and Williams, 1962). In these instances some form of temperature control would be desirable. Examples of temperature changes in fluorescence are shown in Figure 8. (Temperature control of the cell compartment has very recently become available for the Aminco-Bowman, Farrand and Zeiss spectrofluorimeters.)

PHOTO-DECOMPOSITION The stability of a compound when it is irradiated by ultraviolet light is an important consideration in fluorimetry. Most fluorescence assays are carried out on highly dilute solutions and there are several fluorescent compounds which photo-decompose in dilute solutions although they may be more stable in more concentrated but still dilute solutions. Thus highly dilute solutions of quinine bisulphate (0.01 $\mu\text{g./ml.}$) are unstable in ultraviolet light whilst more concentrated solutions (1 $\mu\text{g./ml.}$) are stable. The extent of photo-decomposition depends upon the intensity of the light source, and, whilst a very intense light source may greatly enhance the sensitivity of an instrument, it may at the same time cause increased photo-decomposition. However, even though photo-decomposition does occur, rapid measurements can be carried out before much of the compound is decomposed. Thus we

FIG. 9. The effect of ultraviolet light (295 $m\mu$) on the fluorescence intensity of 3-pyridol at 5 $\mu\text{g./ml.}$ and pH 6.5 (○—○) and of pyridoxine at 5 $\mu\text{g./ml.}$ and pH 6.8 (●—●). The fluorescence $\lambda_{\text{max.}}$ was 400 $m\mu$ for both compounds (Aminco-Bowman spectrofluorimeter.)

have found that sulphanilamide (5 $\mu\text{g./ml.}$) in water undergoes photo-decomposition when exposed to light of wavelength 275 $m\mu$ to non-fluorescent products. However, decomposition is only appreciable after 30 seconds and the intensity of fluorescence of sulphanilamide can be measured within this period. It has also been found that pyridoxine, pyridoxal and its 5'-phosphate, and pyridoxamine (2 $\mu\text{g./ml.}$) undergo photo-decomposition in ultraviolet light. These compounds are derivatives of 3-hydroxypyridine which itself is stable in light. The rate of photo-decomposition of some of these compounds is shown in Figure 9.

It should be mentioned that photo-decomposition does not always lead to loss of fluorescence and in some cases it leads to the enhancement of production of fluorescence. Thus coumarin, 4-aminoquinolines, and certain flavine derivatives are weakly fluorescent, but on exposure to ultraviolet light of suitable wavelengths, they photo-decompose to highly fluorescent products which may be utilized for their detection and estimation (Feigl, Feigl, and Goldstein, 1955; Brodie, Udenfriend, Dill, and Chenkin, 1947; Kuhn, Wagner-Jauregg, and Kaltschmitt, 1934).

FLUORESCENCE QUENCHING

When one compound diminishes or abolishes the fluorescence of another, it is said to quench the

fluorescence. The quenching of fluorescence can be brought about in many ways and the mechanism of quenching is complicated. Furthermore, the quenching of one compound by another is often unpredictable. In this section, an attempt has been made to reduce the theoretical aspects of quenching to simple terms. On these terms, it can be said that quenching may be brought about in four ways, namely, (1) by absorption by the quencher of the exciting light; (2) by absorption of the fluorescence; (3) by degradation of the energy of the excited molecule so that this energy cannot be emitted as light; and (4) by a chemical change in the fluorescent molecule.

QUENCHING BY INNER FILTER EFFECTS The absorption of the excitation (1 above) and the absorption of the fluorescence (2 above) by the quencher are known as 'inner filter' effects, and they are not easily distinguishable. In fact, a quencher could do both. Such effects are found with solvents which absorb light of wavelengths close to those of the exciting or fluorescent light. Examples of such solvents are acetone, benzene, and phenol, which absorb in the ultraviolet and are unsuitable for work in this region. It is clear that when such solvents are used for extraction procedures, they must be carefully removed before the final solution is examined fluorimetrically. The quenching effect of acetone, however, has been suggested as the basis of a fluorimetric method for measuring acetone and organic acids which can be decomposed into acetone. The fluorescence of β -naphthol in aqueous solution is quenched by acetone and the extent of quenching is proportional to the concentration of acetone (Hynie, Večerek, and Wágner, 1960).

Another instance of the inner filter effect is concentration quenching or self-quenching, a point which has already been discussed on page 377.

Light scattering due to very fine particles can also be regarded as a kind of quenching and this can be avoided by filtration or, if the particles are very fine, by centrifugation at high speed.

The quenching effect of traces of dichromate has already been mentioned (see p. 378) and this effect may be partly due to the absorption of light by dichromate. The dichromate ion has absorption peaks at 275 and 348 $m\mu$ which overlap, for example, the excitation and fluorescence peaks of tryptophan (Udenfriend, 1962).

QUENCHING BY ENERGY DEGRADATION The degradation of the energy of the excited state by the quencher can occur in several ways, for example, by conversion of the excited molecule into the triplet state, by electron transfer, or by energy transfer.

These forms of quenching are often known as 'true' quenching.

The quenching molecule may affect the fluorescent molecule in such a way as to convert it from the singlet excited state to the triplet state so that it no longer emits its energy as fluorescence (see p. 372). The quenching effect of oxygen, certain iodo, bromo, and nitro compounds, and probably pyridine may be due to this effect. In the case of the halogen compounds the large magnetic fields of the halogen atoms may be responsible for the conversion. In the case of oxygen, it may be due to the fact that the ground state of the oxygen molecule is a triplet, which, when in contact with the singlet excited state of the molecules of some compounds, exchanges its triplet state for the singlet state of the other molecule:

The quenching effect of oxygen can be avoided by bubbling nitrogen through the solution during the measurement of fluorescence. However, oxygen is not a universal quencher of fluorescence, but it does quench the fluorescence of several aromatic compounds. This observation has been used for the determination of oxygen, employing the fluorescence of a borate-benzoin complex which is quenched by oxygen in proportion to the concentration of the latter (Parker and Barnes, 1957).

The degradation of the energy of the excited molecule can also occur by electron transfer. The excited molecule, but not the corresponding ground molecule, may be able to donate or accept an electron from the quenching molecule and in this way the excited state may be destroyed. Thus ferrous ions destroy the fluorescence of excited methylene blue by electron donation, but have no effect on the unexcited dye. Some fluorescent molecules are quenched by electron-donating anions such as I^- , Br^- , SCN^- , and $S_2O_3^{2-}$, and others by electron-accepting anions such as IO_3^- , NO_3^- , and $S_4O_6^{2-}$, the direction of electron transfer depending upon the redox potential of the excited molecule which is different from that of the unexcited molecule (Bowen and Wokes, 1953). The fluorescence of tryptophan is quenched by thiosulphate or nitrate ions and this effect has been used for identifying tryptophan in tissue fluids (Duggan and Udenfriend, 1956).

For quenching to occur by energy transfer, the energy level of the excited quencher must be just below that of the excited fluorescent molecule. Usually the quencher molecule is one which, on excitation, is converted into the triplet state, so that when the fluorescent molecule is excited, its energy is immediately taken up by the quencher, which then assumes a non-radiative triplet state and the energy

absorbed by the fluorescent molecule cannot now be emitted as fluorescence. In effect, the quencher has robbed the excited fluorescent molecule of its energy because of the proximity of their energy levels. This type of quenching can readily occur in solids where the fluorescent and quenching molecules are close together, but it is more difficult to produce in solution unless high concentrations are used, and then, of course, energy transfer quenching may be difficult to distinguish from concentration quenching. An example of energy transfer quenching is shown by phenazine in solid anthracene. Anthracene has a blue fluorescence, but if it contains one part in a thousand of phenazine, the fluorescence is quenched. The energy level of the triplet state of excited phenazine is just below that of the singlet excited state of anthracene (Bowen and Wokes, 1953).

Energy transfer mechanisms are thought to play an important role in biological systems, particularly those involving proteins (Porter and Weber, 1959; Szent-Györgyi, 1957). The fluorescence of NADP is thought to involve an energy transfer mechanism (see p. 389).

QUENCHING BY CHEMICAL CHANGE If a fluorescent compound undergoes a chemical change as a result of the presence of a second compound it could be converted into a non-fluorescent product. Although the fluorescence of the compound is quenched by the addition of the second compound, this form of quenching is to be distinguished from 'true' quenching during which no net chemical change occurs. Quenching by chemical change (cf. photo-decomposition, p. 382) can occur in many ways but the end product is usually a non-fluorescent compound. Furthermore this type of quenching can be very specific as the examples given below will show.

Quenching can occur as a result of pH changes in the solution. A simple case is that of aniline. If acid is added to an aqueous solution of aniline, the fluorescence begins to be quenched at pH 6, and at pH 2 the solution is non-fluorescent. This is due to the conversion of aniline into the non-fluorescent anilinium ion which is a different chemical species from aniline:—

The fluorescence of phenol is quenched when the solution is made alkaline and in this case phenol is converted into the non-fluorescent phenoxide ion.

An example of specific quenching by an acid is that of quinine. In 0.1N- H_2SO_4 , quinine (1 $\mu g./ml.$) is highly fluorescent but in 0.1N-HCl, quinine is non-fluorescent (Bowen and Wokes, 1953). Quinine

in the excited state probably forms some kind of compound with HCl which is non-fluorescent.

An extensive study of the quenching effects of purines was made by Weil-Malherbe (1946) who found xanthine, hypoxanthine, caffeine, uric acid, and *N*-methylxanthines to quench the fluorescence of several compounds, probably by forming molecular compounds or complexes. Thus caffeine in acid solution quenches the fluorescence of polycyclic hydrocarbons, whilst in neutral solution it quenches the fluorescence of riboflavin, and intensifies that of thiochrome, mepacrine, and eosin. In the case of tryptophan its fluorescence is quenched by ascorbic acid, the nitrate ion, and the thio-sulphate ion, and it is probable that tryptophan is oxidized by these quenchers.

ADSORPTION AND QUENCHING Adsorption of the fluorescent substance on to glassware (including fluorimeter cells) and precipitates, especially protein, frequently occurs in dilute solutions especially with non-polar solvents such as hexane. Such adsorption results in the loss of fluorescent material and consequently in low recoveries. There are many ways in which adsorption can be avoided and these include suitable treatment of new glassware, changing the protein precipitants, changing the solvent, adding a small amount of a polar solvent such as alcohol, etc., but each case has to be dealt with on its merits (see Udenfriend, 1962).

FLUORIMETERS

A fluorimeter is an instrument used for the measure-

ment of the intensity of fluorescence. If the instrument has means of measuring the wavelength of the fluorescence and of the excitation, it is then called a spectrofluorimeter or more accurately, a fluorescence spectrometer. All fluorimeters, simple or complex, contain three essential units, namely, a source of exciting light, a sample container, usually a cell or cuvette of square section, and a detecting unit for measuring the fluorescence emitted by the sample. To increase the specificity of both the exciting and fluorescent light, most fluorimeters contain two sets of light filters, and the spectrofluorimeters, two monochromators, one to select the exciting light and the other to analyse the fluorescent light. The monochromators are devices consisting of either a diffraction grating or a prism and the necessary slits, lenses, and mirrors, which can be moved mechanically to select a desired wavelength or band of light, which is measured on a wavelength scale or drum attached to the monochromator. A simplified outline of a fluorimeter is shown in Figure 10.

When the exciting light strikes the cuvette fluorescence is emitted from all its faces, but most fluorimeters measure only the fluorescence which is emitted from one face at right angles to the direction of the exciting light. The faces in the direction of the incident light emit fluorescence and exciting light, but even at right angles there is some scattered exciting light to be contended with and this shows up on recording spectrofluorimeters at the wavelength of the exciting light on the fluorescence side of the instrument. In filter instruments, this scattered light can be eliminated to a large degree by suitable secondary filters (see Fig. 10). Filters can also be used in spectrofluorimeters, if scattered light is troublesome as a result of a slight impurity or turbidity in the sample.

LIGHT SOURCES The source of exciting light is an important factor in fluorimeters, and the lamps now in use are the xenon-arc, the high pressure mercury, the tungsten filament, and zinc lamps. Modern spectrofluorimeters utilize the xenon-arc lamp which emits a continuous spectrum from 200 to 800 μ . Its brightness in the ultraviolet region is much greater than the tungsten filament lamp and it does not develop line spectra as does the mercury vapour lamp. The mercury lamp gives a discontinuous spectrum consisting of high intensity lines, the

FIG. 10. A simplified outline of a fluorimeter.

main ones being at 365, 405, 436, and 546 $m\mu$. If a compound is maximally excited in the region of these lines, the mercury lamp is then very suitable for exciting such a compound, but it is less satisfactory for compounds maximally excited at other wavelengths. The fluorimetric estimation of vitamin B₁ as thiochrome, of adrenaline as adrenolutine or as a quinoxaline derivative, and of riboflavin are excellent examples of compounds whose maximum excitations almost coincide with one of the mercury lines, thus:—

Mercury Line	Maximum Excitation for
366 $m\mu$	Thiochrome from vitamin B ₁ , 370 $m\mu$
405 $m\mu$	Adrenolutine from adrenaline, 405 $m\mu$
	Riboflavin, 440 $m\mu$
436 $m\mu$	Quinoxaline derivative from adrenaline, 435 $m\mu$

Although the xenon-arc gives a continuous spectrum over the ultraviolet and visible regions its intensity varies with wavelength and tends to

FIG. 11. The absorption spectrum (---), uncorrected excitation spectrum (●—●), and corrected excitation spectrum (○—○) of sulphanilamide at 2 $\mu\text{g./ml.}$ and pH 8.4. The abscissa is given in wavelengths ($m\mu$) and wave numbers or frequencies (in reciprocal microns, i.e., μ^{-1}).

diminish at short wavelengths. This means that instrumental excitation spectra are distorted versions of the true spectra, and instrumental excitation maxima appear at longer wavelengths than the true maxima. For most analytical purposes this is unimportant, but, if necessary, corrections can be made and the corrected excitation spectra then coincide fairly well with the absorption spectra. An example of this type of correction, which is a tedious process, is shown in Fig. 11 which shows the absorption spectrum, the instrumental and corrected excitation spectra, and the fluorescence spectrum of sulphanilamide (Bridges, 1963). No light source has yet been obtained which does not give distorted excitation spectra.

Most filter fluorimeters use the mercury lamp as a light source, since it is easier to isolate the high intensity lines of the mercury spectrum with filters than any part of the continuous spectrum of the xenon-arc lamp, but intense excitation wavelengths from the mercury lamp are limited in number.

CELLS The cells or cuvettes used in fluorimeters are made of glass or silica. If the exciting wavelengths are above 320 $m\mu$, glass cells can be used, but below 320 $m\mu$ cells of quartz or synthetic silica should be used. It has been found that synthetic silica cells tend to be more satisfactory than quartz, which is a natural silica and is more likely to contain impurities than synthetic silica. All cells should be checked that they have no fluorescence of their own and rejected if they have (Parker and Rees, 1962).

DETECTORS The detection of fluorescence is now made with photo-multiplier tubes, but the details of these devices will not be discussed except to mention that with fluorimeters which measure ultraviolet and visible fluorescence two photomultiplier tubes, one 'ultraviolet sensitive' and one 'visible-sensitive' may be used. Although these phototubes are interchangeable for the greater part of the spectrum, the ultraviolet-sensitive tube is to be preferred below 330 $m\mu$, whilst the visible-sensitive tube is more sensitive at the red end of the spectrum (see RCA Tube Handbook).

INSTRUMENTS There are two kinds of fluorimeters commercially available, the filter fluorimeters and the spectrofluorimeters, and the main consideration in deciding which of these instruments to acquire may be an economic one, since the former are much cheaper than the latter. However, spectrofluorimeters are much more versatile than filter instruments for not only is their range much greater, but they also allow maximum excitation and fluorescence wavelengths to be determined and excitation

and fluorescence spectra to be drawn. In fact, they give enough data to the operator to allow analyses to be carried out under optimal conditions. But for many routine analyses, they offer no great advantages over good filter instruments. However, if an institution has a number of filter instruments, a spectrofluorimeter can be very useful in indicating what filters ought to be used for a specific analysis in the filter fluorimeters.

There are several good filter instruments commercially available including those made by the Beckman, Coleman, EEL, Farrand, Hilger-Watt, Locarte, Turner, Photovolt, and Zeiss Instrument companies. Their basic design is similar, a mercury lamp being employed as a light source in most cases. (These instruments generally cost between £200 and £500.)

The main commercial spectrofluorimeters are the Aminco-Bowman, Farrand, and Zeiss instruments. The first two employ diffraction gratings as monochromators whilst the third, a recently introduced instrument, uses quartz prisms. The use of prisms results in a loss of sensitivity, which is, however, compensated by an increased resolving power, and wavelengths can be determined more accurately. The light sources in all three instruments are xenon-arc lamps, the Aminco-Bowman and Farrand instruments employing a 150 watt lamp and the Zeiss instrument a 500 watt lamp whose higher light intensity is intended to compensate for the loss of sensitivity due to the prism optical system. (The basic cost of these instruments is between £1,250 and £3,000.)

FLUORESCENT MOLECULES OF BIOLOGICAL INTEREST

It should now be clear what types of chemical structures are likely to be fluorescent and why simple non-aromatic amino-acids, saturated lipids, steroids (except oestrogens), and carbohydrates are non-fluorescent. There is no reason, however, why it should not be possible to convert the above types of non-fluorescent compounds into fluorescent products by suitable chemical reactions (Udenfriend, 1962). There are a large number of biologically important compounds which contain the necessary conjugated double bond systems and are, therefore, potentially or actually fluorescent. These include aromatic amino-acids, certain proteins, carotinoids, porphyrins, purines, indoles, phenols, oestrogens, vitamins (A, K, pyridoxine, thiamine, riboflavin, tocopherol), hormones (adrenaline, serotonin), and co-enzymes. Furthermore, there are a large number of drugs which are fluorescent *e.g.*, quinine, salicylates, barbiturates, quinoline, antimalarials, cou-

marins, tetracyclines, chlorpromazine derivatives, etc. One can usually tell from the structural formula of a compound whether it is likely to be fluorescent, by examining its structure for conjugated double

TABLE VII
SOME FLUORESCENT NATURAL COMPOUNDS¹

Compound	Excitation Maximum (m μ)	Fluorescence Maximum (m μ)	pH or Solvent
Adenosine	285	395	1
Adenylic acid	285	395	1
Adrenaline	285	325	1
<i>p</i> -Aminobenzoic acid	295	345	11
Anthranilic acid	300	405	7
ATP	285	395	1
Folic acid	365	450	7
Folinic acid	370	460	7
Homogentisic acid	290	340	7
Homovanillic acid	270	315	7
5-Hydroxyindolacetic acid	300	355	7
Kynurenine	370	490	11
NADP	340	435	7
Oestradiol	285	330	Ethanol
Oestrone	285	325	Ethanol
Pyridoxal	330	385	7
Pyridoxine	340	400	7
Riboflavin	270	520	7
	370		
	445		
Serotonin	295	340	2
	295	540	0
Tocopherol	295	330	Ethanol
Tryptophan	285	365	11
Tryptamine	290	360	7
Tyrosine	275	310	7
Tyramine	375	310	1
Vitamin A	325	470	Ethanol
Vitamin B ₁₂	275	305	7

¹Taken from Duggan, Bowman, Brodie, and Udenfriend (1957); the maxima quoted are instrumental values.

TABLE VIII
SOME FLUORESCENT DRUGS¹

Compound	Excitation Maximum (m μ)	Fluorescence Maximum (m μ)	pH or Solvent
Aminopterin	280, 370	460	11
Cinchonine	320	420	1
Chloroquine	335	400	11
Chlorpromazine	350	480	11
Dromoran	275	320	1
Harmin	300, 365	400	1
Lysergic acid diethylamide (L.S.D.)	325	465	7
Menadione	335	480	Ethanol
Neocinchophen	275, 345	455	7
Phenylephrine	270	305	1
Piperoxane	290	325	7
Pamaquin	300, 370	530	13
Podophyllotoxin	280	325	11
Procaine	275	345	11
Procainamide	275	385	11
Quinine	250, 350	450	1
Reserpine	300	375	1
Oxytetracycline	390	520	11
Yohimbine	270	360	1

¹Taken from Udenfriend, Duggan, Vasta, and Brodie (1957); the maxima quoted are instrumental values.

bonds and aromatic systems and for the side chains which contribute so much to fluorescence intensity; and also noting the presence of deactivating atoms or systems in the molecule and the possibility of ionization if pH changes were to be introduced into the medium. A list of some fluorescent natural compounds is given in Table VII, and of some drugs in Table VIII.

It is not possible, within the limits of the review, to deal with the fluorescent properties of all the compounds of importance in biochemistry and medicine, although several such compounds have already been referred to earlier in this text. However, in what follows some examples have been selected to illustrate the possibilities of fluorescence.

PYRIMIDINES AND PURINES These compounds are of considerable interest because of their occurrence in nucleic acids and nucleotides. If the structure of pyrimidine and purine is examined (see below), it will be seen that both molecules contain doubly bound nitrogen ($=N=$), and as stated earlier (see p. 375), such compounds might be expected to be non-fluorescent. However, as also stated earlier, the effects of these nitrogens could be overcome by

suitable substituents and by ionization. Udenfriend and Zaltzman (1962) have examined a number of pyrimidines⁶ for fluorescence and found only thymine (2,4-dihydroxy-5-methylpyrimidine) to be fluorescent. The fluorescence of thymine was very weak and only occurred at pH 11 to 13 where thymine exists as an anion.

Purine, however, is weakly fluorescent as an anion, and presumably the $>NH$ group when ionized is sufficiently electron-donating to overcome the effect of the doubly bound nitrogens in the ring system. Hypoxanthine, xanthine, and uric acid are

non-fluorescent, but adenine and guanine and the nucleotides derived from them are fluorescent at certain pH values. Adenine is fluorescent only as the mono-cation. Adenosine, adenylic acid, adenosine diphosphate (ADP), and adenosine triphosphate (ATP) are also weakly fluorescent in

*Börresen (1963) has recently claimed that pyrimidine fluoresces weakly at high concentrations. This finding is contrary to accepted theory although Börresen has attempted to explain it.

dilute acid solution, and in $5N-H_2SO_4$ they are more fluorescent than adenine itself. Guanine is the most fluorescent of the natural purines, for its mono-cation and mono-anion are fluorescent. Its nucleoside and nucleotides, guanosine, guanylic acid, guanosine diphosphate, and guanosine triphosphate, are also fluorescent (Udenfriend and Zaltzman, 1962; Börresen, 1963). Guanosine and guanylic acid are fluorescent in acid solution, but they have very little fluorescence at pH 11 where guanine is appreciably fluorescent. These observations have been used as the basis of a fluorimetric assay of guanine in nucleic acid hydrolysates (Udenfriend and Zaltzman, 1962).

BARBITURATES An examination of the structural formula of a simple barbiturate (see below) shows that the molecule does not contain conjugated double bonds and is therefore unlikely to be fluorescent. In fact, barbiturates are not fluorescent in aqueous solution, but in 0.1N or $N-NaOH$ some of them show appreciable fluorescence. Phenobarbitone, pentobarbitone, amylobarbitone, and the thiobarbiturates, thiopental and surital, are fluorescent at pH 13 to 14 (Udenfriend, Duggan, Vasta, and Brodie, 1957). Barbiturates have two pK_a's, one

in the region of 7 to 8 corresponding to the formation of a mono-anion, and the other in the region of 12 to 13 corresponding to the formation of the di-anion (Butler, Ruth, and Tucker, 1955). Since the fluorescence of the barbiturates occurs at pH 13 to 14, the fluorescent species is probably the di-anion. Probably the two ionized OH groups opposing the two doubly bound nitrogens are the main contributors in producing the conditions responsible for fluorescence. The thiobarbiturate fluorescence is also probably due to a di-anion (see above). It occurs at a

longer wavelength (530 $m\mu$) than the barbiturate fluorescence (440 $m\mu$). *N*-Substituted barbiturates such as *N*-methyl-phenobarbitone are unable to form a di-anion and are probably not fluorescent.

PYRIDINE NUCLEOTIDES Nicotinamide is non-fluorescent and this agrees with the fact that its molecule contains doubly bound nitrogen and the *meta*-directing carbamyl group both of which would tend to produce a non-fluorescent molecule. The

oxidized forms of the pyridine nucleotides (NAD⁺ and NADP⁺) are also non-fluorescent, but the reduced forms (NADH and NADPH) are fluorescent, a property which has been used for the assay of a number of dehydrogenases, including lactic, glutamic, and malic dehydrogenases, and to study the oxidation-reduction mechanisms of intact cells and subcellular particles (Lowry, Roberts, and Kapphahn, 1957; Lowry, Roberts, and Lewis, 1956; Duysens and Ames, 1957; Duysens and Sweep, 1957; Chance and Baltscheffsky, 1958; Chance and Legallais, 1959). The components of these coenzymes (NADH and NADPH) are adenine, reduced nicotinamide, ribose, and phosphate. The reduced form of *N*-methylnicotinamide is fluorescent (excitation, 360 $m\mu$, and fluorescent, 468 $m\mu$) and the fluorescent form of this molecule as proposed by Weber (1958) is shown above. Adenine is also fluorescent. The corrected excitations of NADH in aqueous solution are at 260 and 340 $m\mu$, and the fluorescence is at 457 $m\mu$ (Weber, 1957). The 260 $m\mu$ excitation is due to the adenine moiety and the fluorescence resulting from it is due to energy transfer from the purine portion of the molecule to the substituted dihydronicotinamide portion, the excitation of which is at 340 $m\mu$. The fluorescent form of NADH proposed by Weber is shown above.

The oxidized forms of the pyridine nucleotides can be induced to fluoresce by heating with alkali (Kaplan, Colowick, and Barnes, 1951; Lowry *et al.*,

1957), and this procedure can be adapted to measure both the oxidized and reduced coenzymes fluorimetrically. Fluorimetric methods have also been devised for *N*-methylnicotinamide, by condensation with ketones in the presence of alkali to give highly fluorescent products (Udenfriend, 1962, for references).

PORPHYRINS The basic ring system in the porphyrins is that of porphin, which, as can be seen from its structure, contains the necessary conjugated double bonds which give rise to fluorescence. Porphyrins in acid solution and in organic solvents are, in fact, highly fluorescent, the fluorescence

occurring in the red and infrared regions of the spectrum. However, it is important to note that the biologically active forms of porphyrins in animals, that is, the metalloporphyrins containing iron and copper, are non-fluorescent. When these metals are removed fluorescence appears. Chlorophyll, on the other hand, is a magnesium porphyrin which is fluorescent in water and alcohol (for details of porphyrin fluorescences, see Vannotti, 1954; and Udenfriend, 1962).

Porphyrins show several fluorescent bands but the principal bands in pyridine are the following:—

	$m\mu$
Protoporphyrin	634
Coproporphyrin	620
Uroporphyrin	623
Haematoporphyrin	625
Mesoporphyrin	621
Aetioporphyrin	621

When haemoglobin undergoes degradation in the body, one of the products is urobilinogen. In this compound, the methine bridges ($-\text{CH}=\text{}$) which occur in the original porphin structure have been reduced so that the conjugated double bond system has been destroyed. Urobilinogen is, therefore, non-fluorescent. However, if it is oxidized to urobilin, conjugation is partly restored, and urobilin is fluorescent.

PYRIDOXINE AND RELATED COMPOUNDS Pyridoxine (vitamin B₆), pyridoxal, pyridoxal 5-phosphate, and pyridoxamine play an important role in the activity of several enzymes, *e.g.*, transaminases, decarboxylases, etc. These compounds are derivatives of 3-pyridol and they show very interesting fluorescence changes at different pHs (Bridges *et al.*, 1963). The fluorescence characteristics of the

pyridoxine series formulated above are similar to those of 3-pyridol and it will be simpler to describe the fluorescences of the latter than to describe the detailed fluorescences of the pyridoxine compounds. 3-Pyridol can occur at different pH values in four different forms each having a characteristic fluorescence:—

The cation, neutral molecule, and dipolar ion of 3-pyridol are only weakly fluorescent, but the anion is strongly fluorescent, its intensity being 50 times that of the cation and 16 times that of the dipolar ion. The cation shows excited state ionization, so that at pH values more than -1 up to 7, it shows the fluorescence of the dipolar ion. The true fluorescence of the cation, *i.e.*, at 340 mμ, is only shown at pH -1 (10N-HCl) and less. The unexcited cation ionizes to the dipolar ion at pH 4 to 5 (pKa 4.86), so that in the excited state (pKa -0.95) the cation is about a million times weaker as a base than in the unexcited state.

The phenomenon of excited state ionization is also shown by the cations of pyridoxine, pyridoxal and its phosphate, and pyridoxamine. However, all the forms of these compounds are more fluorescent than the corresponding forms of 3-pyridol. The most fluorescent forms of pyridoxine and of pyridoxamine are the corresponding dipolar ions (see Table IX) whereas those of pyridoxal and its phosphate are the anions. In the case of pyridoxal the most fluorescent form is probably the anion of the cyclic hemiacetal of pyridoxal, and in the case of

pyridoxal phosphate it is probably the tri-anion of the aldehyde hydrate of pyridoxal phosphate (see Table IX). At high pH values (12 to 14) pyridoxal phosphate shows two fluorescences, one at 370 mμ corresponding to the tri-anion of the hydrate, and another, eight times less intense, at 525 mμ which appears to correspond to the tri-anion of the free aldehyde form of pyridoxal phosphate. 3-Pyridol is

stable in ultraviolet light but pyridoxine and its three congeners show photo-decomposition so that measurements of fluorescence have to be made within 30 seconds of exposure to the exciting light.

The fluorimetric assay of these compounds in biological materials is carried out by oxidation to the highly fluorescent pyridoxic acid which is eventually converted into its lactone which, at pH 9, is some 25 times more fluorescent than the acid (Huff and Perlzweig, 1944; Reddy, Reynolds, and Price, 1958).

PROTEIN FLUORESCENCE AND FLUORESCENCE POLARIZATION The study of the fluorescence of proteins is a difficult but interesting field and up to the present it has hardly been exploited. Here it is intended but to mention a few of its simpler aspects and for further information the reader is referred to Chapter 6 of the book by Udenfriend (1962) and to reviews and papers by Weber and his associates (*e.g.*, Weber, 1953; Weber, 1960 a, b; Teale, 1960; see also Udenfriend, 1962, for other references).

Simple proteins absorb light in the region 270-300 mμ and this absorption is due mainly to the aromatic amino-acids, tyrosine and tryptophan. Proteins containing these amino-acids could therefore be expected to fluoresce. In addition to these amino-acids, proteins frequently contain phenylalanine which is also fluorescent, but its fluorescence is much weaker than that of the other two. The fluorescence maxima and quantum efficiencies (Q.E.) of these acids in neutral aqueous solution are: tryptophan, λmax. 348 mμ, Q.E. 20%; tyrosine, λmax. 303 mμ, Q.E. 21%; and phenylalanine, λmax. 282 mμ, Q.E. 4% (Teale and Weber, 1957). A large number of proteins have been found to fluoresce (Shore and Pardee, 1956; Konev, 1957). Those containing phenylalanine and tyrosine but no tryptophan show a maximum emission at 303 mμ,

TABLE IX
MOST INTENSELY FLUORESCENT FORMS OF PYRIDOXINE AND ITS DERIVATIVES
(5 μ g./ml.) AND EXCITED pKa's OF THE CATIONS
(BRIDGES, DAVIES, AND WILLIAMS, 1963)

Compound	Mos: Fluorescent Form	Excitation ¹ Maximum (m μ)	Fluorescence ¹ Maximum (m μ)	pH of Maximum Fluorescence	pKa of Cation	
					Unexcited ²	Excited ³
3-Pyridol	anion	309	365	10-14	4.86	— 0.95
Pyridoxine	dipolar ion	332	400	6-8	4.72	— 0.8
Pyridoxal	anion of cyclic hemiacetal	310	365	ca. 12	4.22	— 0.95
Pyridoxal 5'-phosphate	tri-anion of aldehyde hydrate	315	370	12-14	4.14	< — 0.3
Pyridoxamine	Tripolar ion	337	400	ca. 5	3.4	— 0.8

¹Uncorrected instrumental values.

²The pKa taken from Metzler and Snell (1955) and Albert (1960).

³Excited pKa's are approximate and deduced from pH/fluorescence intensity curves.

whilst those containing tryptophan show a maximum in the region of 320 to 350 m μ (Teale, 1960; see Table X). The 303 m μ emission is due to tyrosine and the 320 to 350 m μ emission to tryptophan. The tryptophan-containing proteins apparently do not show an emission due to tyrosine which is in some way quenched for there is no energy transfer from tyrosine to tryptophan in the protein. No fluorescence due to the phenylalanine residue has been observed.

A large number of natural proteins contain prosthetic groups, which can have a profound influence on the fluorescence of the protein. These groups include metals, porphyrins, coenzymes, and carbohydrates. If the prosthetic group is capable of absorbing light, it can either enhance or diminish the fluorescence of the protein or it can endow the conjugated protein with its own fluorescence. Usually, haem proteins are non-fluorescent because the excitation energy absorbed by the protein is transferred to the haem portion of the molecule

TABLE X
NATIVE FLUORESCENCE OF PROTEINS
IN WATER (TEALE, 1960)

Protein	λ Maximum (m μ)
<i>A Containing phenylalanine and tyrosine but no tryptophan</i>	
Insulin	304
Ovomucoid	304
Ribonuclease	304
Zein	304
<i>B Containing phenylalanine, tyrosine, and tryptophan</i>	
Edestin	328
Bovine γ -globulin	332
Ovalbumin	332
Trypsin	332
Trypsinogen	332
Chymotrypsin	334
Chymotrypsinogen	331
Haemoglobin globin	335
Fumarase	335
Fibrinogen	337
Human serum albumin	339
Carboxypeptidase	340
Lysozyme	341
Bovine serum albumin	342
Pepsin	342

where it is degraded (Weber and Teale, 1959) (see p. 384). In the case of proteins conjugated with the reduced pyridine nucleotides (*e.g.*, alcohol, lactic, glutamic and malic dehydrogenases) the effect of the prosthetic group is to endow the protein with its own fluorescence which is often intensified and shifted to shorter wavelengths (Shifrin and Kaplan, 1960).

Fluorescent molecules can also be combined with proteins to give highly fluorescent conjugates which can be used for many purposes such as the assay of serum proteins and the distribution of antigens and various proteins in tissues (Udenfriend, 1962).

One aspect of fluorescence which is of particular application to the study of proteins is fluorescence polarization. This phenomenon can be explained as follows. Light consists of waves whose amplitude in all planes is at right angles to the light path. Light is plane polarized if its waves are in one plane only and this polarization can be achieved with a Nicol prism. If molecules are excited by plane polarized light, then those molecules whose axes happen to be orientated in a direction parallel to the light path are preferentially excited. If these molecules are in a rigid medium, the fluorescence they emit will be plane polarized, *i.e.*, the waves are in one plane only. However, if the medium is not rigid, the excited molecules may be able to rotate about their axes before the fluorescence is emitted. In this case the fluorescence will be partially polarized or non-polarized depending upon the rate at which the

excited molecules rotate, the life time of the excited state, and the viscosity of the medium or solvent. The time taken for these excited molecules to disorientate completely is called the relaxation time. Each fluorescent molecule has a characteristic relaxation time for a given solvent under given conditions. For small molecules in low viscosity solvents the relaxation time is short compared with the life time of the excited state (10^{-8} second) so that no polarized fluorescence is observed with such molecules when they are excited by plane polarized light. Large molecules such as proteins, however, may emit polarized fluorescence since their relaxation times are long compared with the life time of the excited state. The degree of polarization can be measured with a suitable Nicol prism analyzer and from this the relaxation time of the molecule can be estimated. The relaxation time gives useful information about the physical characteristics, *e.g.*, shape and size, of a macromolecule. If the macromolecule is one which has no native fluorescence, its relaxation time could still be measured provided it can be conjugated with a small fluorescent molecule (see Weber, 1953; Laurence, 1957).

ADDITIONAL REFERENCES ON COMPOUNDS OF BIOLOGICAL INTEREST Many references to the applications of fluorescence methods in biology and medicine are given in the book by Udenfriend (1962). In addition to these, the following list of recent references may be quoted.

NATURAL PRODUCTS AND THEIR DERIVATIVES

Estrone, β -estradiol, cortisone and related compounds	Ichii, Forchielli, Perloff, and Dorfman (1963)
Rudd, Cowper, and Crawford (1961)
Stewart, Albert-Recht, and Osman, 1961
De Moor, Osinski, Deckx, and Steeno (1962)
Mattingly, (1962)
17 α substituted steroids	Steinetz, Beach, Dubnick, Meli, and Fujimoto (1963)
Methandrostenolone	Tishler, Sheth, Giaimo, and Mader (1962)
Bile acids	Levin and Johnston (1962)
Coproporphyrin	Mentz and Grotepass (1960)
Phospholipids	Harris and Gambal (1963)
Glycerol	Mendelsohn and Antonis (1961)
Acetone	Hynie, <i>et al.</i> , (1960)
Malonaldehyde	Sawicki, Stanley, and Johnson (1963)
Coumarins	Crosby and Berthold (1962)
Anthranilic acid	Sanders and Parks (1962)
Hippuric acid	Ellman, Burkhalter, and LaDou (1961)
Carbohydrates	Towne and Spikner (1963)
Adrenaline, noradrenaline	Merrills (1963)
Jacobs, Sobel, and Henry (1961)
Total catecholamines	Mahler and Humoller (1962)
5-Hydroxy- and 5-methoxy-indoles	Small (1963)
Serotonin	Quay (1963)
Histamine	Crosti and Luchelli (1962)
Tetrahydrofolate and related compounds	Crawford and Rudd (1962)
Pyridoxamine	Noah and Brand (1961)
NADP	Zachariae (1963)
Phenylalanine	Uyeda and Rabinowitz (1963)
2-Dimethylamino-6-hydroxypurine	Toeffer, Polansky, and Hewston (1961)
Guanine	Estabrook (1962)
	McCaman and Robins (1962)
	Udenfriend, Zaltzman-Nirenberg, and Cantoni (1963)
	Udenfriend and Zaltzman (1962)

γ -Globulins
Plasma proteins
Fibrinolysin
Lactic dehydrogenase
Lipase
 Δ^5 -3-Ketosteroid isomerase

Sokol, Hana, and Albrecht (1961)
Hiraoka and Glick (1963)
Pappenhausen, Koppel, and Olwin (1962)
Laursen (1962)
Kramer and Guilbault (1963)
Wang, Kawahara, and Talalay (1963)

Drugs
Digitoxin and digoxin
Emetine hydrochloride
Reserpine
Chlordiazepoxide (librium)
Benzoquinolizines
Phenothiazines
Tetracyclines
Oxytetracycline
Acriflavine
Mechlorethamine (mustargen)
Isoniazid

Jakovljevic (1963)
Ichimura (1961)
Tishler, Sheth and Giaimo (1963)
Koechlin and D'Arconte (1963)
Schwartz and Rieder (1961)
Mellinger and Keeler (1963)
Worsley, McKenna, and Beck (1963)
Ibsen, Saunders, and Urist (1963)
Bičan-Fišter (1960)
Mellett and Woods (1960)
Peters (1960)

Metals in biological material
Selenium

Dye, Bretthauer, Seim, and Blincoe (1963)
Kepner and Hercules (1963)
Wallach and Steck (1963)
Hofman (1961)
Watanabe, Frantz, and Trottier (1963)
Schachter (1961)

Calcium

Magnesium, calcium, and zinc
Magnesium

REFERENCES

- Albert, A. (1960). *J. chem. Soc.*, p. 1020.
Bičan-Fišter, T. (1960). *Acta pharm. jugosl.*, 10, 161. (*Chem. Abstr.* (1961), 55, 14820c.)
Börresen, Hans Chr. (1963). *Acta chem. scand.*, 17, 921.
Bowen, E. J., and Wokes, F. (1953). *Fluorescence of Solutions*. Longmans, Green, London
Bowman, R. L., Caulfield, P. A., and Udenfriend, S. (1955). *Science*, 122, 32.
Bridges, J. W. (1963). Ph.D. Thesis, University of London.
—, Creaven, P. J., and Williams, R. T. (1963). Unpublished data.
—, —, Davies, D. S., and Williams, R. T. (1963). *Biochem J.* 88, 65p.
—, Davies, D. S., and Williams, R. T. (1963). Unpublished data.
—, and Williams, R. T. (1962). *Nature (Lond.)*, 196, 59.
—, —, (1963). Unpublished data.
Brodie, B. B., Udenfriend, S., Dill, W., and Chenkin, T. (1947). *J. biol. Chem.*, 168, 319.
Butler, T. C., Ruth, J. M., and Tucker, G. F. (1955). *J. Amer. chem. Soc.*, 77, 1486.
Chance, B., and Baltschewsky, H. (1958). *J. biol. Chem.*, 233, 736.
—, and Legallais, V. (1959). *Rev. sci. Instr.*, 30, 732.
Cleveland, F. F. (1955). *Raman Spectra In Determination of Organic Structures by Physical Methods*, edited by E. A. Braude and F. C. Nachod, p. 231. Academic Press, New York.
Cowgill, R. W. (1963). *Arch. Biochem.*, 100, 36.
Crawford, N., and Rudd, B. T. (1962). *Clin. chim. Acta*, 7, 114.
Crosby, D. G., and Berthold, R. V. (1962). *Analyt. Biochem.*, 4, 349.
Crosti, P. F., and Lucchelli, P. E. (1962). *J. clin. Path.*, 15, 191.
De Moor, P., Osinski, P., Deckx, R., and Steeno, O. (1962). *Clin. chim. Acta*, 7, 475.
Duggan, D. E., Bowman, R. L., Brodie, B. B., and Udenfriend, S. (1957). *Arch. Biochem.*, 68, 1.
—, and Udenfriend, S. (1956). *J. biol. Chem.*, 223, 313.
Duysens, L. N. M., and Ames, J. (1957). *Biochim. biophys. Acta (Amst.)*, 24, 19.
—, and Sweep, G. (1957). *Ibid.*, 25, 13.
Dye, W. B., Bretthauer, E., Seim, H. J., and Blincoe, C. (1963). *Analyt. Chem.*, 35, 1687.
Ellman, G. L., Burkhalter, A., and LaDou, J. (1961). *J. Lab. clin. Med.*, 57, 813.
Estabrook, R. W. (1962). *Analyt. Biochem.*, 4, 231.
Feigl, F., Feigl, H. E., and Goldstein, D. (1955). *J. Amer. chem. Soc.*, 77, 4162.
Förster, T. (1950). *Z. Elektrochem.*, 54, 531.
Hagins, W. A., and Jennings, W. H. (1959). *Disc. Faraday Soc.*, no. 27, 180.
Harris, R. A., and Gambal, D. (1963). *Analyt. Biochem.*, 5, 479.
Hercules, D. M., and Rogers, L. B. (1959). *Spectrochim. Acta*, 14, 393.
Hiraoka, T., and Glick, D. (1963). *Analyt. Biochem.*, 5, 497.
Hofman, J. (1961). *Čas. Lék. čes.*, 100, 1171.
Huff, J. W., and Perlzweig, W. A. (1944). *J. biol. Chem.*, 155, 345.
Hynic, I., Večerek, B., and Wágner, J. (1960). *Čas. Lék. čes.*, 99, 88.
Ibsen, K. H., Saunders, R. L., and Urist, M. R. (1963). *Analyt. Biochem.*, 5, 505.
Ichii, S., Forchielli, E., Perloff, W. H., and Dorfman, R. I. (1963). *Ibid.*, 5, 422.
Ichimura, Y. (1961). *Bunseki Kagaku*, 10, 623. (*Chem. Abstr.* (1962), 56, 1530c.)
Jacobs, S. L., Sobel, C., and Henry, R. J. (1961). *J. clin. Endocr.*, 21, 305.
Jakovljevic, I. M. (1963). *Analyt. Chem.*, 35, 1513.
Kaplan, N. O., Colowick, S. P., and Barnes, C. C. (1951). *J. biol. Chem.*, 191, 461.
Kasha, M. (1960). *Radiat. Res.*, suppl., 2, 243.
Kepner, B. L., and Hercules, D. M. (1963). *Analyt. Chem.*, 35, 1238.
Kerr, V. N., Hayes, F. N., and Ott, D. G. (1957). *Int. J. appl. Radiat.*, 1, 284.
Koechlin, B. A., and D'Arconte, L. (1963). *Analyt. Biochem.*, 5, 195.
Kohn, K. W. (1961). *Analyt. Chem.*, 33, 862.
Konev, S. V. (1957). *Dok. Akad. Nauk SSSR Otd. Biokh.*, 116, 594.
Kramer, D. N., and Guilbault, G. G. (1963). *Analyt. Chem.*, 35, 588.
Kuhn, R., Wagner-Jauregg, T., and Kaltschmitt, H. (1934). *Ber. dtsh. chem. Ges.*, 67, 1452.
Laurence, D. J. R. (1957). In *Methods of Enzymology*, edited by S. P. Colowick and N. O. Kaplan, vol. IV, p. 174. Academic Press, New York.
Laursen, T. (1962). *Scand. J. clin. Lab. Invest.*, 14, 152.
Levin, S. J., and Johnston, C. G. (1962). *J. Lab. clin. Med.*, 59, 681.
Lippert, E., Lüder, W., and Moll, F. (1959). *Spectrochim. Acta*, 10, 858.
Livingston, R., Watson, W. F., and McArdle, J. (1949). *J. Amer. chem. Soc.*, 71, 1542.
Lowry, O. H., Roberts, N. R., and Kappahnn, J. I. (1957). *J. biol. Chem.*, 224, 1047.
—, —, and Lewis, C. (1956). *Ibid.*, 220, 879.
Mahler, D. J., and Humoller, F. L. (1962). *Clin. Chem.*, 8, 47.
Mattingly, D. (1962). *J. clin. Path.*, 15, 374.
McCaman, M. W., and Robins, E. (1962). *J. Lab. clin. Med.*, 59, 885.
Mellett, L. B., and Woods, L. A., (1960). *Cancer Res.*, 20, 518.
Mellinger, T. J., and Keeler, C. E. (1963). *Analyt. Chem.*, 35, 554.
Mendelsohn, D., and Antonis, A. (1961). *J. Lipid Res.*, 2, 45.
Mentz, H. E. A., and Grotepass, W. (1960). *S. Afr. J. Lab. clin. Med.*, 6, 43.
Merrills, R. J. (1963). *Analyt. Biochem.*, 6, 272.
Metzler, D. E., and Snell, E. E. (1955). *J. Amer. Chem. Soc.*, 77, 2431.
Noah, J. W., and Brand, A. (1961). *J. Allergy*, 31, 236.
Ott, D. G., Hayes, F. N., Hansbury, E., and Kerr, V. N. (1957). *J. Amer. chem. Soc.*, 79, 5448.

- Pappenhausen, A. R., Koppel, J. L., and Olwin, J. H. (1962). *J. Lab. clin. Med.*, **59**, 1039.
- Parker, C. A. (1959). *Analyst*, **84**, 446.
- , and Barnes, W. J. (1957). *Ibid.*, **82**, 606.
- , and Rees, W. T. (1962). *Ibid.*, **87**, 83.
- Peters, J. H. (1960). *Amer. Rev. resp. Dis.*, **81**, 485.
- Porter, G., and Weber, G. (1959). *Nature (Lond.)*, **184**, 688.
- Quay, W. B. (1963). *Analyt. Biochem.*, **5**, 51.
- R.C.A. Tube Handbook, HB-3. Radio Corporation of America, Electron Tube Division, Harrison, New Jersey.
- Reddy, S. K., Reynolds, M. S., and Price, J. M. (1958). *J. biol. Chem.*, **233**, 691.
- Rosen, A., and Williams, R. T. (1961). *Bull. photoelect. Spectrom. Grp*, no. 13, 339.
- Rudd, B. T., Cowper, J. M., and Crawford, N. (1961). *Clin. chim. Acta*, **6**, 686.
- Sanders, P. P., and Parks, L. W. (1962). *Analyt. Biochem.*, **3**, 354.
- Sawicki, E., Stanley, T. W., and Johnson, H. (1963). *Analyt. Chem.*, **35**, 199.
- Schachter, D. (1961). *J. Lab. clin. Med.*, **58**, 495.
- Schwartz, D. E., and Rieder, J. (1961). *Clin. chim. Acta*, **6**, 453.
- Shifrin, S., and Kaplan, N. O. (1960). *Advanc. Enzymol.*, **22**, 337.
- Shore, V. G., and Pardee, A. B. (1956). *Arch. Biochem.*, **60**, 100.
- Small, N. A. (1963). *Clin. chim. Acta*, **8**, 803.
- Sokol, F., Hana, L., and Albrecht, P. (1961). *Folia Microbiol. (Praha)*, **6**, 145.
- Steinetz, B. G., Beach, V. L., Dubnick, B., Meli, A., and Fujimoto, G. (1963). *Steroids*, **1**, 395.
- Stewart, C. P., Albert-Recht, F., and Osman, L. M. (1961). *Clin. chim. Acta*, **6**, 696.
- Szent-Györgyi, A. E. (1957). *Bioenergetics*. Academic Press, New York.
- Teale, F. W. J. (1960). *Biochem. J.*, **76**, 381.
- , and Weber, G. (1957). *Ibid.*, **65**, 476.
- Tishler, F., Sheth, P. B., and Giaimo, M. B. (1963). *J. Ass. off. agric. Chem.*, **46**, 448.
- , —, —, and Mader, W. J. (1962). *J. pharm. Sci.*, **51**, 1175.
- Toepfer, E. W., Polansky, M. M., and Hewston, E. M. (1961). *Analyt. Biochem.*, **2**, 463.
- Towne, J. C., and Spikner, J. E. (1963). *Analyt. Chem.*, **35**, 211.
- Udenfriend, S. (1962). *Fluorescence Assay in Biology and Medicine*. Academic Press, New York.
- , Duggan, D. E., Vasta, B. M., and Brodie, B. B. (1957). *J. Pharmacol. exp. Ther.*, **120**, 26.
- , and Zaltzman, P. (1962). *Analyt. Biochem.*, **3**, 49.
- , Zaltzman-Nirenberg, P., and Cantoni, G. L. (1963). *Ibid.*, **5**, 258.
- Uyeda, K., and Rabinowitz, J. C. (1963). *Ibid.*, **6**, 100.
- Van Duuren, B. L. (1963). *Chem. Rev.*, **63**, 325.
- Vannotti, A. (1954). *Porphyrins—Their Biological and Chemical Importance*, translated by C. Rimington, p. 20. Hilger and Watts, London.
- Wallach, D. F. H., and Steck, T. L. (1963). *Analyt. Biochem.*, **6**, 176.
- Wang, S., Kawahara, F. S., and Talalay, P. (1963). *J. biol. Chem.*, **238**, 576.
- Watanabe, S., Frantz, W., and Trotter, D. (1963). *Analyt. Biochem.*, **5**, 345.
- Weber, G. (1953). *Advanc. Protein Chem.*, **8**, 415.
- (1957). *Nature (Lond.)*, **180**, 1409.
- (1958). *J. Chim. Phys.*, **55**, 878.
- (1960a). *Biochem. J.*, **75**, 335.
- (1960b). *Ibid.*, **75**, 345.
- , and Teale, F. W. J. (1959). *Disc. Faraday Soc.*, no. 27, 134.
- Weil-Malherbe, H. (1946). *Biochem. J.*, **40**, 363.
- White, A. (1959). *Ibid.*, **71**, 217.
- Williams, R. T. (1959). *J. roy. Inst. Chem.*, **83**, 611.
- Worsley, G. H., McKenna, R. D., and Beck, I. T. (1963). *Canad. med. Ass. J.*, **88**, 1272.
- Zachariae, H. (1963). *Scand. J. clin. Lab. Invest.*, **15**, 173.