RECENT ADVANCES IN

3

CLINICAL BIOCHEMISTRY

Edited by

C. P. Price

K. G. M. M. Alberti

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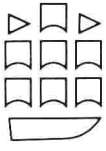
NUMBER THREE



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Preface

It is sometimes said that the development of clinical biochemistry has been very closely associated with advances in technology. Indeed there are many examples to support this view; which came first—biochemical profiling or the multichannel analyser? The renewed interest in the measurement of electrolytes by way of ionic activity, it may be argued, is a result of advances in the design and manufacture of ion selective electrodes.

The view that the repertoire of biochemical analyses is defined by technology is unfair on those who have pioneered the development of clinical biochemistry. However, there is no doubt that advances in technology play a key role in the contribution that fundamental science makes to our discipline. Monoclonal antibodies, for example, whilst making a contribution to the analytical techniques used in routine clinical biochemistry, have probably made a far greater contribution in purification technologies and also in the development of molecular probes.

The Recent Advances series has always attempted to present developments in clinical biochemistry, both in the technology and the fundamental science recognising that the two are inseparable. The first three chapters present three very different technologies that have risen to prominence in the last decade. Each of these technologies has yet to reach its full potential in laboratory medicine and this is a unique opportunity to review their impact to date.

The middle section of the book reviews seven established subjects drawn from the diagnostic and fundamental aspects of clinical biochemistry. The 'acute phase reaction' is a well-recognised but little-understood phenomenon; the chapter on the so-called acute phase proteins provides a valuable insight into some of the features of this phenomenon. The chapter on bile acids leads the reader logically from bile acid metabolism through to the use of bile acid measurement in the diagnosis and management of disease.

The chapter on hyponatraemia continues the strategy linking basic physiology with a discussion of pathological change through to diagnosis and management. Basic physiology is at the core of the next chapter which reviews the role of opiate receptors in the control of reproductive functions.

Respiratory disease is often limited to a discussion of acid base status; this approach is clearly wrong as acid base status is maintained through involvement of several organs of the body. This review of the biochemical investigation of respiratory disease is broader in that it considers the wider aetiology of respiratory disease.

A chapter on burn injury reviews the widespread biochemical changes that occur and discusses the physiological changes that take place in this condition. The emphasis on physiology is maintained in a review of the biochemical assessment of small intestinal function.

The final two chapters return to advances in technology in a sphere that embraces the challenge of clinical biochemistry. Two decades have past since the concept of a centralized analytical facility was proposed. It is clear where this became established, that the gap that grew up between those requesting and those providing biochemical investigations was to the detriment of the discipline. The opportunity now exists for the doctor to perform his/her own analyses; measuring a blood glucose or theophylline may be as easy as taking a temperature.

When this technology becomes established many of the concepts in the provision of a clinical biochemistry service will be challenged.

1985

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1. Chemiluminescence in medical biochemistry

Anthony K. Campbell Mary E. Holt Ashok Patel

Analytical techniques based on chemiluminescence have been in use for some time in research laboratories measuring ATP, pyridine nucleotides or intracellular calcium. The unique characteristics of chemiluminescence are now attracting much attention from clinical biochemists, particularly those interested in developing immunoassay technology and those studying the pathological significance of oxygen radicals. Before exploring the clinical potential of chemiluminescence it is first necessary to examine the phenomenon in order to identify characteristics uniquely suited to the investigation of medical problems.

WHAT IS CHEMILUMINESCENCE?

The phenomenon

Most animals and plants respond to light. Man is no exception, for phenomena causing the emission of light, collectively known as luminescence, have fascinated the layman and scientist alike for centuries. The various types of luminescence are distinguished semantically by a prefix (chemi-, photo-, electro-, radio-, sono-, tribo-, thermo-) which designates the source of energy ultimately responsible for generating molecules in an electronically excited state. Some of these excited molecules will emit a photon as decay to ground state. In chemiluminescence the energy arises as the result of a chemical reaction, and is distinguished from photoluminescence such as fluorescence and phosphorescence where the excited molecules are generated by absorption of electromagnetic radiation, often visible light. The physical nature of the excited product molecules of a chemiluminescent reaction may be identical to that induced by conventional fluorescence. However, in several chemiluminescent reactions the molecular species actually emitting the light is very unstable chemically and thus only exists transiently during the reaction. The result is that the fluorescence spectrum, if it exists, of the stable reaction products may be different from that observed during the chemiluminescent reaction.

The chemiluminescence of lophine oxidation has been known for more than a century (Radziszewski, 1877). Chemiluminescence has since been observed as the result of the oxidation of many synthetic organic compounds (see Johnson & Haneda, 1966; McCapra, 1966, 1973, 1982; Gunderman, 1974; Campbell & Simpson, 1979; Carter & Kricka, 1982 for references). Two groups of compounds have attracted considerable interest in the research and clinical laboratory during the past 10 years, phthalazine diones such as luminol characterised by Albrecht in 1928, and acridinium salts such as the esters of N methylacridinium salts discovered by McCapra (see Fig. 1.1).

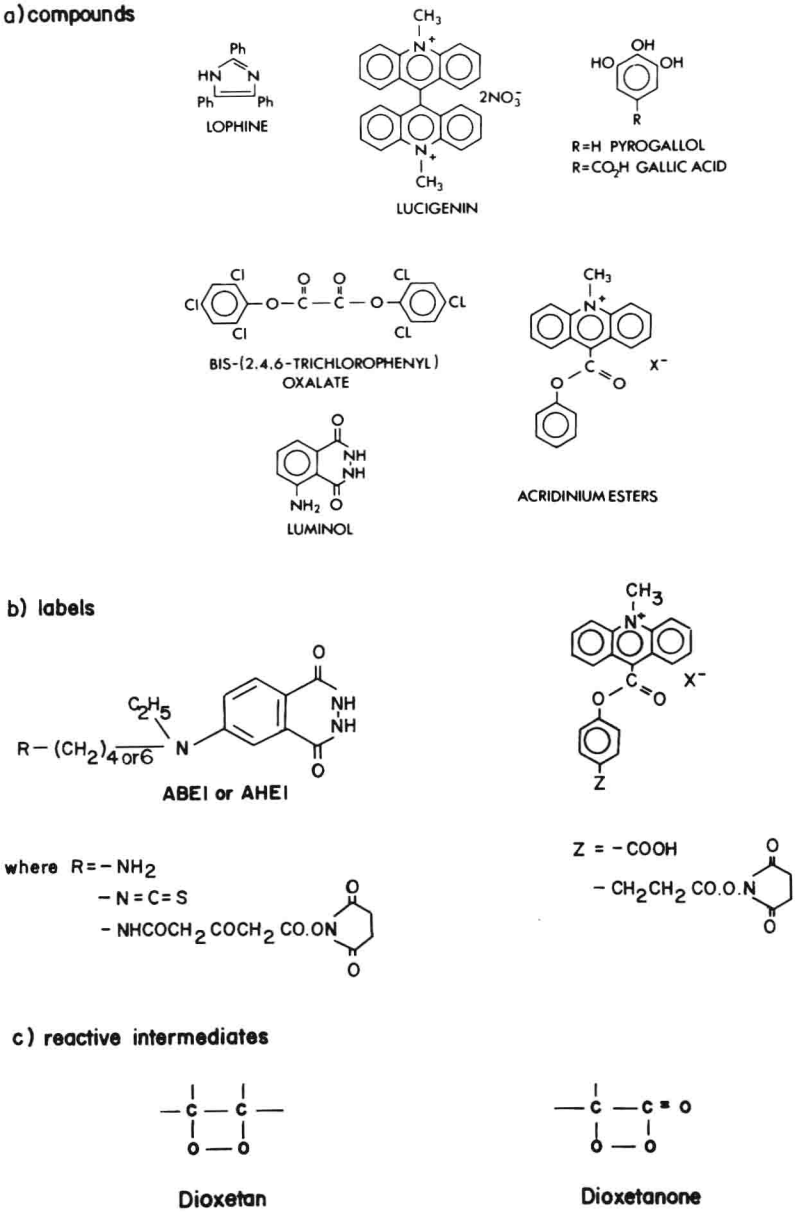

Fig. 1.1 Some chemiluminescent compounds. This includes labelling groups such as isothiocyanate and N-hydroxysuccinimide and reaction intermediates such as dioxetans and dioxetanones responsible for producing excited carbonyl groups.

At night spectacular displays of some of nature's examples can be seen, including the yellow-green luminescence of fireflies and glow-worms, the blue luminescence of decaying fish caused by certain bacteria, or the blue luminescence from a ship's wake due to luminous dinoflagellates or jelly-fish. Most of this bioluminescence is marine

though a few genera such as fireflies are terrestrial (Harvey, 1940, 1952; Herring, 1978).

Chemistry

The chemiluminescence of most synthetic compounds is an oxidative process requiring either O_2 or H_2O_2. The two groups currently of most clinical interest, namely phthalazine dione derivatives and acridinium compounds (Fig. 1.1), react best and with greatest light yield at alkaline pH, though luminescence can be observed at physiological pH. They emit blue light. Phthalazine diones also require a 'catalyst' for maximum rate of light emission, though several of these compounds may not be true catalysts but rather act as substances supplying electrons. Peroxidases and microperoxidase (Feder, 1970), a degradation product of cytochrome c, are commonly used. The optimal pH with microperoxidase for greatest light yield is 11 for luminol and 13 for isoluminol, with more than 90% of the light being emitted within 10–40 s (Patel, 1983).

Robert Boyle in 1668 showed that the luminescence of decaying fish and fungi was extinguished in a vacuum. The luminescence of most other bioluminescent systems, with the exception of certain luminous coelenterates and radiolarians, has since been shown to require molecular oxygen (Harvey, 1952; Herring, 1978; Campbell et al, 1980). The luminescence of marine bacteria has been used in a highly sensitive probe for oxygen in the nmol/l range (Lloyd et al, 1980) with useful application in the study of anaerobes. Luminous radiolarians such as *Thalassicola* and coelenterates such as *Aequorea* and *Obelia* contain photoproteins which emit light when they bind Ca^{2+} (Shimomura et al, 1962; Campbell et al, 1981; Hallett & Campbell, 1982a). No O_2 is required since the prosthetic group of the protein already has a hydroperoxide group attached to it. These Ca^{2+}-activated photoproteins have provided unique information about the concentration of free Ca^{2+} inside cells (Ridgway & Ashley, 1967; Ashley & Campbell, 1979; Campbell, 1983).

The major breakthrough in understanding the chemistry of luminous animals came as the result of the experiments of Raphael Dubois (1885, 1887, 1892, 1914) working first with a luminous beetle, *Pyrophorus* and then with a rock-boring mollusc, *Pholas dactylus*. He showed that the luminous organs contained a heat stable factor and a heat labile factor which, when added together in the presence of O_2, produced light. He called these factors *luciférine* and *luciférase* respectively. In 1947 McElroy showed, using the firefly *Photinus pyralis*, that another cofactor is sometimes required in other organisms, in this case Mg ATP. This means that if a bioluminescent reaction is to be used to measure a substance of biological or clinical interest it must be coupled to one of the four possible components of the reaction namely O_2, luciferase, luciferin or the cofactor(s).

Non-radiative energy transfer

Some luminous coelenterates, for example *Aequorea*, *Obelia* and *Renilla*, emit a greener light (λ_{max} *ca* 510 nm) than that from the luminescent proteins (λ_{max} *ca* 460–480 nm) extracted from these organisms. A similar phenomenon occurs in some luminous fish, for example, *Malacosteus*, where light emission shifts from blue into the red. The reason for this is that the cells containing the luminescent protein also have another protein containing a fluorophore capable of excitation by the chemilu-

minescent chromophore without the direct transfer of a photon. The efficiency of this non-radiative energy transfer occurring by dipole–dipole resonance is predicted by the Förster equation (1948):

$$\text{Efficiency} = E = d^{-6}/(d^{-6} + R_o^{-6}) \qquad \text{(Eqn 1)}$$

where d = distance between the centres of the donor and acceptor chromophores
$R_o = (JK^2\, Q_o n^{-4})^{1/6} \times 97 \times 10^3$ Å

This contains the spectroscopic variables

J = spectral overlap integral
n = refractive index of medium between the donor and acceptor
k_F = the rate constant for photon emission by the donor (the chemiluminescent compound)
Q_o = the quantum yield of the chemiluminescent energy donor in the absence of the acceptor.

The rate of energy transfer = $k_T = J \times \text{constant} \times d^{-6}\ s^{-1}$

$$\text{constant} = K^2.\ n^{-4}.\ k_F \times 8.71 \times 10^{23} \qquad \text{(Eqn 2)}$$

K^2 = the orientation factor for a dipole–dipole interaction.

As a general rule energy transfer is negligible if the donor and acceptor molecules are more than 50–100 Å apart (Stryer, 1978). The possibility therefore exists that ligand–ligand interactions could be quantified by chemiluminescence energy transfer. Since an antibody molecule has a radius of about 40 Å it should be possible to quantify antibody–antigen reactions using the change in quantum yield, kinetics or wavelength resulting from non-radiative energy transfer, without the need to separate bound and free antigen (Campbell et al, 1980a; Hallett & Campbell, 1982a; Patel et al, 1983). Other homogenous ligand–ligand interactions should also be able to be studied in this way, for example hormone-receptor interactions.

Dim chemiluminescence

No mammals have luminous organs visible to the naked eye. However, during the late 1920s A. G. Gurvich reported that dividing cells of onion root, the regenerating cornea of the eye of a frog, dividing cells of malt yeast and blood cells produce a dim emission in the ultraviolet region (190–326 nm). This was detected by biological indicators such as onion root or yeast cells. In spite of the controversial nature of these initial observations several other workers, using more reliable light detectors such as photomultipliers, have also reported UV emission during mitosis (see Baremboim et al, 1969 for references).

Dim chemiluminescence in the visible region was first observed in higher plants (Colli et al, 1955). In 1961 Tarusov and co-workers discovered that mouse liver in situ produces a very weak light emission not visible to the eye but detectable with a sensitive photomultiplier connected to a photon counting device. They extended their observations to brain, muscle, intestine, as well as to tissue homogenates and lipid extracts (Tarusov et al, 1961, 1962). Some 28 years earlier (Ado, 1933; Baldridge & Gerard, 1933) it was observed that a burst of O_2 uptake occurs in phagocytic cells when activated. This 'respiratory burst' has since been observed in polymorphs,

monophages and monocytes (Karnovsky, 1962; Cline & Lehree, 1968; Babior et al, 1973; Klebanoff & Clark, 1978), and several other cells including some fertilised eggs (Foerder et al, 1978). It is not inhibited by CN^- and is therefore not caused by increased mitochondrial respiration. It appears to be caused by activation of an NADPH oxidase on the cell membrane which, coupled apparently to a unique b cytochrome (Segal et al, 1978; Segal & Jones, 1979), reduces O_2 to superoxide anion, O_2^-. This radical, together with other active oxygen species ($\cdot OH$; OCl^-; H_2O_2; $^1O_2$), plays a key role in the killing of endocytosed bacteria. In 1972 Allen showed that this production of oxygen radicals by phagocytes results in a weak luminescence from these cells (Fig. 1.2). Polymorphonuclear leukocytes (polymorphs), macrophages and

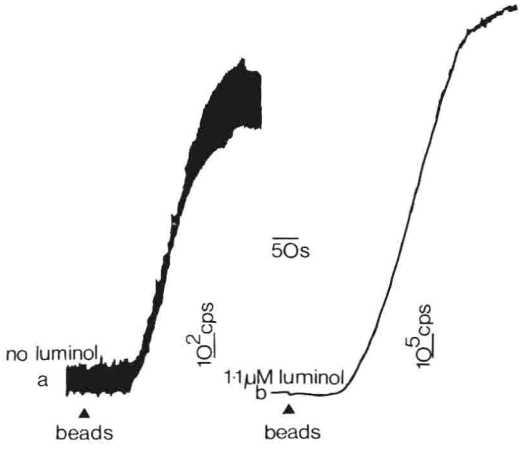

Fig. 1.2 Polymorph chemiluminescence in the presence and absence of luminol (from Hallett et al, 1981)
1.2×10^7 rat polymorphs/ml Krebs-Ringer-Hepes medium + 0.1% w/v bovine serum albumin.
Stimulus = 2×10^{10} latex beads (↑).
(a) No additions.
(b) Luminol 1.1 μmol/l in dimethyl-sulphoxide (final concentration 0.1% v/v).
37°C, c.p.s. = luminescence counts/s.

monocytes were subsequently shown to exhibit such chemiluminescence (Trush et al, 1978). This weak chemiluminescence provides a convenient method of monitoring oxygen radical production. The light emission can be increased some 1000–10 000 times by adding substances such as luminol or lucigenin (Fig. 1.2).

The dark-adapted human eye requires approximately 30 000 photons s^{-1} cm^{-2} hitting the retinal surface to perceive a light signal (Pirenne & Denton, 1952) at 510 nm, the optimal wavelength for sensitivity. The dim chemiluminescence produced by certain oxidative reactions in mammalian cells (Table 1.1) is often less than 100 photons s^{-1} per cell, some 10^3 times less than that necessary for perception by the naked eye. The light emission itself is therefore not thought to be of any functional significance to the organism. In contrast the luminous cells from certain fish, invertebrates and protozoa is some 10^3–10^6 times brighter than this. It is visible to the eye and probably serves a function, attracting food, a mate, scaring predators or acting as a camouflage. Chemiluminescence from cells can therefore be divided into

two classes (Table 1.1). The one bright and functional, the other dim and non-functional. Both have important applications for studying reactions in living cells.

How do chemical reactions produce light? In spite of the structural diversity of synthetic and biological substances which produce light when oxidised, two general chemical mechanisms have emerged which generate photons (McCapra, 1966, 1973, 1982; Boveris et al, 1981). Four membered peroxides (dioxetans and dioxetanones

Table 1.1 Two classes of chemiluminescence from living cells

1. Bright*	— functional (at least 10^6–10^8 photons/s/cell)	
		marine bacteria
		protozoa (dinoflagellates, radiolarians)
		coelenterates
		echinoderms
		molluscs
		arthropods
		polychaetes
		vertebrates (pisces only)
2. Dim	— non-functional (< 1000 photons/s/cell)	
		whole blood phagocytes (amoeba, polymorphs, eosinophils, monocytes and macrophages)
		some killer cells (e.g. NK cells)
		platelets
		some fertilised eggs (e.g. echinoderm and fish)
		yeast
		liver
		lung
		brain
		tissue homogenates
		subcellular particles (mitochondria and submitochondrial particles, microsomes, seminal vesicles)

For references, see Harvey (1952); Tarusov et al (1961, 1962); Allen et al (1972); DeLuca (1978); Foerder et al (1978); Herring (1978); Mills et al (1978); Lloyd et al (1979); Cadenas et al (1980a, 1980b); Boveris et al (1980, 1981)
*See Harvey (1952) and Herring (1978) for other *Phyla*

Fig. 1.1) appear to be common intermediates in most bioluminescence systems, as well as in lipid peroxidation and the oxidation of the synthetic compounds luminol, lucigenin and acridinium compounds by H_2O_2 and oxygen radicals. The dioxetan or dioxetanone then breaks down to form an excited carbonyl group which emits the light. The light emission is usually blue, in the range 400–500 nm, though green and yellow emitters are known, for example firefly luciferin. In contrast, the other major source of chemiluminescence in living systems is singlet oxygen which emits red light ($\lambda_{max.}$ 634 and 703 nm; $'\Delta g^- + {}'\Delta g^- \rightarrow 2\,{}^3\Sigma^- g$); (Kahn & Kasha, 1970; Nakano et al, 1975; Cadenas et al, 1981).

The broad spectrum light (400–700 nm) emitted by mammalian cells (Table 1.2) makes it difficult to identify the precise chemical origin of the endogenous chemiluminescence. The addition of luminol or lucigenin, whilst massively increasing the light yield, may help to clarify this problem, though these substances may react with several 'peroxide species' including O_2^-, HO_2^-, $^1O_2$ and $\cdot OH$.

HOW IS CHEMILUMINESCENCE MEASURED?

Light can be detected in any one of four ways:

(a) thermal, e.g. bolometers and thermopiles;

(b) chemical, e.g. photographic paper, photosensitive solutions;
(c) biological, e.g. the eye, photosensitive pigments;
(d) electrical, e.g. photoelectric devices and photomultiplier tubes.

The key to developing the research and clinical applications of chemiluminescence has been the development of photomultiplier tubes capable of generating a detectable pulse of charge from an average of one in every five photons hitting the photocathode.

Table 1.2 Some commercial luminometers

Apparatus	Company
Digital II	Ryall, Medical Biochemistry, Welsh National School of Medicine
Berthold Biolumat LB9500 (also LB503, LB9505 and auto Biolumat LB9500)	Berthold Laboratorum, Wildbat, West Germany
LKB 1250 & 1251	LKB Wallac, Turku, Finland
Packard Pico Lite 6100 & Auto Pico Lite 6200	Packard Instrument Co Inc, Downers Grove, Illinois
Automated Luminescence Analyser	Alpkem Corp, Clackamas, Oregon
Moonlight Model 201	Analytical Luminescence Laboratory Inc, San Diego, California
Lumac luminometers 1010, 1030, 1070, 2000, 2010, 2080	Lumac BV Schaesberg, The Netherlands
Marwell Kinetic Luminescence Analyser 302 and 304/50	Marwell International AB, Solna, Sweden
SAI ATP–Photometers 100 & 2000	SAI Technology Inc, San Diego, California
Skan Bioluminescence Analyser XP–2002–2	Skan AG, Basel, Switzerland
Turner ATP-Photometer 20	Turner Designs, Mountain View, California
Vitatect IIs	Vitatect Corp, Alexandria, Virginia

This has been made possible by the discovery of secondary electron emission by Stepian in 1923 leading to the production of the first photomultiplier tubes by RCA in 1935. For maximum sensitivity these tubes have a voltage of about 1000 volts across them resulting in an amplification (gain) of some 10^6 for each electron produced at the photocathode. The spacial distribution of light emitted from cells can be analysed using image intensification or charge coupled devices (Reynolds, 1972, 1978).

Until relatively recently apparatus for detecting and quantifying chemiluminescence was unavailable commercially. Most researchers in this field therefore constructed their own devices. Now, however, a wide range of commercial luminometers is available (Table 1.2). Before choosing a luminometer the biochemist needs to consider four questions:

(a) Can I use a scintillation counter?
(b) Do I need a digital or analogue device?
(c) What parameters do I need to measure?
(d) Do I need automation?

Most commonly used isotopes (e.g. ^{3}H, ^{14}C, ^{125}I) produce bursts, sometimes several hundreds, of photons from the scintillant for each α or β particle emitted. Scintillation counters therefore are designed with two photomultipliers which, in coincidence, result in one count being registered when both tubes respond simultaneously. Chemiluminescent molecules, however, usually emit at most one photon for each molecule reacting. If a scintillation counter is to be used then it should be switched to the 'out of coincidence' mode. Although the optics of scintillation counters result in very efficient trapping of photons, it is not possible to add reagents whilst the sample is in front of the photomultiplier, nor can they be used at 37°C. In spite of these problems ATP measurements can be carried out quite satisfactorily.

Most of the commercial luminometers, with some exceptions (e.g. Berthold), measure the mean current from the photomultiplier and display this on a digital display or on to a chart recorder. The electrical background is substracted by a 'back-off' device. In contrast, we have found that a digital pulse counter, analogous to a scintillation counter, is more sensitive than an analogue system. This is because the background is removed by a discriminator which assesses the energy level of each pulse from the photomultiplier. At relatively high light intensities there is little to choose between analogue and digital. However, higher signal to noise ratios can be achieved with digital devices, and thus they are usually more suitable for detecting low light intensities. A further feature of our own luminometers (Ryall & Campbell, unpublished) is that the counter generates a square wave pulse rather than having a true time constant, and has a resolution down to 2 ns. The signal from the scalar is interfaced to a computer (Digital Equipment LSI 11, 64 K, RT 11 operating system, dual floppy disc) for analysis of data.

Since most chemiluminescent reactions obey first order kinetics the equation fundamental to quantification of substances by this technique is:

$$\text{light intensity} = dhv/dt = Q.k.X_0e^{-kt} \qquad \text{(Eqn 3)}$$

where Q = overall quantum yield of the reaction
k = apparent rate constant
X_0 = total amount of chromophore at time 0
t = time.

The measured rate of photon emission will be less than the true dhv/dt by a factor, f, incorporating the efficiency of light collection on to the photomultiplier tube surface and the efficiency of the electronics. Most systems are at best 1% efficient, being limited ultimately by the sensitivity of the photocathode (*ca* 10–20% max.). This latter sensitivity is critically dependent on the wavelength. Most photomultipliers used for photon counting are blue-sensitive and decrease markedly in sensitivity above 520 nm, though more expensive red-senstive tubes can be purchased. The optimum high voltage and discriminator setting must be assessed for each photomultiplier. The sample housing should be absolutely light tight and thermostatable. Since the dark current of the photomultiplier increases with temperature, maximum sensitivity is obtained by cooling the photomultiplier to < -20°C.

This lowers the background on our apparatus to < 10 cps. Reduction of the surface area of the photocathode or the use of a magnetic focusing ring can reduce this figure by a further factor of 10.