Pure & Appl. Chem., Vol. 57, No. 3, pp. 523–529, 1985.
Printed in Great Britain.

Chemiluminescence immunoassay

J. Stuart Woodhead and Ian Weeks

Department of Medical Biochemistry, University of Wales College of Medicine, Cardiff CF4 4XN, Wales, UK

Abstract - The use of chemiluminescent molecules as non-isotopic alternatives to ^{125}I in immunoassay permits the development of sensitive, stable assay systems.

INTRODUCTION

The phenomenon of bioluminescence occurs widely in nature and has been studied for many years. Analytical applications of bioluminesence have been comparatively recent and followed the discovery that ATP was a cofactor for the luciferase catalysed luminescence of firefly luciferin (1). This observation has led to the development of methods for quantitating ATP both as an indicator of the presence of living cells and as a product of various enzyme systems (2). More recently, there has been considerable interest in synthetic molecules which can be oxidised to undergo chemiluminescent reactions. Both luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) and the bis-acridinium salt lucigenin will dramatically enhance the naturally occurring luminescence which accompanies phagocytic activity of granulocytes (3), a property which can be used to monitor acute phase disturbances of the immune system.

One of the most significant applications of chemiluminescent molecules has been as labels in immunoassay. The basic techniques of immunoassay derive from observations such as those of Berson and Yalow (4) who demonstrated the specific binding of ^{125}I-labelled insulin by endogenous antibodies circulating in insulin-treated diabetic subjects. The high specific activity of the labelled reagent is a key factor in allowing the formation of soluble immune complexes to be recognised even at very high dilution. The quantitative inhibition of binding of labelled antigen to antibody by increasing concentrations of unlabelled antigen forms the basis of radioimmunoassay (RIA). The fact that label distribution can be effectively monitored at high dilution has given the technique considerably greater sensitivity than can be achieved using immunological reactions which have a directly quantifiable end point (e.g. those relying on immuno-precipitation).

Two fundamental aspects have made RIA one of the most important techniques in diagnostic and therapeutic medicine in recent years. Firstly, the specificity of the immune reaction means that analysis can be carried out in complex mixtures such as serum. Secondly, the realisation that antibodies can be produced not only to high molecular weight proteins but also low molecular weight compounds such as drugs and steroids provided they are coupled to a suitable carrier.

In 1968 Miles and Hales introduced an alternative procedure to RIA which used the same type of immunological reaction but was based on a different principle (5). The fundamental difference between RIA and the immunoreadiometric assay (IRMA) of Miles and Hales is illustrated by reactions (1) and (2).

$$Ab + Ag + Ag^* \rightleftharpoons AbAg + AbAg^* \quad (1)$$

$$Ag + Ab^* \rightleftharpoons AgAb^* \quad (2)$$

While the former technique involves competition between labelled antigen and unlabelled antigen (as standards or unknowns) for a limited number of antibody binding sites, the latter relies on the conversion of antigen to a labelled derivative by reaction with labelled antibody. The derivative is then measured directly.

The main advantage of IRMA is that performance is optimal when excess labelled antibody is used, the consequence being that all antigen is rapidly derivatized. These rapid reaction kinetics contrast with those seen in RIA where high sensitivity is achieved by diluting out the antibody with the result that extended reaction times may be required to provide the desired sensitivity.

Until recently both RIA and IRMA have relied on the use of ^{125}I as the label. This isotope is a moderately high-energy γ-emitter with a half-life of 60 days. While its sensitivity of detection is excellent, approximately 5×10^{-18} mol being the practical limit, damage caused by its incorporation into protein results in a restricted shelf-life and frequently compromised immunological performance. One potential advantage of labelled antibody techniques arose from the possibility of labelling antibodies to very high specific activities. It has been claimed theoretically that this should provide improved performance since in the reagent excess mode, sensitivity is not ultimately limited by antibody affinity as in RIA but by the ability to sensitively monitor uptake of labelled antibody (5).

Because of the damaging consequences of radioiodination it has not been possible to assess this theory. Moreover, the majority of non-isotopic immunoassays so far developed have

used labels with detection limits which are poor by comparison with ^{125}I. In this paper we describe the preparation of chemiluminescence labelled antibodies which have long-term stability and which form the basis of analytical procedures with improved performance when compared with conventional immunoassays.

CHEMILUMINESCENCE AND ITS MEASUREMENT

Chemiluminescence occurs when the vibronically excited product of an exogenic chemical reaction reverts to its ground state with the emission of photons. The reactions are invariably oxidative and are biphasic in nature. There is a rapid initial phase in which the excited state is populated, the rate being dependent on reagent concentration. This is followed by a decay phase which is characteristic of the chemiluminescent derivative. Quantitation of light emission is most reliably carried out by integration of the intensity/time profile.

Quantitative techniques based on the use of chemiluminescent molecules rely on reproducible measurements of low levels of emitted light. The problems of light measurement are more akin to those encountered in β-counting rather than in γ-counting. Visible radiation and reaction systems generating it are more naturally abundant than γ-radiation so that great care is required to eliminate non-specific luminescence from the assay system. Moreover, since the emission is brought about by a chemical reaction rather than being a spontaneous physical process, there is also the potential for interference in the measurement itself.

Since the analytical potential of luminometry has not long been appreciated, these problems have only recently been addressed. However, developments have been rapid and several commercially manufactured luminometers are now available. However, the variable characteristics of these machines means that some degree of standardization is required to ensure that chemiluminescent systems will yield consistent results regardless of instrumentation.

The high efficiency photomultiplier tubes used in these machines are capable of recording up to 20% of the emitted photons. Events detected by the photomultiplier tube may be treated in either a digital or analogue manner according to the type of instrument. In the case of the former each photon arriving is treated as an individual event and yields an electrical pulse, the number of pulses per unit time being a function of the light intensity. Analogue systems involve the treatment of the photomultiplier output as a photocurrent or photovoltage the magnitude of which is proportional to the intensity of emission. Integrated luminescent reaction profiles are thus expressed as photon counts in digital systems and as millivolt seconds in analogue systems.

However, in addition to signal measurement there is the equally important consideration of automation of sample processing if a system is to be applied to diagnostic testing. The only automated photon counter presently available which has been designed for immunoassay application is the Autobiolumat LB950 manufactured by Laboratorium Berthold (D7547, Wildbad, FRG). This system is comparable to radioactivity counters providing a sample transporation mechanism. This is achieved by means of a flexible interlocking plastic belt with is propelled by motorized cogs. Sample tubes are introduced into the light-tight measuring chamber by means of an elevator. The total operating system together with automatic injection of reagents is controlled by means of an Apple II Processor utilizing the manufacturer's software. The system has been designed to operate a range of luminometric techniques including immunoassays and carries all the appropriate data reduction facilities. The throughput is approximately 250 sample tubes per hour.

CHEMILUMINESCENT LABELS

While instrumentation is a crucial factor in determining the success of any non-isotopic immunoassay, so is the chemistry of labelling and of label quantitation. The need for high specific activity has already been stressed and a consequent requirement is the high sensitivity of detection of the labelling material itself. Conventional enzyme labels or fluorescent derivatives have detection limits in the region of 10^{-14}mol and consequently have been successfully applied only to low sensitivity assay systems. However, both luminol and acridinium salts have detection limits of approximately 10^{-18} mol using available luminometers, thus matching and even surpassing the sensitivity of ^{125}I detection.

Not unexpectedly the constraints arise from the need to couple the chemiluminescent molecules to the analytes of respective antibodies in such a way that the properties of the label are not disturbed. In our early studies for example we found it possible to diazotise luminol and incorporate 22 mol of this reactive product into immunoglobulins (6). However, the derivative had less than 1% of the luminescence of native luminol, possibly due to polymerization of the diazonium salt.

More recently, however, it has become apparent that the association of luminol with protein can drastically affect the quantum yield of the reaction. This is evidenced by

work with derivatives of isoluminol, in particular aminobutylethyl isoluminol (ABEI) which can be coupled to low molecular weight compounds without loss in quantum yield (7). As a result ABEI and related molecules have been used as labels for a variety of steroid immunoassays which have levels of performance comparable to those of the corresponding radioimmunoassays (8). A universal problem of these methods arises from the extensive quenching of the signal when the label is associated with antibody. So great is the loss in quantum yield under these conditions that dissociation of the complex prior to luminometry has been adopted as an obligatory step. A similar loss in quantum yield was observed when aminohexylethyl isoluminol was used to label antibodies to hepatitis B surface antigen. This was attributed to quenching by certain amino acid residues (9) though once again polymer formation may have been a contributing factor.

Our own studies have concentrated on the use of acridinium salts. The potential advantages of these compounds as compared with luminol derivatives can be seen by reference to figure 1 which summarizes their light-emitting reactions. Both reactions are oxidative

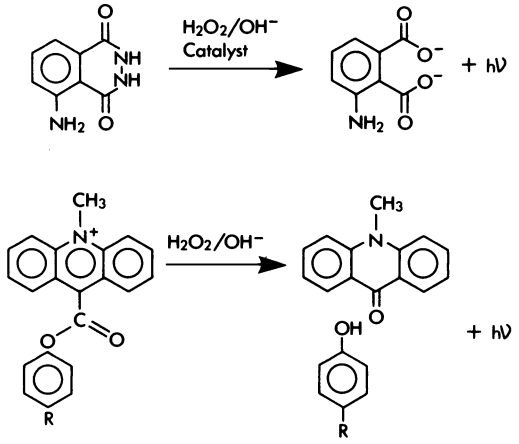

Fig. 1. Chemiluminescent reactions of luminol and an acridinium ester

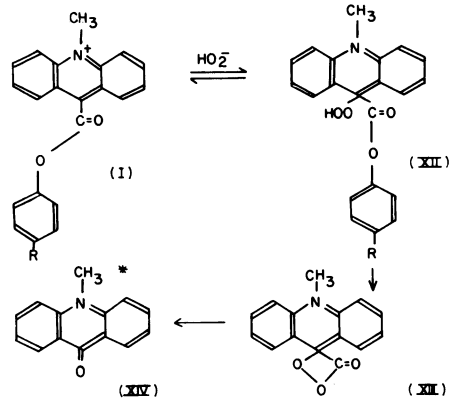

Fig. 2. Mechanism of acridinium ester chemiluminescence

and occur in alkaline conditions, though in the case of luminol there is the absolute requirement for a catalyst. This can be a simple transition metal cation such as Mn^{++} or Ni^{++} but may also be a complex macromolecule such as horseradish peroxidase and cytochromes (10). The most popular catalyst is microperoxidase since this yields an efficient reaction under relatively mild conditions. This requirement for a catalyst renders the reaction susceptible to interference from materials present in biological samples while the powerful oxidizing conditions may result in high background effects.

In contrast, the mild conditions required for acridinium ester reactions produce relatively low background chemiluminescence. Moreover, the reaction itself involves an oxidative cleavage to yield a dioxetanone intermediate before the formation of the excited product molecule N-methyl acridone (fig.2). As a result of the cleavage the N-methyl acridone is dissociated prior to the light emission so that quenching by associated protein is avoided.

Initial studies with acridinium esters as labels for antibodies were disappointing in that specific activities were low mainly as a result of unsatisfactory coupling procedures (11,12). However the recent synthesis of a succinimidyl ester of an acryl acridinium salt (fig.3) has enabled us to label antibodies reproducibly to a high predetermined specific activity (13). Under aqueous conditions at pH8, the succinimidyl ester reacts with primary and secondary aliphatic amines thus providing an efficient labelling mechanism. The reaction proceeds rapidly as illustrated in fig. 4 which shows elution profiles of labelled antibodies subjected to gel filtration on a 10 x 1 cm column of Sephadex G-25 after different reaction times. Reactions were allowed to proceed for 0.5 - 15 min and were stopped by addition of excess lysine, column chromatography being carried out in the presence of 0.1% bovine serum albumin to reduce non-specific label interactions. A significant amount of labelling compound is incorporated into protein within 0.5 min while the reaction is virtually complete after only 5 min. The consistent properties of IgG from various sources has meant that it has been possible to label a range of antibodies to the desired incorporation level.

The fluorescent properties of N-methyl acridone have meant that it has been possible

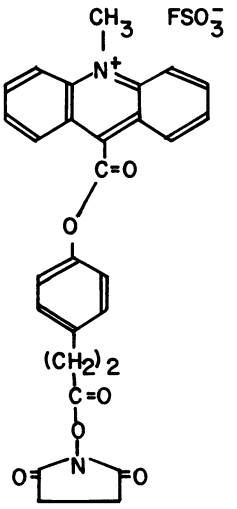

Fig. 3. N-succinimidyl derivative of an acridinium salt

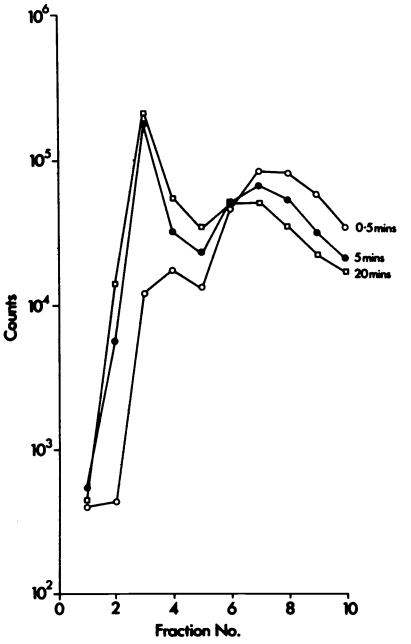

Fig. 4. Gel filtration profiles of chemiluminescent labelled antibodies

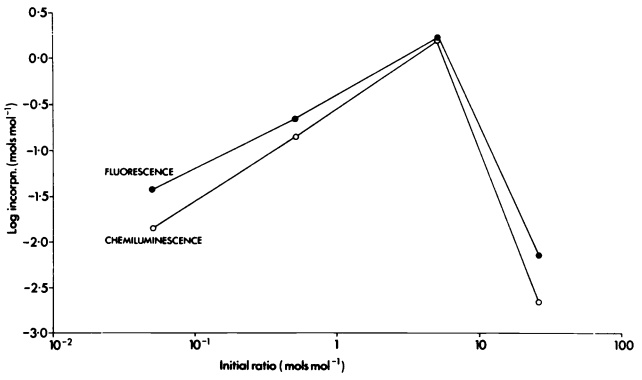

Fig. 5. Incorporation of acridinium ester into antibodies

to monitor the incorporation of label and compare this with yields of chemiluminescence. In this way we have been able to demonstrate that there is indeed no loss of quantum yield as a result of covalent association of the acridinium salt with protein. At present, the factor limiting incorporation appears to be the solubility of the labelled protein. As shown in fig. 5, the rise in chemiluminescence activity of labelled antibody rises with incorporation up to approximately 3 mol ester/mol antibody and then falls. This pattern is reflected by the recovery of N-methyl acridone as monitored by fluorescence and also by the recovery of protein, suggesting that heavily labelled derivatives are in fact insoluble. Work is at present under way in our laboratory to produce more soluble compounds for labelling which will enable higher incorporations to be achieved. Nevertheless, an incorporation of 3 mol/mol yields a derivative with 1.5 x 10^6 photon counts/ng protein giving it a detection limit almost five times better than can be achieved using ^{125}I, where incorporation of more than one atom/antibody molecule is invariably destructive. At these levels of incorporation there is no detectable effect of label on the immunological behaviour of the antibody. An equilibrium exists between the acridinium salt and its non-chemiluminescent pseudobase. At high pH pseudobase formation is favoured and there is also spontaneous oxidation of the acridinium salt. For this reason labelled antibodies are stored, and assay procedures carried out at pH 6.3 to minimise loss of activity. Under these conditions labelled antibodies are stable for periods in excess of 1 year when stored at 4°.

IMMUNOASSAYS WITH CHEMILUMINESCENT LABELS

A unique advantage of radioactive labels is that quantitation is unaffected by their environment. This is not true in the case of non-isotopic compounds. Luminescent molecules are susceptible to quenching of the signal by constituents of biological fluids such as

serum. In general, this problem has been avoided by the use of solid-phase antibody technology. As a result, the antibody bound fraction can be isolated from and washed free of potentially interfering compounds before signal detection.

As indicated above, the majority of chemiluminescent hapten immunoassays so far described have been based on ABEI or similar labels. These assays include plasma estradiol-17β(14), testosterone(15) and progesterone (16) as well as urinary conjugated steroids such as pregnanediol-3α-glucuronide (17) and estrone glucuronide (18). Dissociation of the label prior to signal detection has been facilitated by means of strong alkali often at high temperature (8). Though this results in satisfactory light emission, it does introduce a step into the analytical procedure which is operationally complex and which may affect the overall performance of the assay itself.

It is possible that acridinium esters will provide simpler alternatives, though as yet there is only one report of their use on steroid immunoassay (19). In this case a synthetic acridinium derivative of estradiol was prepared which could be detected with high sensitivity. Assay performance was however limited by the low affinity of the antibody for the labelled derivative once again illustrating the dependence of hapten systems on suitable immunochemistry.

Our own work has centred on the development of assay methods for polypeptides using acridinium ester labelled antibodies. We have employed almost exclusively two-site assay procedures in which the analyte is reacted either sequentially or simultaneously with labelled antibodies and solid-phase antibodies (20). In this procedure, the uptake of labelled antibody on to solid-phase antibody is a function of the quantity of analyte bound and hence the analyte concentration in the sample. The most satisfactory solid-phase antibodies developed so far are prepared by reacting antibody preparations with the diazonium salt of reprecipitated aminoaryl cellulose (21). This preparation has the advantage that it remains in suspension during the reaction without agitation. It is also extremely efficient as an immunoadsorbent since it has a high binding capacity for protein as well as very low non-specific uptake of protein.

A typical procedure which exploits this methodology to yield a highly sensitive assay for circulating human thyroid stimulating hormone (TSH) is shown diagrammatically in fig. 6. In this procedure standards or serum samples (100 µl) are reacted with a monoclonal antibody

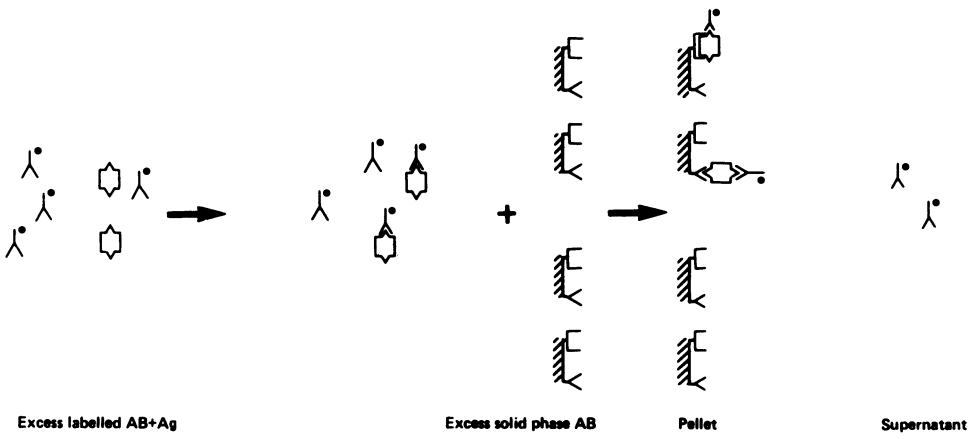

Fig. 6. Two-site immunochemiluminometric assay (ICMA) of human thyrotropin (TSH)

(100 µl, 1.5 ng) labelled with 3 mol/mol acridinium ester. After 2 h, solid-phase antibody suspension (100 µl, 50 µg) is added and after a further 1 h, 1 ml of buffer is added and the mixture centrifuged. After one further 1 ml wash, the chemiluminescent activity associated with the solid-phase is quantified luminometrically following injection of 200µl water to resuspend the pellet followed by 200 µl 0.1% (v/v) H_2O_2 in 0.1 M NaOH. The photon counts are integrated over a 2 s period and the bound counts related to the dose of analyte present.

The use of reagent excess methodology together with an acridinium ester label of high specific activity combine to make this the most sensitive immunoassay yet described for TSH (22). A composite standard curve based on 5 sequential assays is shown in fig. 7. The dose response is actually linear from 0.015 to 60 mU/l and in view of its stability offers the potential for removal of the normal requirement for a calibration curve with each individual assay. This will produce a significant reduction in cost and the labour

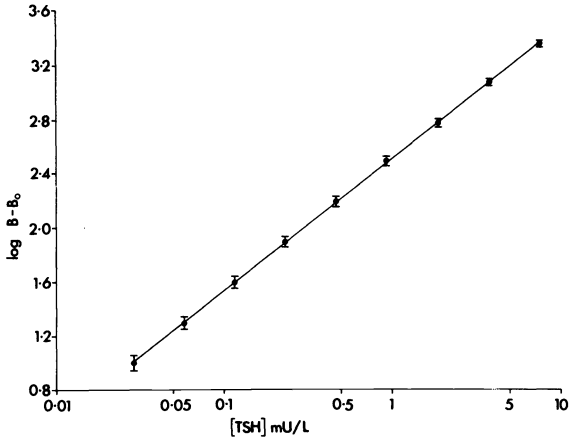

Fig. 7. Dose-response curve of TSH-ICMA

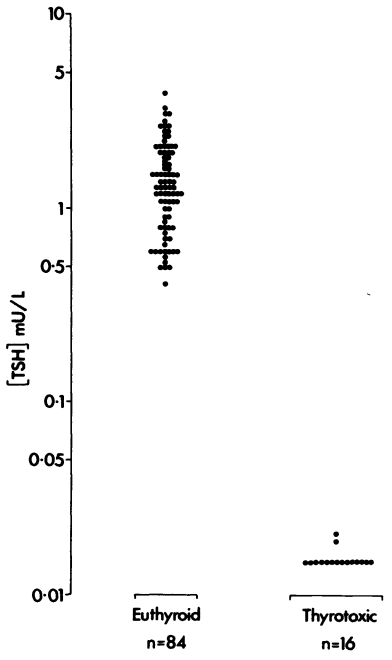

Fig. 8. TSH concentrations in the serum of euthyroid and thyrotoxic subjects as measured by ICMA

involved in assay procedures adopting this approach. Based on precision profiles the assay has an absolute sensitivity of 0.003 mU/l and a working range from 0.015 to >60 mU/l (22).

From the clinical point of view this assay offers a remarkable breakthrough in diagnostic and therapeutic testing. Pituitary TSH secretion plays a central role in the control of thyroid function. Since it is under negative feedback control by the thyroid hormones whose production it stimulates, it provides a sensitive indicator of thyroid status. However, the relative insensitivity of presently available procedures does not allow discrimination of the suppressed TSH levels of hyperthyroid patients from normal situations. The main applications of TSH measurement being in the confirmation of hypothyroidism where levels are elevated and in monitoring the pituitary response to thyrotropin release hormone (TRH).

While this immunochemiluminometric assay (ICMA) is at present under evaluation, some idea of its potential is shown in fig. 8 which compares TSH levels in 16 hyperthyroid patients with those in 84 normal subjects. The clear discrimination seen between the groups suggests that this test will play an increasingly important role as a test of thyroid function, not only from the diagnostic point of view but also in the monitoring of patients being treated for hypo-or hyperthyroidism.

Based on the same methodology, assays have also been developed for α_1-fetoprotein (23) and ferritin (24). The performance of these procedures compares very favourably with existing methods of immunoassay based on ^{125}I as the label and indicates the general applicability of the technology.

CONCLUSIONS

Chemiluminescence immunoassay offers a viable and logical alternative to a range of analytical procedures based on the use of radioisotopes. Its feasibility has been demonstrated for a range of analytes including both high and low molecular weight species. The stability of the reagents and their analytical performance has important implications in improving and maintaining high quality assay performance. Most importantly the development of ICMA technology based on labelled antibodies has the potential for increasing assay sensitivity. It is likely that this technology will prove valuable in the detection and monitoring of infective agents as well as tumour markers where conventional immunoassay technology provides insufficient sensitivity.

REFERENCES

1. W.D. McElroy, Proc. Natl. Acad. Sci. USA. 33, 342-345 (1947).

2. A. Lundin, In 'Clinical & Biochemical Luminescence' L.J. Kricka and T.J.N. Carter (eds), Marcel Dekker, New York, 1982, pp.44-74.

3. R.C. Allen and L.D. Loose, Biochem. Biophys. Res. Comm. 69, 245-252 (1976).

4. S.A. Berson and R.S. Yalow, Advances Biol. Med. Phys. 6, 349 (1958).

5. L.E.M. Miles and C.N. Hales, Nature (Lond.) 219, 186-189 (1968).

6. J.S.A. Simpson, A.K. Campbell, M.E.T. Ryall, et al., Nature (Lond.) 279, 646-647 (1979).

7. H.R. Schroeder, R.C. Boguslaski, R.J. Carrico, et al., In 'Methods in Enzymology' Vol. 57, M.A. DeLuca and W.D. McElroy (eds.), Academic Press, New York, 1978, pp.424-455.

8. W.P. Collins, G.J. Barnard, J.B. Kim, et al. In 'Immunoassay for Clinical Chemistry', 2nd ed. , W.M. Hunter and J.E.T. Corrie (eds.), Churchill Livingstone, Edinburgh, 1983, pp.373-397.

9. H.R. Schroeder, C.M. Hines, D.D. Osborn, et al., Clin. Chem. 27, 1378-1384 (1981).

10. H.R. Schroeder and F.M. Yeager, Anal. Chem. 50, 1114-1120 (1978).

11. J.S.A. Simpson, A.K. Campbell, J.S. Woodhead, et al., In 'Bioluminescence and Chemiluminescence - Basic Chemistry and Analytical Applications', M.A. De Luca and W.D. McElroy (eds.), Academic Press, New York, 1981, pp.673-679.

12. J.S. Woodhead, J.S.A. Simpson, I. Weeks, et al, In 'Monoclonal Antibodies and and Developments in Immunoassay', A. Abertini and R.P. Ekins (eds.), Elsevier/North Holland, 1981, pp.135-145.

13. I Weeks, I. Beheshti, F.McCapra, et al, Clin. Chem. 29, 1474-1479.

14. J.B. Kim, G.J. Barnard, W.P. Collins et al, Clin. Chem. 28, 1120-1124 (1982).

15. J.K. Kim, G.J. Barnard, W.P. Collins, et al, J. Steroid Biochem. 18, 625-629 (1983)

16. F. Kohen, J.B. Kim, H.R. Lindner, et al, Steroids 38, 73-88 (1981).

17. Z. Eshhar, J.B. Kim, G.J. Barnard, et al, Steroids 38, 89-109 (1981).

18. D.A. Weerasekera, J.B. Kim, G.J. Barnard, et al, Acta Endocrinologica 101, 254-263, (1982).

19. A. Patel, J.S. Woodhead, A.K. Campbell, et al, In 'Luminescent Assays: Perspectives in Endocrinology & Clinical Chemistry', M. Serio and M. Pazzagti (eds.), Raven Press, 1982, pp. 181-189.

20. C.N. Hales and J.S. Woodhead, In 'Methods in Enzymology', vol. 70, M. Van Vanukis, and J.J. Langone (eds.), Academic Press, New York, 1980, pp.334-355.

21. H.A. Kemp, J.S.A. Simpson and J.S. Woodhead, Clin. Chem. 27, 1388-1391 (1981).

22. I. Weeks, M. Sturgess, K. Siddle, et al, Clin. Endocrinol. 20, 489-495 (1984).

23. I. Weeks, A.K. Campbell and J.S. Woodhead, Clin. Chem. 29, 1480-1483.

24. I. Weeks, and J.S. Woodhead, Clin. Chim. Acta. In press.