Meeting Report

APPLICATIONS OF FLUORIMETRIC ENZYME ASSAYS

The first Workshop Meeting on this subject was held at the Lecture Rooms of the Zoological Society, London, on 14 April 1976

Organised and reported by
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Introduction

The past decade has seen an explosive growth in the development and use of fluorimetric methods for the assay of enzyme activities. A few years ago fluorimetric methods for this purpose might fairly have been described as interesting novelties. Today there is an increasing number of important problems in biology and medicine for which the fluorimetric assays are necessary or are the analytical methods of choice. This is a report of a meeting organised partly to provide an up-to-date account of the range of recent applications of the methods but also to provide a forum where current problems and possible future developments might be discussed.

Introductory lectures

After a welcome by P. G. Walker (Institute of Orthopaedics), D. H. Leaback outlined the attractive features (simplicity, convenience and sensitivity) of fluorimetric methods compared with the corresponding absorptiometric procedures and went on to show that these features are a consequence of the nature of the processes involved, and of the way that the measurements are carried out. These attractive features of the fluorimetric methods have probably been realised best in procedures employing synthetic fluorogenic substrates – such as those based upon fluorogenic derivatives of 4-methylumbelliferone. Dr Leaback attributed the present success of the latter substances as substrates principally to the almost ideal spectral characteristics of 4-methylumbelliferone for analysis in ordinary filter fluorimeters. The use of 'unnatural' fluorogenic substrates of this kind does, however, entail a relatively low specificity in the assay procedures concerned. While this can be an advantage (in that the method permits a range of 'isoenzymes' to be assayed), it is now clear that the relationship between the results of such assays and the activities of the component enzymes against natural substrates in vivo can be extremely complex. D. Page-Thomas (M.R.C. Rheumatism Unit, Taplow, Berks.) spoke of the largest class of enzymes acting upon naturally fluorogenic materials – that is, of the oxidative–reductive enzymes involving the puridine nucleotides. Even here, the methods can often be improved by chemical treatment of the products on the catalysed reactions. Dr Page-Thomas especially favoured those procedures in which the resorufin or homovanillic acid fluorogenic systems are coupled to oxido-reductase enzymes. H. N. Fernley (Institute of Orthopaedics) described some advantageous applications of fluorogenic procedures to the continuous assay of enzyme activities – with special reference to his own experience with certain phosphohydrolases. Applications to the determination of the kinetic parameters of the catalysis and to the analysis of two-component enzyme mixtures by thermal inactivation were outlined, together with some interesting active-site titrations of alkaline phosphatases using as

substrates, methylumbelliferone phosphate and the corresponding phosphothioate. Dr Fernley considered that active-site titrations have a number of advantages over estimations of enzyme activities and that fluorogenic methods might be usefully employed more extensively for this purpose. J. W. Bridges (University of Surrey, Guildford) gave a brief but authoritative account of the very different kinds of enzymes involved in the metabolism of foreign substances (such as drugs) that gain access to living tissues. Fluorogenic methods are playing an important part in the study of such enzymes and are usually based on oxidative cleavage of ethoxy derivatives of umbelliferone or resorufin; hydroxylation of automatic or heterocyclic compounds like biphenyl or benzpyrene; conjugation with glucuronic, sulphuric or other acid moieties; or reductive or de-alkylative generation of primary amines that can be detected by the fluorogenic reagent 'Fluorescamine'. Dr Bridges attributed the recent surge in interest in this field partly to the fact that almost every chemical and drug manufacturer must now consider the effects of their products on enzyme induction or inhibition, but reports such as those indicating that carcinogens specifically induce systems hydroxylating biphenyl in the 2-position, and that persons at risk to bronchio-carcinoma can be detected by benzpyrene hydroxylase estimations on lymphocytes have also stimulated much interest. In some of these applications the high sensitivity of the fluorimetric procedures is important (e.g. in the studies on cultured lymphocytes) but in others the assays depend upon the 'excited-state ionization' phenomenon (e.g. in the measurement of 2-hydroxy-biphenyl in the presence of the 4-analogue) and are therefore not accessible to absorptiometric measurements. Dr J. F. Jongkind (Rotterdam) described the impressive range of techniques that he and his colleagues have perfected at the Cell Biology and Genetics Department at Erasmus University for the handling of very small samples of living cells (including single cells) and for fluorogenic measurement of enzymes in such samples. The measurements are often carried out in microscope fluorimeters, but there are also advantages in employing very small reaction volumes in conjunction with conventional fluorimeters. The use of such techniques in genetic studies (including prenatal diagnosis of genetic defects) was also described.

Workshop discussion

In this session certain speakers introduced an aspect of the applications of fluorimetric methods and each topic was then discussed more generally. D. A. Hopkinson explained how the fluorogenic methods were used extensively at the Galton Laboratory, University College, London for the detection of polymorphism in human isoenzymes (esterases, carbonic anhydrase, acid phosphatases, glycosidases etc.) after separation by electrophoresis. For the population studies involved, a qualitative assessment of the isoenzyme patterns is usually sufficient. D. A. Gibbs (Clinical Research Centre, Northwick Park, Harrow) spoke of how fluorogenic methods are being used to study inborn errors of metabolism in samples of human hair follicles. Dr Gibbs pointed out the advantages of hair follicles as a source of biopsy material, but also made a plea for the development of certain fluorogenic substrates. R. Ellis (Institute of Child Health, London) described how he has developed new procedures for the automated ion-exchange chromatographic separation of β-*N*-acetyl-D-hexosaminidases using fluorimetric methods for the enzyme assays. The procedures permit the determination of up to about 40 'isoenzyme' patterns per day with accuracy and reproducibility. The procedures could, in principle, be adapted for the study of a variety of iso-enzyme patterns using other chromatographic absorbents for the separation. Dr Ellis made special mention of the difficulties in the use of the term 'isoenzyme' in this context. Although the activities may be measured using the same synthetic substrate, there is abundant evidence that such activities can be directed in vivo towards a variety of natural substrates. He prefers the term 'enzyme component'. M. L. Morgan (Clinical Research Centre, Northwick Park, Harrow) described how automated fluorogenic assays have been developed for the study of lysosomal and cytoplasmic enzymes from cells in culture. Such studies can be used to give insight into cellular processes in normal and pathological conditions. B. G. Wraxall (Metropolitan Police Laboratories, London, S.E.1.) spoke of the way that fluorogenic methods for the study of isoenzymes esterase and phosphatase can be invaluable in helping to solve certain forensic problems. Thus, esterase and phosphatase 'isoenzyme' patterns in tiny samples of blood

have been used in the characterization of the samples after electrophoresis, and acid phosphatases have been characterised similarly as of male or female origin in samples from vaginal fluids. R. C. W. Berkeley (Medical School, University of Bristol) outlined the synthesis and separation of a new fluorogenic β-*N*-acetyl-muramide substrate and how this had been used in the study of a bacterial exo-β-*N*-acetyl-D-muramidase. The sensitivity of the assays using this material made possible the miniaturization of the enzyme assay reaction mixtures and had thereby conserved a very scarce and valuable substrate. R. G. Price (Queen Elizabeth College, London, W.6.) explained how fluorogenic assays of β-*N*-acetyl-D-hexosaminidase in urine can be used as a sensitive method for the early detection of kidney damage. These findings have excited great interest and are now being used widely in the detection of kidney graft rejection and in the detection of potential nephrotoxicity in drugs. T. J. Peters (Royal Postgraduate Medical School, Hammersmith, London) spoke of the application of the fluorogenic assays to the detection of enzymes in milligram samples of tissue subfractionated by centrifugation. Results obtained in this way have led to evidence of lysosomal fragility in certain pathological conditions. G. V. Sengbusch (Batielle Institut, Frankfurt, Germany) outlined techniques for the measurement of the intracellular turnover of fluorogenic substrates using bacterial cells in a novel flowthrough 'microfluorimeter'. The apparatus and a theoretical model for the system were described and results of experiments designed to distinguish between diffusion and enzyme-mediated effects were presented. D. Graham-Knight (Strangeways Laboratory, Cambridge) gave a description of how Sephadex LH-20 can be used very effectively in reducing the (often troublesome) amount of free 4-methylumbelliferone in fluorogenic substrates based upon this material.

Poster and instrument exhibition

A session of poster displays allowed participants to examine and discuss in greater detail aspects of techniques and applications presented in earlier sessions. An associated exhibition of biochemicals, fluorimeters and accessories enabled participants to see what is commercially available at present, and enabled those on the commercial side to acquaint themselves with the particular requirements of those in this specialized but growing aspect of fluorimetry.

Some trends and conclusions

Although fluorimetric methods have other attractions for the estimation of enzyme activities, their potential for a very high sensitivity continues to be their special feature of interest. This feature fits in with the present trend in biology, medicine and other specialities to investigate tiny quantities of (or poorly-active) body fluids and tissue samples. There are signs that manufacturers of instruments are beginning to heed the requirements of biochemical applications of fluorimetry, e.g. to provide adequately thermostated sample compartments, rather than elaborate instrumentation (e.g. that necessary to produce 'corrected' emission spectra) allegedly required by chemists. However, it became clear at this meeting that there is still room for a simple but effective microfluorimeter capable of handling much smaller samples volumes – such as those appropriate for conserving valuable substrates or the assay of enzymes in samples from a few living cells.

In the period immediately ahead we can expect to hear of further applications of existing assay methods to the study of enzymes, metabolising drugs and carcinogens and the results of further searches for the signs of pathological conditions (or predisposition to disease) in readily available clinical samples. There was general agreement at this meeting of the urgent need for fluorogenic methods for the assay of certain enzymes (e.g. for α-L-iduronidase) and for the extension of the methods to a yet wider range of enzymes. Clearly, there is plenty of scope for further chemical innovation here.

Acknowledgements

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