Shedding light on lysosomes — applications of fluorescence techniques to cell biology and diagnosis of lysosomal disorders

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Introduction

The basic principles of fluorimetry have been evident since the middle of the last century when Stokes outlined the relationships of activating and emitted light [1], but quantification was largely restricted to those substances that have a natural fluorescence or later to chemically derived products such as thiochrome. The methodology was recognized as being inherently very much more sensitive than spectrophotometry, but was limited by the performance of photodetectors and the availability of good stable intense and polychromatic light sources.

The first examples of enzyme assays using this technique appeared in the 1950s and depended on measuring changes in coenzyme redox state. Lowry and co-workers described a number of assays for dehydrogenases based on the rate of reduction of NAD(P) as measured by fluorescence [2, 3]. The idea of custom-designed substrates synthesized with the intention that enzymic conversion would produce a fluorescent product in a stoichiometric amount was probably first realized with the appearance of the glycoconjugates of 4-methylumbelliferone (4-MU) (Fig. 1).

In the course of studies on the metabolism of coumarins, Mead et al. noted that the β-glucuronic acid conjugate of 4-MU was virtually non-fluorescent on chromatograms of urinary metabolites and could be converted to the fluorescent aglycone with ease by samples of β-glucuronidase from various sources. They proceeded to develop and describe a fluorimetric assay method for β-glucuronidase based on this principle using the biosynthetic substrate isolated from the urine of rabbits dosed with the aglycone [4]. They also attempted to demonstrate an analogous procedure for arylsulfatase using synthetic samples of the other major metabolite of 4-MU, the ‘ethereal’ sulphate. The background fluorescence at this time proved unsatisfactory though subsequently this method has also been widely adopted.

Unfortunately, these methods won a cool reception from the acknowledged experts on β-glucuronidase who found them ‘too exacting for general use’. Meanwhile Robinson synthesized a number of analogous glycosides and used them to demonstrate the presence of glycosidases in a number of tissues that were otherwise too weakly active for the then current colorimetric procedures based on the liberation of nitrophenols [5].

Fortuitously, these developments coincided with two historic events in cell biology: the development of the operon theory which stimulated the requirement for the assay of β-galactosidase with some precision and sensitivity, and the...
discovery of a new cellular organelle, the lysosome and its biochemical identification by DeDuve and co-workers as a repository of acid hydrolases of which the glycosidases constituted a major component. The fluorogenic substrates offered considerable advantage in speed and sensitivity for the assay of the sequestered contents of the organelle and for determination of the degree of integrity of the lysosomal membrane. It was, however, the pathological aspects that produced the main demand for these substrates, and this can be attributed to the observations of Hers and co-workers, also working in the Louvain laboratory, who noted the absence of \( \alpha \)-glucosidase in Pompe's disease [6] and propounded the hypothesis that the storage diseases such as the mucopolysaccharidoses resulted from the loss of function of some specific lysosomal enzyme or other that would normally be required for the smooth sequential degradation of these heteropolymers [7]. To this extent this was that the chemical nature of the partial degradation products that constituted the storage material would be those naturally occurring substrates of the enzyme in question curtailed at the linkage that was no longer able to be attacked. In other words, the terminal moieties should correspond to the defective enzyme. There rapidly followed from various laboratories confirmation of the general correctness of Hers' hypothesis and substrates including glycolipidoses such as Gaucher's disease [8, 9]. The fluorogenic substrates thus fortuitously came into commercial demand as diagnostic agents for the confirmation of enzyme deficiencies in patients, and later for the identification of carriers of the autosomal recessive examples by studying the gene-dose relationships.

**Tay–Sachs' disease and related disorders**

The accumulation of the \( N \)-acetylglactosamine-terminal ganglioside GM2 characteristic of Tay–Sachs' disease proved not to be accompanied by the expected diminution of the \( \beta \)-\( N \)-acetylhexosaminidase for which the corresponding 4-MU substrate had been synthesized by Leaback & Walker [10] and to this extent the disease stood out as an exception to the general rule of lysosomal enzyme defects. The reason for this anomaly became apparent with the observation that two major forms of this enzyme existed in human spleen and other organs [11]. These could be readily separated and observed on gel electrophoresis as a rapidly moving anodic form Hex A and a slower less positively charged species Hex B occurring in roughly equal levels so that both made significant contributions to the overall activity assayed by the fluorogenic substrate. Patients with Tay–Sachs' disease proved to have a profound deficiency of the A form while showing compensating elevations of the B form so that the overall activity levels were not significantly depressed.

The distinctive charged nature of the two enzyme species allowed us to separate them by ion-exchange and to separately characterize them. While kinetically they were not easily distinguished, the A form proved to be highly susceptible to denaturation by mild heating under conditions that left the B form virtually unaffected. It was thus possible to propose an arbitrary set of conditions for a two-step differential assay measuring total activity followed by residual B activity remaining after heat denaturation of the A component.

Using such methods, Okada & O'Brien [12] successfully demonstrated classical 'Tay–Sachs' disease as a lesion in the A form of hexosaminidase, and were able to apply the differential assay to the detection of heterozygote carriers of this autosomal recessive disease. The high frequency of the mutation in Jews of Ashkenazi origin in comparison to the non-Jewish population at large identified a population at risk where mass screening for carrier detection and diagnosis \textit{in utero} of pregnancies in mothers who already had an affected child was economically feasible. With the co-operation of all sections of the Jewish community the incidence of new cases of Tay–Sachs' disease worldwide has been dramatically reduced over the past 10–15 years as a result of the availability of this simple diagnostic procedure.

**Multiple enzymes and variant disease states**

It is now well established that the structural relationships of Hex A and B lie in a subunit structure that can exist as a homopolymer of \( \beta \)-units in Hex B or as a heteropolymer of \( \beta \)-units associated with distinctive \( \alpha \)-units in Hex A [13]. A genetic mutation affecting the function of the \( \alpha \)-unit results in the inability to express the Hex A form as in classical Tay–Sachs' disease, while a defective \( \beta \)-unit effectively disturbs both forms resulting in the substantial loss of activity seen in the Sandhoff 'O' variant of GM2 gangliosidosis that affects patients of non-Jewish origin.

**Activator proteins**

The hydrolysis of an amphipathic ganglioside in an aqueous environment presents particular problems for the correct orientation and substrate. \textit{In vitro}, considerable difficulties are experienced in obtaining significant hydrolysis rates without the addition of artificial or natural detergents. It has emerged that in many cases there exists accessory proteins that function as activators for the hydrolysis of glycolipid substrates while having no significant effect on the reactions with synthetic or natural water-soluble conjugates.

The existence of some patients exhibiting the symptoms of Tay–Sachs' disease and showing accumulation of undegraded GM2 in their tissues, but having apparently a normal complement of the two enzyme forms, is attributed to a deficiency of a natural activator protein for Hex A [14]. In these variants the assay of enzyme activity by the fluorogenic substrate offers minimal information.

**Other isoenzyme forms**

Elevation of hexosaminidase activity in the serum of pregnant women was an early observation that made Tay–Sachs' carrier testing equivocal in this important group [15]. This was shown to be due to the appearance of a characteristic form of the enzyme Hex P that rose to major proportions during pregnancy and fell to insignificance at parturition [16]. In addition, other forms of the enzyme intermediate in their ion-exchange chromatographic behaviour compared to the A and B forms were consistently present in serum and varied in quantity in certain pathological conditions [17]. The subunit relationships of these 'P' and 'I' forms and their physiological function has until recently been subject to speculation, but their existence has undoubtedly complicated the precision of differential assay for diagnostic purposes.

**Specificity of Hex A**

Although the effects of enzyme deficiency are most apparent in their consequences for ganglioside metabolism, the same sugar moieties occur in a range of other natural polymers ranging from chitin through hyaluronic acid to keratin sulphate. Early attempts to seek some indication of preferential substrates for the various forms of hexosaminidases were suggestive of a relationship between cationic centres in the proximity of the hydrolysis site and susceptibility to Hex A attack. Thus asialo-GM2 is known to be hydro-

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lysed by Hex B and its accumulation becomes significant in Sandhoff’s disease, while the salated GM2 is clearly not effectively degraded by the ample amounts of Hex B remaining in the tissues of Tay–Sachs patients. Similarly, studies on the hydrolysis of chitin oligosaccharides and degradation products of hyaluronic acid suggested that these were differentially attacked by the two isoenzymes [18, 19].

On the other hand, it has been somewhat dogmatically supposed that the degradation of sulphated polysaccharides proceeds by the preliminary removal of esterified sulphate by the sulphatases that undoubtedly exist in the lysosomal armoury followed by cleavage of the unesterified sugar from the main chain.

A search for the putative sulphatase that de-sulphates the N-acetylgalactosamine of keratan sulphate before hexosaminidase attack revealed that the glycosidic link was capable of being cleaved without this agency [20]. The enzyme responsible proved to be Hex A. Hexosaminidase B on the other hand has an apparent requirement for desulphation under these circumstances. The outcome of these studies has been the derivation of a sulphated 4-MU-N-acetylgalactosaminide (Fig. 2) that has a high specificity for Hex A and offers a one-step direct assay for use in Tay–Sachs screening as well as being a powerful tool for the assessment of the presence or absence of functional α-units in the various intermediate forms of this enzyme family [21, 22].

**Fig. 2.** 4-Methylumbelliferyl-β-N-acetylgalactosaminide-6-sulphate (a specific substrate for Hex A)

Fabry’s disease

This α-galactosidase deficiency that results in the accumulation of galactosylceramide and renal malfunction presents a number of special problems. It is one of a small number of lysosomal disorders that are X-linked, being expressed in roughly half the male offspring of carrier mothers. Until recent developments in molecular biology, the unequivocal identification of a female carrier of such a disease before she had produced an affected offspring was a difficult diagnostic procedure. This stems from the random inactivation of one of the X chromosomes in the female, the so-called Lyon hypothesis, and the consequent variability of the levels of gene product that might be expected in various organs. Because of this mosaicism, unless there are other factors affecting the proportion of normal and mutant genes that are expressed, the enzyme levels in multicellular biopsy samples and in body fluids may range widely from near normal to approaching hemizygote levels. Thus while some carriers can be identified by clearly subnormal levels or by selective inhibitors, but it is now known that while α-galactosidase A (the enzyme implicated in this disorder) is a highly specific agent, α-galactosidase B is in fact an α-N-acetylgalactosaminidase and can be selectively assayed by the corresponding 4-MU substrate in the presence of variable amounts of α-galactosidase A [23].

In view of the cellular mosaicism that is implied and the corresponding variable expression of the Fabry enzyme in female carriers, it might be supposed that the only unambiguous detection of heterozygotes at a diagnostic enzymology level would require the daunting prospect of a statistical sample of single cell analyses and examination for the occurrence of some cells with the characteristics of the hemizygote. Gartler’s studies on the classic X-linked disorder of G-6-PDH indicated the value of using hair roots as pseudolines of X+ and X− cells [24]. Because hair originates from a very small number of progenitor cells and there is little migration of the daughter cells in the scalp during subsequent development, there is a high probability that a single hair root will prove to consist of cells originating from a single ancestral cell and thus be in that respect monochlonal. There is therefore a reasonable chance that in a small number of individuals hair root samples can be treated as a single X-linked trait, some of them will express the characteristics of the hemizygote. The problem then is reduced to being able to carry out the analyses on samples as small as a single hair root, to have a satisfactory baseline to compensate for variable sample size and to eliminate those which give negative values due to lack of viability.

It is well within the capacity of the fluorimetric procedures to enable such assays to be carried out and the problems of sample size and viability can be accommodated simultaneously by comparing the indicator enzyme levels with those of an unaffected enzyme of the same class that is also susceptible to loss of activity at the onset of morbidity. Thus in a survey of a large family with the Fabry trait we compared the α-galactosidase levels of hair root samples with the corresponding levels of hexosaminidase and found them to fall into three distinct groups [25]. In particular, the putative female carriers inevitably produced some samples with values corresponding to the hemizygote level and showed a trimodal distribution overall owing to the occurrence of some hair roots of mixed (X+/X−) ancestry. While the method is capable of identifying carriers whose overall enzyme levels are within the normal range, the confidence with which one can predict the absence of a defective chromosome is limited by the size of the statistical sample it is feasible to analyse.

More recently, the same approach has been used in a study of the genetics of Hunter’s syndrome, another X-linked lysosomalopathy, this time involving α-L-iduronate sulphate sulfatase [26].

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Until suitable gene probes are readily available, the most definitive approach to estimating carrier status in X-linked disorders of this kind would appear to be single cell analysis. Single cells freeze-dried on to plastic film and incubated in oil-entrapped microdroplets of substrate have been successfully assayed by fluorimetry through an inverted fluorescence microscope [27]. With the advent of the ‘Cytospin’ centrifuge it has been possible to prepare monolayers of unfixed cells on a microscope slide and to cover each with a discrete microdroplet of substrate solution. After incubation these can be treated with an organic base to reveal the fluorescence of 4-MU and quantified. For X-linked carriers the number of enzymically active cells can be compared with the total counted under phase-contrast and the scatter diagram of activity levels reveals those cells with hemizygote characteristics and a second population with the range of activities corresponding to that of the normal subject [28].
The multiple forms of β-galactosidase that are seen are largely a result of this enzyme's propensity to aggregate. The monomeric form around 64 kDa in size is frequently seen to dimerize in response to ionic and pH conditions, while a variable amount of the total activity of any biological sample is in a high molecular mass aggregated form at least ten times larger than the constituent monomers [29]. This lysosomal acid hydrolase is distinct from another enzyme of lower molecular mass with a neutral pH optimum and a broad specificity that includes activity towards β-glucosides, β-xylanosides and α-arabinosides, and is principally a glucosidase of non-lysosomal origin [30].

The absence of lysosomal β-galactosidase is associated with GM1 gangliosidosis, a severe neurological disorder of which several variants have been identified in the unrelated cases that have been recorded worldwide. The classic infantile cases have been well documented as being devoid of any gene product resembling the enzyme and the question arises whether the variants represent allelic mutations of the same gene [31].

The technique of somatic cell hybridization offered the opportunity to test whether the combination of any two of the variant cell lines was able to complement each other and restore activity, thereby suggesting the possibility of further genetic factors involved in the expression of this monomeric enzyme. In one particular combination, that of cells from the classic infantile case with a milder variant expressing some residual activity, there was a significant restoration of activity after fusion [32].

It became apparent subsequently that this second variant was also deficient in neuraminidase [33]. The residual β-galactosidase activity was kinetically and immunologically normal and biologically half-life studies attributed the low amounts of steady-state activity to a normal ratio of biosynthesis coupled with an excessive rate of degradation. It was shown that the effect could be temporarily alleviated in cell cultures by promoting the uptake of protease inhibitors, particularly leupeptin, which allowed the steady-state levels inside the cells to rise [34] (Table 1). Immunoprecipitation and electrophoresis of β-galactosidase–related antigens from radiolabelled cultures [Fig. 3] revealed a hitherto unsuspected low molecular mass glycoprotein factor that was present in the classical galactosidase-deficient cells (lane 2), but missing from the variant (lane 3). Further studies showed this factor to be secreted into the medium of classical gangliosidosis fibroblasts under the stimulus of ammonium chloride in the form of a higher molecular mass precursor, but it was not seen in the corresponding medium from the variant cells (lanes 4 and 5). This non-enzymic agent of molecular mass 54 kDa is transformed intracellularly to the 32 kDa active principle and in this state causes a tenfold increase in the half-life of the enzyme by raising its resistance to proteolysis [35]. At the same time, the residual activity which was previously seen to be in the monomeric state is now found to be aggregated [36]. The protective factor is found in association with this aggregated form of the enzyme which in vivo constitutes the major β-galactosidase component and also expresses neuraminidase activity.

This novel biological system for the control of steady-state levels of enzyme activity may not be unique. For example, other lysosomal enzymes such as α-fucosidase and α-iduronidase are also known to show the phenomenon of aggregation under appropriate conditions, while at least one other example of more than one enzyme being apparently affected by a single defect is seen in multiple sulphatase deficiency [37].

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<th>Table 1. Effects of modulators on β-galactosidase activity in normal and GM1 gangliosidosis fibroblasts</th>
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<tr>
<td>Representative data from [32, 34, 35].</td>
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<td>Activity (nmol of 4-MU released h⁻¹ mg⁻¹ protein)</td>
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<tr>
<td>1. Control</td>
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<tr>
<td>2. Infantile form</td>
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<td>3. Galactosidosis</td>
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<td>4. Fused cells 2 x 3</td>
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<td>5. Adult form</td>
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Prolate substrates

The fluorimetric assay of lysosomal cathepsins and other proteases has been made possible by the synthesis of peptide...
analouges of the 4-MU glycosides in which a 7-amino-
coumarin substitutes for the phenolic derivative [38]. These
peptides are designed to mimic the susceptible bond of a
given protease with N-terminal blocking if such is required
by a specificity for endoprotease attack. The liberated
7-amino-4-methylcoumarin has the advantage of being
maximally fluorescent above pH 4, when the amino group is
virtually unprotonated. The peptides frequently need to
resemble a substantial sequence of amino acids upstream of
the cleaved bond and specificity on the C-terminal side
cannot be accommodated. These substrates have significant
fluorescence before cleavage and to keep signal-to-
background noise at acceptable levels, as well as for
economy, they may need to be used at sub-optimal concen-
trations while the activating and fluorescent wavelengths for
assay are chosen to maximize the slight differences between
the substrate and product in favour of the latter.

An alternative system of general application that has not
been greatly exploited is the use of fluorescently labelled
proteins as substrates and the subsequent measurement of
solubilized fluorescent peptides [39]. This is surprising in
view of the continued use of denatured haemoglobin for
spectroscopic assay in this fashion and the availability of
dyed proteins for similar colorimetric procedures. An
advantage of the fluorometric approach is that it is possible to
use an electrophoretic peptide fingerprint of the terminal
hydrolys products as an additional adjunct for the specific
identification of a given protease. Because only those
peptides that carry a fluorescent label are visualized, the
fingerprint pattern is simpler and easier to identify than it
would be if the total peptides were stained. One may
speculate in passing on the potential of the same approach
for the production of fluorescent peptides for molecular
mass or iso-electric point markers.

Fluorescent protein labelling

Identifying the location of specific cell components by
specific antibodies or other binding proteins labelled with
fluorescent reporter groups is a well-established histochemi-
tical technique. Of the various reagents that have been
proposed for this purpose the isothiocyanates of fluorescein
and rhodamine have become the established standards and
allow two-colour fluorescence microscopy to show the rela-
tive locations of different cell components [40]. More
recently, the use of naturally occurring phycobilins has been
introduced to challenge their 20 year superiority [41]. There
are, however, certain fundamental deficiencies in all these
reagents as fluorophores, the principal of which is the small
Stokes shift between optimal activation and emission wave-
lengths (Fig. 4). Further, the emission characteristics of
fluorescein seriously overlap the activation wavelengths of
rhodamine, so that when both are used on the same sample
care must be taken not to introduce an energy transfer
situation whereby the stimulated fluorescence of the former
is quenched by the presence of the latter. Under the intense
illumination used in these techniques, photodecomposition
becomes a serious problem and various oxygen-consuming
or free-radical quenching agents may be required in the
system [42]. An additional hazard is the fact that both these
fluorophores introduce a new cationic centre in substituting
an amino function on the protein and thus can cause a
considerable shift in isoelectric point which may eventually
affect the solubility or binding affinity of the protein ligand.

Our immunochemical studies of enzyme-related antigens
in normal and mutant cells [43] and the identification of
specific carbohydrate polymeric storage products by fluores-
cent lectin binding [44] have led us to formulate the desirable
characteristics of such fluorescent labels for our purpose.
Ideally, they should not affect the isoelectric point of the
labelled protein, should be photostable and should have a
considerable Stokes shift. For practical purposes an activa-
tion wavelength that coincides with a strong emission band in
the mercury arc would facilitate the applications in micro-
scopy. An emission spectrum that is discriminated from
those of fluorescein and rhodamine would be an added
advantage in that it would allow three-colour immuno-
fluorescence techniques.

The outcome of these considerations has been the
development [45] of aminomethylcoumarin acetic acid
N-hydroxysuccinimide ester (AMCA-NHS). This reagent re-
acts smoothly with lysine residues in proteins under mild
conditions substituting an intense blue fluorescent group
with a shift of about 100 nm between the activating and
fluorescing wavelengths (Fig. 5). Because it substitutes
selected lysine residues by peptide bond formation with a
fluorophore carrying an aromatic amino-function, it pro-
duces little or no change in isoelectric point and the substi-
tuent is remarkably resistant to photodecomposition. Its
absorption characteristics coincide with an intense band in
the mercury arc.

We have used this reagent successfully in a number of
immunochemical applications including the localization of
cell structural elements, the immunostaining of specific
lysosomal enzymes and demonstration of storage material
in mutant cells by fluorescent lectin binding. Other current
applications include diagnostic identification of gut
pathogens, and chromosome probes in situ. Most recent and
perhaps most exciting is the use of AMCA-labelled anti-
bodies in the simultaneous identification and quantification

![Fluorescent protein labelling](image)

**Fig. 4. Structure and spectral characteristics of fluorescein isothiocyanate (FITC)**

Absorption, ——; fluorescence, ·····.
of c-myc and c-fos oncogene products by dual-beam laser cell cytometry being carried out by Dr J. Watson and his team at the M.R.C. Clinical Oncology Unit, Cambridge.

In over 30 years of application to diagnostic enzymology the technology has undergone extensive refinements, from the manually operated null-point Hilger Spekker fluorimeter using samples of 10 ml or more on which the original feasibility studies were carried out, through microfluorimetric assays on single cells reacting in oil drops, to the new laser activated systems that offer the possibility of quantification at the subcellular level. In all this time one factor has remained constant - the remarkable versatility of these coumarin derivatives as quantitative fluorescent agents.


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