Allosteric regulation was a hot topic in the 1960s, but there was very limited structural data on allosteric equilibria, and no solid information on the rates of allosteric conformational changes. In this Biochemical Journal Classic paper from 1969 George Radda and his first D.Phil. student, George Dodd determined the rate of allosteric transition in the regulatory enzyme glutamate dehydrogenase by a method new in the 1960s, the fluorescence of an environmentally sensitive extrinsic probe.

Allosteric regulation was formalized and established as a central regulatory mechanism by Monod and colleagues in their classic 1963 review1. In addition to haemoglobin, the model for all cooperative phenomena, this review cited six bacterial enzymes showing endproduct feedback inhibition, plus three highly regulated mammalian enzymes [phosphorylase b from muscle, acetyl-CoA carboxylase from adipose tissue and l-glutamate dehydrogenase (GDH) from bovine liver]. The last of these was known to be activated or inhibited by a huge array of metabolites (including nucleotides, oestrogens and thyroxine) and to show complex kinetics in relation to its substrates glutamate, 2-oxoglutarate (α-ketoglutarate) and the nicotinamide coenzymes. Monod and colleagues reviewed data on regulation of GDH activity, desensitization and changes in association state that clearly identified GDH as an allosteric enzyme and they commented1 “By contrast with bacterial systems, whose functions are simple and obvious, the physiological interpretation of the multiple sensitivities and activities of GDH appears exceedingly difficult. But... it cannot be doubted that the complex allosteric reactivity of GDH does reflect its central multivalent role in cellular metabolism.”

Following this key review article, allosteric regulation rapidly became a very active research area, with important papers on theoretical models of allostery2,3 and an expanding body of experimental work. It was possible to interpret much enzyme steady-state kinetic data, such as sigmoidal substrate dependence and inhibition by regulatory metabolites, in terms of allosteric equilibria between active (R) and inactive (T) enzyme conformations. But the structural information underpinning such interpretations was extremely limited and there were essentially no data on rates of the key process in which an enzyme interconverted between conformations. Indeed, the limited X-ray diffraction data on proteins at this time tended to make researchers think of proteins as rigid, rather than dynamic, so the idea of determining the rate of a dynamic conformational change was distinctly unconventional.

It was against this mid-1960s background that Radda and colleagues began to analyse the allosteric transition of GDH. This was one of Radda’s earliest projects as an independent researcher. He had come to the U.K. in 1956, escaping from Hungary during the uprising against Soviet domination. He had already begun studying chemistry at the University of Budapest, and, despite his rudimentary English, he was accepted to study chemistry at Merton College Oxford. This was a far-sighted decision by the College which became his base for most of the next 50 years. After graduating with a first-class degree, he stayed in Oxford to do a doctorate in physical organic chemistry with R.O.C. Norman, and then spent a year in California as a postdoctoral researcher in the laboratory of the Nobel Prize-winner Melvin Calvin, working on flavins and flavoproteins and gaining experience in fluorescence methods4. He returned to Oxford in 1964, succeeding Norman as fellow and organic chemistry tutor at Merton College, and with a junior lectureship in Oxford’s Department of Biochemistry, then led by Sir Hans Krebs.

Analysis of the allosteric behaviour of GDH was made especially difficult by complete absence of sequence information and uncertainty about the quaternary structure of the enzyme. (A high-resolution structure was not determined until 30 years later5 and we now know that the enzyme is a hexamer of large, >50 kDa, subunits which shows further concentration and ligand-dependent aggregation.) Nevertheless, it was known that GTP and ADP had reciprocal regulatory effects on GDH activity, the former as allosteric inhibitor and the latter as activator, and Radda soon showed6 that GTP-dependent inhibition was associated with a secondary structure change that could be detected by optical rotatory dispersion (ORD), a technology that has now
be superseded by circular dichroism (CD). He and his students also showed, using chemical modification with group-specific and site-directed reagents, that lysine residues were essential both for GDH catalytic activity and for its response to the inhibitor GTP\textsuperscript{5,6}. But the key tool introduced by Radda’s laboratory for sensitive and rapid detection of conformational change was the fluorescence of the extrinsic fluorescent probe ANS (1-anilino-naphthalene-8-sulfonate).

ANS was popularized as a research tool by Lubert Stryer\textsuperscript{7}. He showed that ANS binds to apo-myoglobin and apo-haemoglobin competitively with haemin, and that the very non-polar haem-binding site in these proteins provides a hydrophobic environment which enhances (200-fold) and blueshifts (by 60 nm) the fluorescence emission of ANS. The paper’s abstract states “The use of anilinonaphthalene sulfonates as fluorescent probes of non-polar regions in proteins is discussed.” The concept was not entirely new, but Stryer’s paper characterized in detail the extreme environmental sensitivity of ANS and similar probes, and ensured that their use became mainstream. Today, ANS is widely used to monitor protein folding and unfolding; the detection of species that greatly enhance the fluorescence of ANS is regarded as diagnostic of the existence of molten globule folding intermediates\textsuperscript{10}.

Dodd began his doctoral studies with Radda in 1965; they soon found that ANS bound to GDH with enhancement of its fluorescence and that there was further 3-fold enhancement when the substrate NADH and inhibitor GTP were added\textsuperscript{11}. This combination of substrate and inhibitor was known to convert GDH into the inactive (T) conformation and the data showed that a titration of GTP into GDH+ANS in the presence of NADH generated a sigmoidal enhancement of ANS fluorescence, as did a titration of NADH into GDH+ANS in the presence of GTP. Hence ANS was a suitable extrinsic fluorescent probe for monitoring the allosteric transition of GDH. Dodd and Radda proceeded to exploit this finding, presenting further data at a Biochemical Society meeting in Birmingham, U.K., in March 1968\textsuperscript{12} and publishing their full results later in the Biochemical Journal\textsuperscript{13}.

To begin, Dodd and Radda confirmed that the process reported by ANS when GTP was titrated into GDH+NADH showed the same GTP-dependence as the ORD change reported previously, and that it could also be followed in the absence of ANS, by using the fluorescence of bound NADH. Hence ANS was a true non-perturbing reporter of an underlying conformational change, with the advantages of sensitivity and specificity. They then used ANS fluorescence to explore the GTP-dependent conformational equilibrium and analysed the data as a classic allosteric transition between R (relaxed, active) and T (tense, inactive) states, showing that the plot of the T function (the proportion of enzyme in the T form) against GTP concentration varied with NADH concentration (Figure 1).

They were then in a position to attempt something ambitious and novel, namely to measure the rate of this allosteric conformational transition. Using stopped-flow mixing and sensitive fluorescence detection, they observed rate processes occurring on mixing GTP (in one syringe) with GDH, ANS and NADH (in the other syringe). Two distinct rate processes could be observed on the millisecond timescale, and the linearity of the two phases in the semi-logarithmic plot of the data showed that these were both first-order processes (Figure 2). Given that the allosteric transition to the T state requires the presence of both GTP and NADH, the kinetic process of conversion into the T state could be measured either by mixing GTP with GDH+NADH or mixing NADH with GDH+GTP and the rates observed were the same in either case, with rate constants of 34±13 ms and 200±60 ms for the two phases. The rates were also independent of whether ANS was present premixed in the syringe with the enzyme or was in the syringe with the component triggering the transition. The rates were also independent of the concentration of GDH across the accessible range from 0.5 to 2.0 mg/ml final concentration.

Dodd and Radda were very conscious that it was important to establish that the ANS probe data were truly reporting the underlying allosteric transition. Their controls included the fact that changes corresponding simply to ANS and NADH binding were complete within the dead-time of their stopped-flow instrument, and that no signal or signal change at these wavelengths was observed in the absence of ANS. Hence they were observing changes in conformation reported by ANS bound to the enzyme. They interpreted the fact that two
rates were observed as indicating changes in tertiary and quaternary structure respectively, i.e. that there was an initial rapid change in the conformation of the individual GDH subunits, followed by a slower rearrangement leading to change in contacts between the subunits in the oligomer.

George Dodd is a Dubliner with a first degree from Trinity College Dublin, who was trained as a perfumer before beginning his academic career. In 1965, he became Radda’s first D.Phil. student, supported by a Wellcome Trust studentship. Subsequently he joined Unilever Research at The Frythe, Welwyn, Hertfordshire, working on the biophysics of lipid membranes and later was one of the first Lecturers in Biochemistry in the School of Molecular Sciences at Warwick University, where he did research on the mechanisms of olfaction through the 1970s and 1980s (e.g. on the effect of concanavalin A on the rat electroolfactogram). He continued to enjoy practical perfumery and at one time had a perfume shop in Stratford-upon-Avon. In 1994, he opened an aroma science research laboratory and perfumery on the West Coast of Scotland where he now lives (see http://www.thesoslab.com/dr-george-dodd.asp), but has maintained academic connections through the University of the Highlands and Islands.

George Radda’s laboratory grew rapidly in the late 1960s and, by the time this Biochemical Journal Classic was published, it comprised more than ten overseas visitors, part II chemistry students and doctoral students (including the authors of this article). We were very aware of the up-to-date equipment for physical biochemistry that was available to us. On the ground floor, in a private air-conditioned room, dwelled a Spinco Model E analytical ultracentrifuge with Schlieren optics, carefully nursed by a full-time technician. On the sixth floor of the Biochemistry tower (now scheduled to be demolished), Dr Rupert Cecil maintained a titrating polarograph for the determination of free thiol groups, based on a ‘dropping mercury electrode’. We used to drink coffee in this laboratory, and the level of mercury vapour in the air gave us headaches, and would give a modern Health and Safety Officer nightmares! Close to the Radda laboratory, was a huge Cary 14 scanning spectrophotometer, at least 1 mitre in every dimension, and equipped with its own in-built chart recorder, a very modern touch. We were also proud of our Zeiss spectrofluorimeter, which had temperature control, attachments for inclusion of polarizers and, best of all, manual slit control plus motor-driven scanning on both excitation and emission monochromators! We even had a fluorimeter with a pulsed light source generating 20 ns pulses at 0.2 ms intervals, with fluorescence output to an oscilloscope, which could be used for time-resolved observations of fluorescence decay.

But Radda’s pride and joy was the stopped-flow rig, based on a design by Quentin Gibson, which was used to generate the data on the rate of the GDH allosteric transition. The core was a home-built stainless steel mixing block comprising a mixing chamber with 2 mm inlet and outlet tubes providing a dead-time of <4 ms. With a high-pressure mercury lamp as light source, grating monochromator on the excitation side, perpendicular optics and a glass filter to select emitted light of wavelength >500 nm, ANS fluorescence was detected by a monochromator linked to an oscilloscope and the oscilloscope traces recording the time course of reaction were photographed with a polaroid camera.

After publishing this Biochemical Journal Classic paper, Radda rapidly extended the application of fluorescent probes to complex membrane systems and his pioneering work in applying novel spectroscopic methods to a wide range of protein and membrane systems was recognized with the award of the Biochemical Society’s Colworth Medal in 1970 (Figure 3). His Colworth Medal Lecture ‘Enzyme and Membrane Conformation in Biochemical Control’ described ongoing work on...
continued in Oxford and did a stint as Head of the Department of Biochemistry before serving as Chief Executive of the Medical Research Council (MRC) from 1996 to 2003. He was knighted in 2000. A less committed researcher might have chosen retirement at the end of his time at the MRC, but Radda returned to his research in Oxford and spent 3 years as Head of the University’s newly merged Department of Physiology and Anatomy and Genetics. At this time, he was also spending one week each month in Singapore, serving as Founding Chair of the Bioimaging Consortium and later Chairman of the Biomedical Research Council of Singapore’s Agency for Science Technology and Research. He moved to Singapore in 2008/2009 to take up this role full-time and continues to provide research leadership in this emerging centre of excellence.

Alan D.B. Malcolm is Executive Secretary to the Parliamentary & Scientific Committee and Expert Adviser to Select Committees in both the House of Commons and the House of Lords. As Chief Executive of the Institute of Biology 1998–2009, he successfully led the merger of many disparate bodies in the life sciences under the umbrella of the Society of Biology. He was Chairman of the Biochemical Society (1993–1995) and (twice) Chairman of the British Nutrition Foundation. email: alan.malcolm1944@googlemail.com

Robert B. Freedman is at the School of Life Sciences, University of Warwick having moved to Warwick in 2002 to become Head of the Department of Biological Sciences. He was at University of Kent for many years, including periods as Head of the School of Biosciences and as Deputy Vice-Chancellor. He is best known for his work on protein folding in the cell and the folding catalyst protein disulphide-isomerase. He was Chair of the Biochemical Society (1996–1998) and served on the Council of BBSRC (2002–2008). email: R.B.Freedman@warwick.ac.uk

Figure 3. George K. Radda. Taken from Radda, G.K. (1971) Biochem. J. 122, 385–396

References