

## CONSTRUCTION OF AN FIA UNIT AND ITS USE IN THE INVESTIGATION OF IMMOBILISED LIGNIN PEROXIDASE

J. STIERLI\*\* M. FAWER S. CLIFFE & A. FIECHTER

Institute of Biotechnology, ETH, Zürich

\*\*Present address : Ismatec SA, Glattbrugg

A flow injection analyser was designed to serve as a tool for the characterisation of enzymes. The original device was constructed from available laboratory equipment, and has subsequently been refined to a fully automated, stand alone unit. The hard- and software have been so conceived as to enable a wide range of applications. All system components are controlled by a microprocessor, which also takes over data acquisition, peak integration and evaluation, and provides a convenient interface to the user via a key-board and display. We have used this FIA system to investigate the enzymatic properties of lignin peroxidase. This enzyme is believed to play a key role in the biodegradation of lignin by white-rot fungi, and has attracted interest for use in various industrial processes. Lignin peroxidase has a very similar mechanism of action to horse radish peroxidase, but differs in its ability to oxidise organic compounds of higher oxidation potential, and in its extreme sensitivity to inactivation by excess  $H_2O_2$ . The enzyme can be successfully immobilised on a variety of carriers. However when investigated in batch assays, the loss of activity is such that repeated experimentation is impossible. FIA is characterised by small sample volumes, and thus low contact times when working with an immobilised enzyme. We have exploited this to enable investigation of an essentially unstable enzyme system.

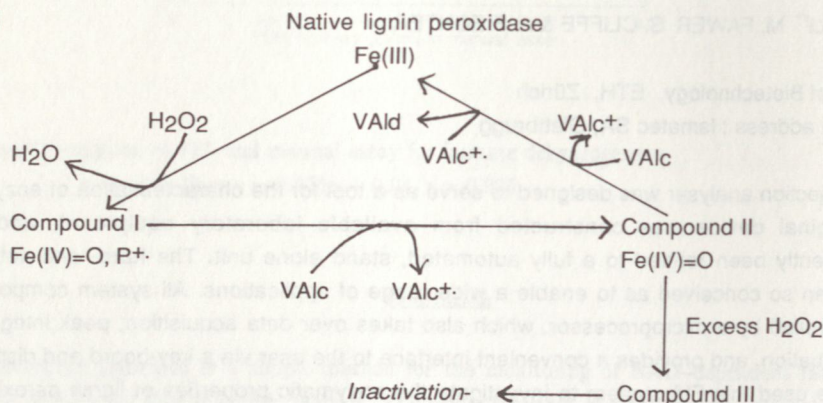
### 1 INTRODUCTION

Whilst excellent FIA systems are currently commercially available, the rapid advance in microelectronics results in the rapid ageing of instrumentation. Thus it is possible to incorporate automatic control and data processing systems into small microprocessors instead of using an external computer. We were interested in using FIA for the investigation of immobilised enzymes in general, and thus required a system with the following specifications : automated sample injection, a programmable sequence including dilutions and stop-flow

analysis, automatic switching between different buffers or calibration solutions, and two-channel data acquisition & processing.

Lignin peroxidase is activated by  $H_2O_2$  (Fig 1) to compound 1, which in turn affects a one electron oxidation of an organic substrate to yield a cation radical and compound II. This radical may then undergo a second enzyme catalysed one electron oxidation, which in the case of veratryl alcohol leads to the formation of veratryl aldehyde. Alternatively the cation radical may undergo chemical reactions with  $H_2O$ ,  $O_2$ , or a second organic species. This accounts for the wide range of reactions reported for the enzyme, and is of paramount importance in the biodegradation of lignin (1). Lignin peroxidase is very susceptible to excess  $H_2O_2$ , reaction with which leads to irreversible enzyme inactivation. Substrates, and in particular veratryl alcohol, have a protective function by reducing the enzyme back to the native state.

Fig. 1



## 2 MATERIALS AND METHODS

### 2.1 Mechanical parts

Two prototype FIA units were constructed. The mechanical parts for the first were taken from available laboratory equipment, whilst for the second unit, two peristaltic pumps (Ismatec) and two low pressure chromatography valves (Rheodyne, one injection valve and one six way valve for buffer and / or calibrant choice, both with electric motor actuators) were fixed in a metal housing. Automated samplers were constructed by attachment of a sampling arm to available fraction collectors (LKB Redirac and Ultrarac), with control via the drop function; drops being simulated by on / off switching of an electric (bicycle) light bulb.

### 2.2 Detectors

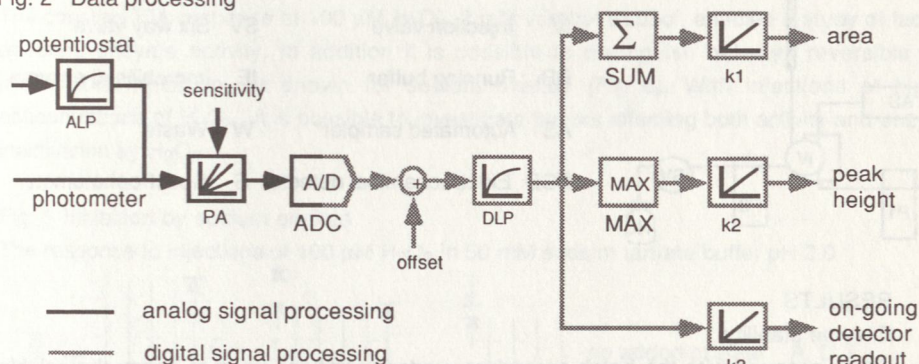
Two detectors were used in series. Firstly an electrochemical wall-jet detector (Metrohm 656), equipped with a gold counter electrode and a Ag / AgCl / 3M KCl reference electrode.  $H_2O_2$  was determined by oxidation at a platinum electrode (+600 mV), whilst oxidised products were quantified by reduction at a glassy carbon electrode (+100 mV). Initially a potentiostat from IMT (Institute of Microtechnology, Neuchâtel) was used, and

subsequently a commercially available unit (Metrohm 641). The outlet from the first detector was lead directly to a spectrophotometer (Shimadzu 120 - 02) equipped with a 100 $\mu$ l flow cuvette ( Hellma), where veratryl aldehyde formation was monitored at 310 nm.

### 2.3 Control and data acquisition unit

This was based on an Intel 8052 AH Basic microcontroller. The unit controls the mechanical parts, acquires and evaluates the data, and provides an interface to the user via a keyboard and visual display. Software for keyboard definition, control functions and data processing was written on a personal computer ( Macintosh SE ), and stored in a non volatile memory in the microprocessor. Via the keyboard it is possible to use the unit in a manual fashion, or to programme a sequence of analyses with defined number of samples, number of injections per sample, analysis time, start and stop of integration, and to select between different running buffers or calibrant. Data processing was achieved as outlined in Fig. 2

Fig. 2 Data processing



The signal from the photometer is directly amplified by the preamplifier [PA] (part of the chart printer). The output signal of the potentiostat is first filtered by an active second order Butterworth low pass filter [ALP] (two selectable rise times: 0.1 and 0.5 s). The amplified signal is digitalized by an analog to digital converter [ADC] (12 bit resolution). To set zero the baseline at the beginning of an integration period, an offset is added. After passing a digital first order filter [DLP], the peak signal is either displayed directly, or processed by a maximum peak height detection algorithm [MAX], or integrated [SUM]. k1, k2 and k3 are constants for standardising the outputs.

### 2.4 Immobilisation of lignin peroxidase

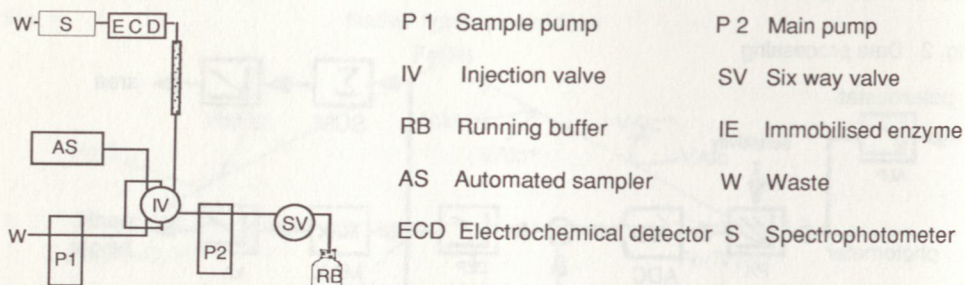
Lignin peroxidase from *Phanerochatae chrysosporium* was immobilised on amino-silane derivitised control pore glass ( Daltosil 500, 0.1 - 0.2 mm particle size, Serva ) by the glutaraldehyde methodology. Batch assays were carried out by suspending a known quantity of beads in 9.0 ml 0.1 M sodium tartrate buffer pH 3.0, 2.2 mM veratryl alcohol. The reaction was started by addition of 1.0 ml H<sub>2</sub>O<sub>2</sub> (0.22 mM) and the suspension agitated by rotation at 30 rpm in an empty chromatography column ( Pharmacia PD-10), which allowed easy

removal of an aliquot after 5 min. The production of veratryl aldehyde was quantified by measuring the absorbance at 310 nm.

### 2.5 Conditions for FIA

For FIA, enzyme laden beads were packed into silicon tubing (1.0 mm or 2.0 mm in diameter and 10-40 mm long), and retained behind a porous polyethylene frit. The running buffers were either sodium tartrate, sodium phosphate, or tartrate-phosphate, typically of pH 3.0, flow rate 1.0 ml min<sup>-1</sup>. Veratryl alcohol was added at the same concentration to both buffer and the injected sample. To stabilise the FIA response, 3 - 4 injections of 5 mM H<sub>2</sub>O<sub>2</sub> were made prior to experimentation. For steady state experiments, hydrogen peroxide was added to the running buffer, whilst for FIA, hydrogen peroxide and any additional substrates were injected together. The two injection valves used had sample loops of 90 µl and 100 µl.

Fig. 3 FIA SCHEMA

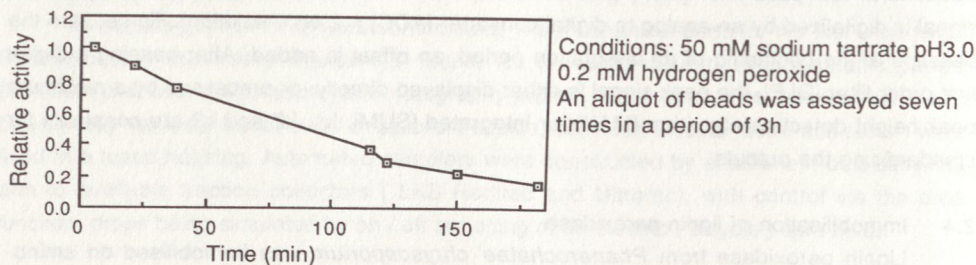


## 3 RESULTS

### 3.1 Enzyme stability

When assayed with the batch procedure, under conditions used to assay the soluble enzyme, the immobilised preparation showed a steady loss in activity ( Fig. 4).

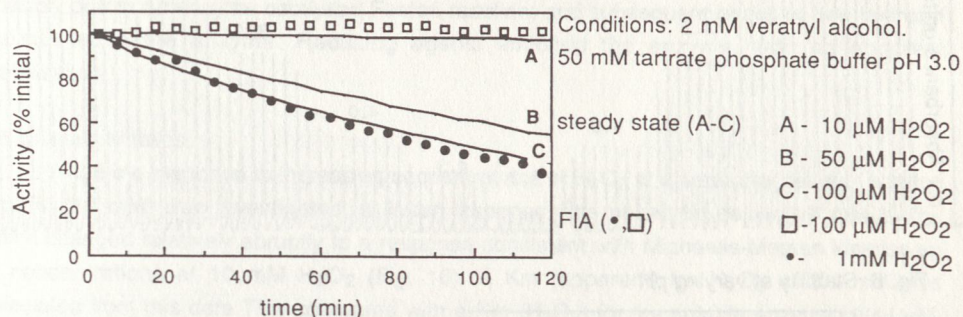
Fig. 4 Stability during sequential batch assays



We made a systematic study of the effect of veratryl alcohol & H<sub>2</sub>O<sub>2</sub> concentrations, in the flow system under conditions where conversion was approx. 10% (Fig. 5). In the steady state the activity remains reasonably constant over a two hour period only at 10 µM H<sub>2</sub>O<sub>2</sub>, at which concentration the signal was at the limits of detection. By contrast, during FIA a stable signal is obtained at 100 µM H<sub>2</sub>O<sub>2</sub>. Decreasing the veratryl alcohol concentration below 2

mM lead to a corresponding decrease in stability, whilst stability increased only slightly when going from 2 mM ( used in most subsequent experiments ), to a maximum effect at 10 mM.

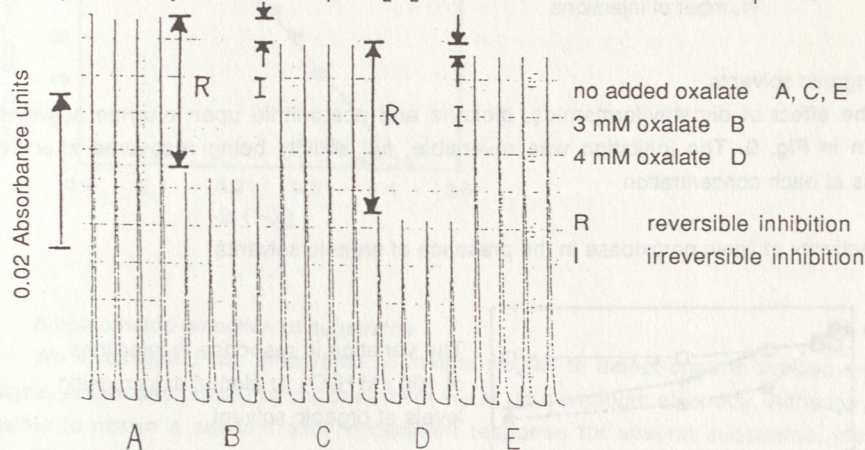
Fig. 5 Stability of immobilised lignin peroxidase in the steady state and during FIA



The constant FIA response at 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$ , 2 mM veratryl alcohol, enabled a study of factors affecting enzyme activity. In addition it is possible to distinguish between reversible and irreversible inhibition, as shown for sodium oxalate (Fig 6). With injections of higher concentrations of  $\text{H}_2\text{O}_2$ , it is possible to investigate factors affecting both activity and enzyme inactivation by  $\text{H}_2\text{O}_2$ .

Fig. 6 Inhibition by sodium oxalate

The response to injections of 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$  in 50 mM sodium tartrate buffer pH 3.0



### 3.2 pH dependence

Soluble lignin peroxidase has an unusually low pH optimum, showing maximal activity at pH 2.0, below which it is rapidly inactivated. A similar pH profile for the immobilised enzyme was observed (Fig. 7). The immobilised enzyme was also rapidly inactivated below pH 2.0. Inactivation by  $\text{H}_2\text{O}_2$  was reduced in going from pH 2.0 to pH 3.0, but no further increase in stability was observed at pH 4.0 (Fig. 8).

Fig. 7 pH profile in sodium tartrate buffers: the response is to injections of 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$

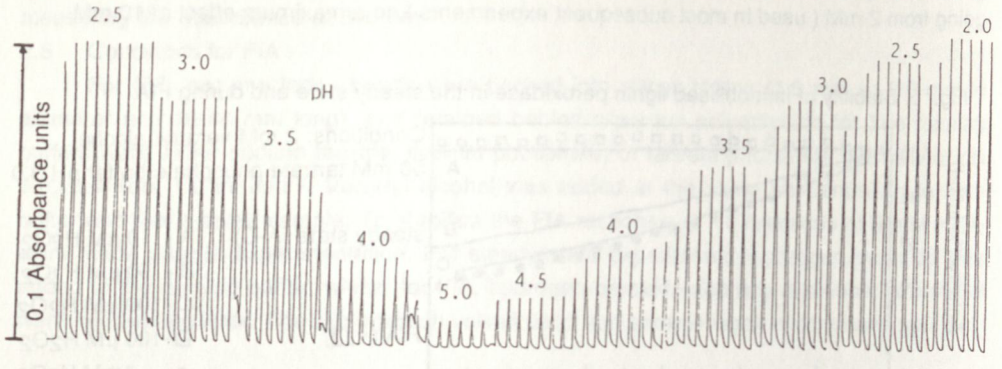
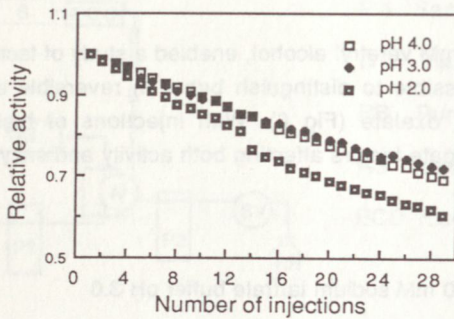


Fig. 8 Stability at varying pH

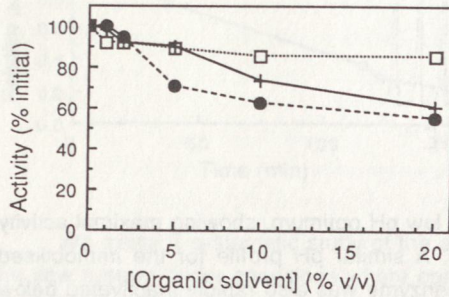


Samples of 1.0 mM  $\text{H}_2\text{O}_2$  were injected at 2.0 mM veratryl alcohol. The initial activity at each pH has been set to 1.0.

### 3.3 Organic solvents

The effect of dimethylformamide, dioxane and acetonitrile upon enzyme activity is shown in in Fig. 9. The inhibition was reversible, full activity being recovered after five injections at each concentration.

Fig. 9 Activity of lignin peroxidase in the presence of organic solvents



The variation in response to injections of 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$  is plotted at increasing levels of organic solvent

- dioxane
- + dimethylformamide
- acetonitrile

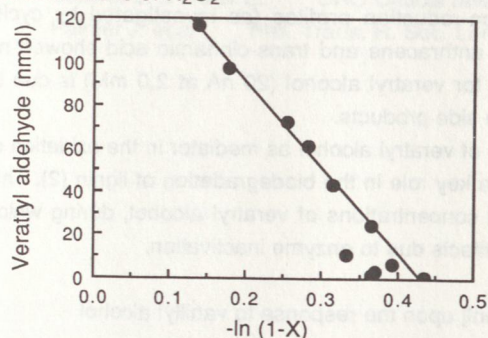
### 3.4 Enzyme activation, inhibition and stabilisation.

A series of compounds was screened for their effect upon enzyme activity and stability. Most, including two initiators of radical formation, tetraacetythylenediamine and tetramethylethylenediamine were without effect. Ferrous chloride caused rapid inactivation, possibly due to an enzyme catalysed Fenton reaction, and subsequent attack of free hydroxyl radicals upon the enzyme. Reducing agents inhibited the enzyme, with no apparent stabilisation.

### 3.5 Enzyme kinetics

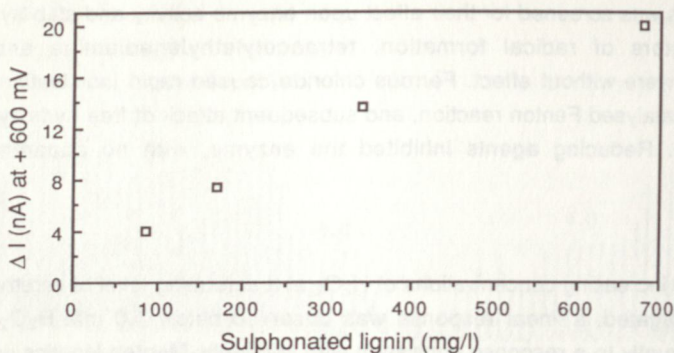
When the response to increasing concentrations of  $H_2O_2$  at a saturating level of veratryl alcohol (10 mM) was investigated, a linear response was observed below 1.0 mM  $H_2O_2$ , which changed relatively abruptly to a response consistent with Michaelis-Menten kinetics up to concentrations of 10 mM  $H_2O_2$  (Fig. 10). A  $K_m$  (apparent) for  $H_2O_2$  of 3.0 mM was calculated from this data. This compares with a  $K_m$  ( $H_2O_2$ ) for the soluble enzyme of 44  $\mu M$ , and, even allowing for dispersion of the injected sample in the enzyme reactor, suggests a large degree of diffusional limitation.

Fig. 10 Integrated Michaelis-Menten rate equation for the FIA response to increasing concentrations of  $H_2O_2$



### 3.5 Amperometric detection of substrates

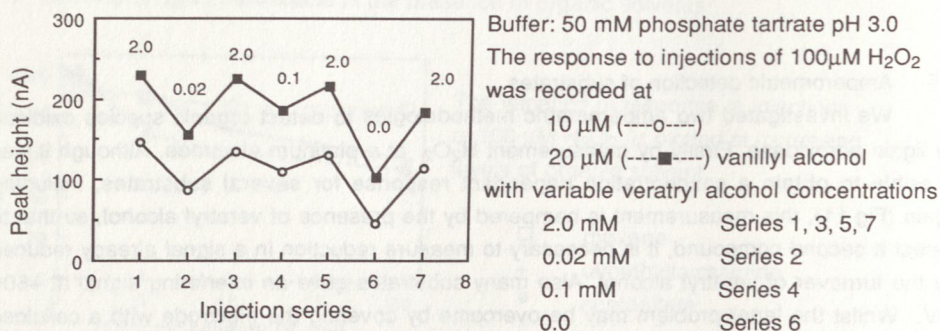
We investigated two amperometric methodologies to detect organic species oxidised by lignin peroxidase. Firstly by measurement  $H_2O_2$  at a platinum electrode. Although it was possible to obtain a concentration dependant response for several substrates, including lignin (Fig.11), this measurement is hampered by the presence of veratryl alcohol, so that to detect a second compound, it is necessary to measure reduction in a signal already reduced by the turnover of veratryl alcohol. Also many substrates gave an interfering signal at +600 mV. Whilst the latter problem may be overcome by covering the electrode with a cellulose membrane, we did not pursue this methodology.

Fig. 11 Detection of lignin by measurement of H<sub>2</sub>O<sub>2</sub> consumption

Alternatively, when the oxidation is at least semi-reversible, oxidised products may be detected by subsequent reduction. We obtained concentration dependant response curves for a series of lignin peroxide substrates, eg. vanillyl alcohol, 3,4 dimethoxyphenol, p-chlorophenol and 1,4 dimethoxybenzene, in the range 2 -100  $\mu\text{M}$  with sensitivities varying between 0.1 - 20  $\text{nA } \mu\text{M}^{-1}$ . Sulphonated lignins and two water insoluble lignin fractions gave positive responses in the range 10 - 200  $\text{mg l}^{-1}$ , with sensitivities between 0.1 and 0.9  $\text{nA l mg}^{-1}$ . Substrates with irreversible oxidation-reduction profiles (as investigated by cyclic voltammetry), eg. 4 -hydroxybenzylalcohol, anthracene and trans-cinnamic acid showed no response. The background signal observed for veratryl alcohol (20 nA at 2.0 mM) is due to the small percentage of quinone and lactone side products.

We have recently been investigating the role of veratryl alcohol as mediator in the oxidation of a third substrate, as this is believed to play a key role in the biodegradation of lignin (2). This involved conducting experiments at varying concentrations of veratryl alcohol, during which particular care had to be taken to exclude artifacts due to enzyme inactivation.

Fig. 12 Effect of variable [veratryl alcohol] upon the response to vanillyl alcohol



From Fig. 12, it can be seen that the activity does indeed fall, particularly in the total absence of veratryl alcohol. However, by returning to a control point (2.0 mM veratryl alcohol) between

each series of injections, it is possible to distinguish the positive effect of increasing veratryl alcohol upon the reductive response to vanillyl alcohol. By reducing the number of injections, and data points, it was possible to run a series of such experiments with negligible loss of activity.

#### 4 Discussion

Using a small microprocessor it was possible to automate an FIA unit with two-channel data processing, which fully met our requirements in the laboratory. The original concept has subsequently been developed (Ismatec SA, Glattbrugg, CH) to form a stand alone unit, with modular exchange of mechanical parts, including detectors. In addition to normal key-board control, it is open to pre-programming via "memory cards". We plan, in collaboration with other groups within the institute, to apply the instrument to the continuous monitoring, and possible feed back control, of bioprocesses.

For the investigation of immobilised lignin peroxidase, FIA has proven to be an invaluable tool, to the extent that it has made possible experimentation not viable by other means.

#### References

- 1 Buswell J. & Odier E. CRC Critical reviews in Biotechnology (1987) 6 1-60
- 2 Palmer J. et al Phil. Trans. R. Soc. London (1987) A 321 495-505