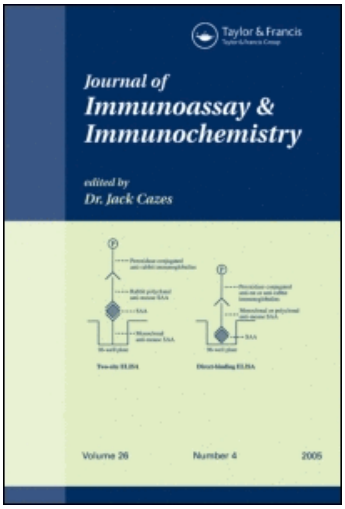

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Clearing of Suspensions of *Micrococcus lysodeikticus* Catalysed by Lysozymes from Hen, Goose, and Turkey Egg Whites, Human Milk, and Phage T4. Assessment of Potential as Signal Generators for Homogeneous Enzyme Immunoassays for Urinary Steroids

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Abstract: Lysozymes (3.2.1.17) from goose (*Anser anser*) egg white, turkey (*Melagris gallopavo*) egg white, phage T4 and human milk were compared with hen egg white lysozyme in their ability to clear a suspension of *Micrococcus lysodeikticus*. All of the lysozymes, except hen egg white lysozyme, catalysed the clearing of the *Micrococcus lysodeikticus* suspension in a biphasic fashion. Compared to hen egg white lysozyme, the total absorbance or transmission change over 5 and 20 minutes was less in all cases, except for human lysozyme. Human lysozyme was, therefore, a potential alternative, more rapid signal generator for the measurement of urinary estrone glucuronide excretion rates because of its structural similarity to hen egg white lysozyme. The apparent K_M values for hen egg white lysozyme increased with the enzyme concentration.

Keywords: Lysozyme, Clearing-curve, Immunoassay, Signal generator, Estrone glucuronide

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INTRODUCTION

Hapten conjugates of hen egg white lysozyme (HEWL)[1–5] have been used as signal generators in homogeneous enzyme immunoassays[6] because of their extensive inhibition (>90%) by anti-hapten antibodies. In particular, estrone glucuronide (E1G) and pregnanediol glucuronide (PdG) conjugates of lysozyme have been used in the Ovarian Monitor[1] to monitor the main urinary estradiol and progesterone metabolites and, thus, define the fertile period of the human menstrual cycle.[1–3,7] The most readily acylated lysine residues in HEWL (K33, K97 and K116) are all situated close to its well-defined active site cleft[4,5] and HEWL is relatively small compared to its bacterial cell substrate *Micrococcus lysodeikticus* (*M. lysodeikticus*).[6] Thus, the extensive inhibition on which the homogeneous enzyme immunoassay depends,[6] is believed to be due to steric hindrance of substrate access by antibody bound close to the active site cleft. No other enzyme conjugate has been reported as having the same degree of inhibition in the presence of an anti-hapten antibody. The inhibition of the HEWL-E1G and HEWL-PdG conjugate lytic activity is prevented in a dose-dependent manner by pre-incubation of the antibody with the appropriate free hapten from either standards or from urine samples.[1] Thus, there is a direct relationship between the lytic activity of the assay system and the steroid glucuronide concentration in a sample. The Monitor utilises an endpoint assay for measurement of E1G which is calculated from the difference in transmission values (ΔT) taken 20 minutes apart[1–3,5,7] as the suspension of *M. lysodeikticus* clears during the lytic reaction. From a simple measurement of ΔT over 20 minutes, the accurate E1G excretion rate[1] can be obtained by reference to the appropriate standard curve.

The first rise in the urinary excretion rate of the estradiol metabolite E1G above an early follicular phase baseline can be used as biochemical proof that a dominant follicle is established and, hence, ovulation is imminent. Consequently, the Monitor assay is an important tool for measurement of this biomarker for the beginning of the potentially fertile period of the menstrual cycle.[8] To achieve the sensitivity required to measure, accurately, the first rise in E1G excretion rates (50–100 nmoles/24 hr), the concentration of the lysozyme conjugate used in the Monitor assay must be kept low,[2] typically being about 21 nM,[7] depending on the specific activity of the conjugate. Because of the relatively low specific activity of HEWL, this leads to the relatively long assay time of 20 minutes for the E1G test.[1] A reduction in the assay time for the E1G assay would improve this accurate and useful fertility detection system[1] so that decisions concerning fertility status can be made rapidly, either in the home or in a clinical setting. One obvious method of decreasing the assay time would be to choose another lysozyme with a higher specific activity than HEWL.

Although the initial rates for lysis of *M. lysodeikticus* catalysed by goose egg white lysozyme (GEWL), turkey egg white lysozyme (TEWL), phage T4

lysozyme (T4L) and human milk lysozyme (HuL) have all been recorded previously,[9–12] very few details are available for the kinetics of the complete clearing curves produced by these enzymes under comparable conditions. The present paper reports a comparison of the lytic clearing of suspensions of *M. lysodeikticus* catalysed by HEWL, GEWL, TEWL, HuL and T4L as a first step in the development of a faster homogeneous enzyme immunoassay for the markers of the fertile period in women.

EXPERIMENTAL

Materials

HEWL (grade VI, 50,000 units/mg), TEWL (80,000 units/mg), and HuL (100,000–200,000 units/mg) were obtained from the Sigma Chemical Co. (St. Louis, MO) and were used in kinetic assays without further purification. *M. lysodeikticus* was also obtained from the Sigma Chemical Co. (St. Louis, MO), as were the CM-sepharose and CM-superose resin. All other chemicals were of analytical grade, or higher, and all water for buffers was Milli-Q quality. The tris-maleate 1 M stock buffer (pH 7) was prepared as described elsewhere[7] and the 40 mM and 75 mM solutions were prepared by dilution.

Equipment

The Ovarian Monitor equipment, including meter, empty assay tubes, and multi-assay heating block, was supplied by St. Michael Natural Family Planning (NFP) Services Pty (Victoria, 3123, Australia). The FPLC equipment was from Pharmacia (Uppsala, Sweden). Enzymes and buffers for the plasmid digests and ligations were supplied by Bethesda Research Laboratories (BRL, MD, USA) and all restriction endonuclease digestion of DNA samples was in accordance with their instructions. All other molecular biology techniques were carried out using standard methodology.[13]

Goose Egg White Lysozyme Purification

GEWL was prepared essentially as described by Canfield and McMurray.[9] Freeze-dried goose egg white (10 g) was mixed with approximately 500 mL of 50 mM ammonium acetate buffer (pH 8.0) and left overnight. The solution was then centrifuged at 15,000 G for 15 minutes and the supernatant adjusted to pH 8.0 before addition to pre-equilibrated CM-Sepharose resin (30 g wet weight). The suspension was stirred at room temperature for 31/2–4 hours and the non-bound fraction removed by filtration before the cake was rinsed with 50 mM ammomium acetate buffer (pH 8.0). The washed resin was then

packed into a glass column (10 × 2 cm i.d.) using the same buffer and the crude GEWL material was eluted with a step of 1.0 M NaCl. After dialysis, the material was reloaded, at pH 9.0, onto a CM-Superose column (20 × 2.6 cm i.d.) attached to a Pharmacia FPLC system and eluted with a linear NaCl gradient (0–0.35 M over 70 minutes) in pH 9.0 ammonium acetate buffer (50 mM) at 2 mL/min. The activity peak coincided with the absorbance peak, and gel electrophoresis of the peak tube showed essentially a single band of protein upon silver staining.

Phage T4 Lysozyme Purification

The general strategy for transferring the T4L gene from the pHS1401e plasmid to the expression vector pHN1403 (both supplied by Brian Matthews[14]) involved cleaving both plasmids at the *Bam* H1 and *Hind* III sites and ligating the 630 bp fragment containing the T4L gene into the pHN1403 plasmid.[14] *E. coli* cells, strain RR1, bearing the T4L plasmid were cultured according to Poteete et al.[14] A clear solution was indicative of successful expression of the T4L gene by showing that cell lysis had occurred. The resulting lysates were centrifuged for 10 minutes at 5,000 G and the supernatant decanted and recentrifuged for 80 minutes at 39,000 G to remove cellular debris. The supernatants were then combined, diluted with water to give a conductivity of less than 4 mmho, and run through a CM-Sepharose column (2.5 × 10 cm i.d.) pre-equilibrated with 50 mM Tris-HCl, pH 7.25, and 1 mM sodium EDTA. Elution was effected using a linear gradient of 50–300 mM NaCl at 2 mL/min in the same buffer. One pass through the CM-Sepharose column resulted in a single protein peak for which the A_{280} and activity peaks coincided and gel electrophoresis of the peak fractions gave a single band upon silver staining.

Measurement of Lysis Rates

In the Ovarian Monitor, lysis rates were determined spectrophotometrically at 40°C by measuring the rate of change in transmission at 650 nm. The transmission values given by the Monitor readout are arbitrary units and will be referred to, henceforth, simply as transmission for convenience. If percentage transmission was required, it was obtained by expressing the arbitrary transmission value for a sample as a percentage of the corresponding value for a buffer only solution. To calculate the lytic rate, a transmission value (T_0) was taken at time zero and a final transmission value (T_{20}) was taken 20 minutes after the addition of the enzyme (Figure 1a). The end point lysis rate was defined as $\Delta T/20\ \text{min} = (T_{20} - T_0)$. The bacterial suspension was prepared fresh each day as follows. Lyophilised *M. lysodeikticus* (15 mg) was triturated in a glass homogeniser with 75 mM tris-maleate pH 7 buffer

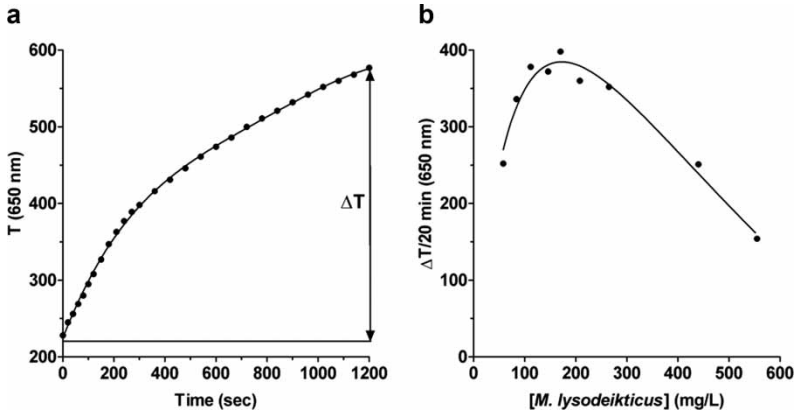

Figure 1. Panel a. The transmission clearing curve for HEWL-E1G conjugate E1 (21 nM). Data obtained on the Ovarian Monitor at a *M. lysodeikticus* substrate concentration of 214 mg/L; Panel b. The relationship between the 20 minute transmission change and the initial substrate concentration as measured on the Ovarian Monitor at a HEWL-E1G conjugate E1 concentration of 21 nM.

(2 mL) and sonicated for 5 minutes to ensure homogeneity. Reactions were initiated by adding 10 μL of the bacterial suspension, via a stepper syringe, to a solution pre-equilibrated with the appropriate concentration of lysozyme in 40 mM pH 7 tris-maleate buffer to give a total volume of 350 μL and a final substrate concentration of 214 mg/L. Lysis rates measured with a Cary I Varian spectrophotometer were determined at 450 nm (unless specifically stated otherwise) using the same methodology, except the final assay volume was increased to 2 mL and the substrate stock was prepared with 45 mg suspended in 2 mL of buffer, which reduced the final concentration of substrate in the assay mixture to 113 mg/L. Initial rates were determined from the clearing curves by drawing a best fit line through the initial data points.

Analysis of Clearing Curves

For analysis of the clearing curves, the data were fitted to a second order equation, as described by McKenzie and White[15] and Bernath and Vieth[16] after conversion of the transmission data into absorbance. For second order decay, a plot of A^0_λ/A^t_λ against time is linear with a slope of $k_2' A^0_\lambda$ where k_2' is a rate constant, A^0_λ is the initial absorbance at λ nm and A^t_λ is the absorbance at λ nm at time t.

$$A^0_\lambda/A^t_\lambda = k_2' A^0_\lambda t + 1$$

Thus, k_2' may be obtained by dividing the slope of the plot by A_λ^0. Although k_2' is expressed in s^{-1}, it is an empirical rate constant,[15] since it contains a proportionality constant between absorbance and mg/L, and the concentration of *M. lysodeikticus* cannot be expressed in molar terms. The true intrinsic rate constant for the enzyme (k_{cat}) is given by the relationship

$$k_{cat} = (k_2'/[E])(K_M/[S] + 1)$$

where K_M is the Michaelis Menten constant and [S] is the *M. lysodeikticus* concentration in mg/L. When $[S] \gg K_M$ the equation reduces to $k_{cat} = k_2'/[E]$. The Monitor readings, in transmission, can be converted into equivalent apparent absorbance readings by the relationship

$$A_\lambda^t = \log T_{buffer} - \log T_t$$

Michaelis Menten Parameters

The apparent Michaelis Menten parameters (K_M and V_{max}) were measured for HEWL, TEWL, HuL, and T4L at 450 nm using a Cary spectrophotometer from the initial rates of absorbance decrease. For T4L, HuL, and TEWL, the concentrations of enzyme in the final reaction mixture which gave a reasonable change in absorbance in 5 minutes, were 7 nM, 7 nM, and 35 nM, respectively. For HEWL, four different enzyme concentrations were used (7, 35, 70, and 105 nM). The bacterial substrate concentrations were varied from 6.3 to 200 mg/L and all assays were performed in triplicate and agreed to within 5%. The Michaelis-Menten parameters were calculated from the initial rate data using the data analysis program PRISM (Graphpad™).

RESULTS

Clearing of *Micrococcus lysodeikticus* Suspensions by a HEWL-E1G Conjugate

Figure 1a shows the clearing curve obtained with the Ovarian Monitor of a suspension of *M. lysodeikticus* (214 mg/L), catalysed by a K13-monoacylated [5, 7] HEWL-E1G conjugate (E1) at an assay concentration of 21 nM. The initial transmission value (T_0) of the suspension was 225 and the transmission change at 20 minutes was 349 ($\Delta T/20$ min). T_0 depends on the initial concentration of the substrate, being lower the higher the substrate concentration (S) but, when $\Delta T/20$ min was measured as a function of the initial S value at a fixed E1 conjugate concentration of 21 nM, it passed through a broad maximum between substrate concentrations of 120 and 200 mg/L and then

declined at S values > 200 mg/L (Figure 1b). At a fixed E1 concentration (21 nM), the amount of substrate lysed over 20 minutes (ΔS), as a function of initial substrate concentration, was calculated from the ΔT/20 min values by reference to the Beer's law curve. The relationship was essentially linear up to 200 mg/L, but was non-linear thereafter (Figure 2a). When ΔT/20 min was determined over a range of different E1 concentrations and a fixed substrate concentration (214 mg/L), the relationship was approximately linear up to an E1 concentration of 8–10 nM, but after this, the relationship was clearly non-linear (Figure 2b).

In the immunoassay, an amount of anti-E1G antibody sufficient to inhibit the control rate of lysis for E1 (21 nM) by 90% in the absence of standard was determined by titration. This amount of antibody was added to each assay tube, giving a typical sigmoidal standard curve (Figure 3a), relating ΔT/20 min to the E1G concentration. The working range of this curve was 30 to 250 nmol/24 hr and the use of this standard curve to give E1G excretion rates during a menstrual cycle has been reported elsewhere.[7] The shape of the standard curve was similar if the data were converted from transmission into absorbance first, since the relationship between ΔAbs/20 min and [E1] was non-linear also, and the clearing curve was similar to Figure 1a (but the mirror image). Equally, if the standard curve was expressed in terms of the amount of substrate lysed over 20 minutes, an almost identical shape to Figure 3a was obtained. Although the clearing curve obtained with the E1 conjugate was

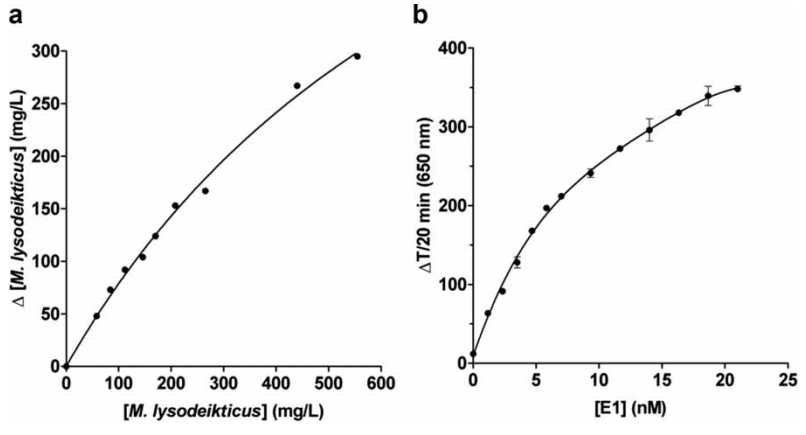

Figure 2. Panel a. The relationship between the amount of substrate lysed and the initial substrate concentration as measured on the Ovarian Monitor for HEWL-E1G conjugate E1 (21 nM); Panel b. The relationship between the 20 minute transmission change and the concentration of HEWL-E1G conjugate E1 in the lytic assay as measured on the Ovarian Monitor at a *M. lysodeikticus* substrate concentration of 214 mg/L.

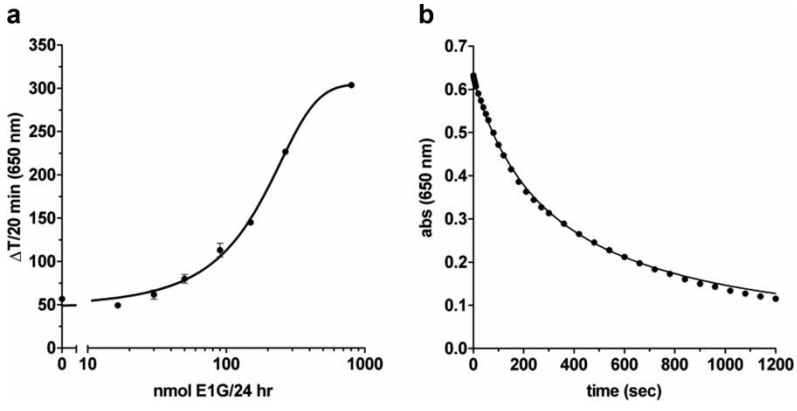

Figure 3. Panel a. A standard curve for E1G using HEWL-E1G conjugate E1 (21 nM) and *M. lysodeikticus* (214 mg/L) as measured on the Ovarian Monitor. The semi-logarithmic plot shows the relationship between rate of lysis and the concentration of E1G in the standard; Panel b. The HEWL (24 nM) transmission clearing curve after conversion to absorbance showing the fit to a second order process. Data obtained on the Ovarian Monitor at a *M. lysodeikticus* concentration of 113 mg/L.

non-linear (Figure 1a), when the transmission data were converted to absorbance and plotted according to the second order kinetic equation of McKenzie and White,[15] a straight line was obtained with a slope of $0.00337 \pm 0.00005\ s^{-1}$ ($r^2 = 0.996$) giving a k_2' value of $0.69 \times 10^{-2}\ s^{-1}$. The specific activity of the E1 conjugate defined by this analysis is given in Table 1.

Control Clearing Curves with Native HEWL

Monophasic transmission clearing curves were also obtained with the Ovarian Monitor using native HEWL. The transmission data obtained by HEWL catalysed lysis (24 nM), after conversion to absorbance (Figure 3b), also followed second order kinetics closely and the deviation was only 0.01 absorbance units from the true second order clearing curve at 20 minutes. The second order plot of the data was linear and when the slope of the plot was divided by A_λ^0 a k_2' value of $0.63 \times 10^{-2}\ s^{-1}$ was obtained thus the specific activity of HEWL was similar to that obtained for E1 (Table 1). A plot of k_2' versus HEWL concentration was linear over the range 0–24 nM. When the HEWL concentration was increased from 24 nM to 35 nM, to act as a Monitor control for comparison, with the lytic behaviour of GEWL, k_2' increased by 78% (Table 1).

Since we were interested in relative clearing rates compared with HEWL as the control, for convenience and comparison with literature data,[15,17,18]

Table 1. Summary of rate data

Lysozyme	[Enzyme] (nM)	[S] (mg L^{-1})	λ (nm)[b]	Fast phase 10^2 ($k'_2 s^{-1}$)	SA[a] ($\times 10^5$)
HEWL	24	214	650	0.63	2.6
E1[c]	21	214	650	0.69	3.3
HEWL	35	214	650	1.12	3.2
GEWL	35	214	650	5.50	15.7
HEWL	7	113	450	0.16	2.3
HEWL	21	113	450	0.68	3.2
HEWL	24	113	450	0.75	3.1
HEWL	63	113	450	2.60	4.1
T4L	7	113	450	–	6.7[d]
HuL	21	113	450	2.80	13.3
TEWL	63	113	450	3.40	5.4

[a]Calculated as $10^2\ k'_2 s^{-1}/[E]$.

[b]650 nm data obtained with the Ovarian Monitor, 450 nm obtained with Cary Spectrophotometer.

[c]HEWL-E1G conjugate substituted on K33.

[d]Determined from the relative initial rates.

the clearing curves with all other lysozymes were monitored in absorbance at 450 nm over 5 minutes using a Cary spectrophotometer. To achieve a decreased time for the lysis reaction, the concentration of HEWL was increased. At an HEWL concentration of 24 nM, the calculated initial absorbance of a control solution of *M. lysodeikticus* (214 mg/L) measured in transmission with the Ovarian Monitor at 650 nm (Figure 3b) was 0.67; this reduced to 0.12 in 20 minutes (18% of the initial value) after addition of the enzyme. In comparison, the initial absorbance measured with the Cary spectrophotometer at 450 nm and a reduced *M. lysodeikticus* suspension of 113 mg/L was 0.47. This was decreased over 5 minutes to 0.07 by addition of HEWL (63 nM), or to 15% of the initial absorbance. The empirical second order rate constants derived from the data for the two experiments are given in Table 1. For comparison with the more active enzymes (T4L and HuL), control clearing curves for HEWL were also determined at 7 nM and 21 nM, respectively, using a Cary spectrophotometer. The derived second order rate constants and specific activities are given in Table 1.

Lysis by Goose Egg White Lysozyme Measured with the Ovarian Monitor

When GEWL (35 nM) was substituted as the lytic catalyst in the Ovarian Monitor end-point assay, the *M. lysodeikticus* substrate was lysed in a

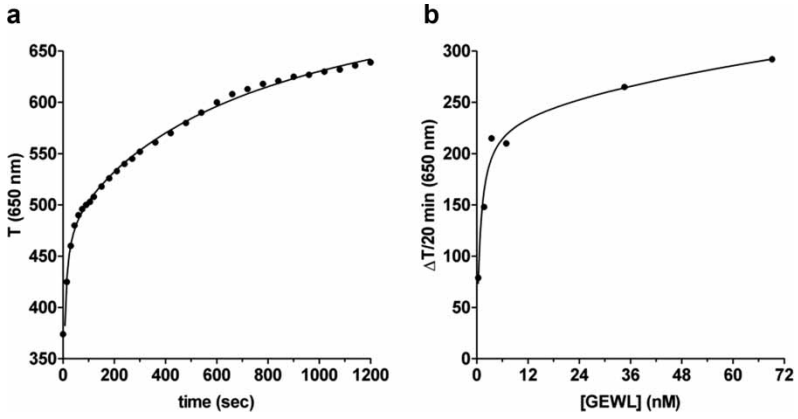

Figure 4. Panel a. The transmission clearing curve for GEWL (35 nM). Data obtained on the Ovarian Monitor at a *M. lysodeikticus* substrate concentration of 214 mg/L; Panel b. The relationship between the 20 minute transmission change and the concentration of GEWL in the lytic assay as measured on the Ovarian Monitor at a *M. lysodeikticus* substrate concentration of 214 mg/L.

markedly biphasic manner to give an end-point lysis rate ($\Delta T/20$ min) of 265 (Figure 4a). This may be compared with the higher lysis rate of 350 $\Delta T/20$ min for an HEWL concentration of only 24 nM. Doubling the GEWL concentration increased the lysis rate, but only by 10% to 292 $\Delta T/20$ min. At very low GEWL concentrations (<6 nM) the increase in $\Delta T/20$ min was almost linear with GEWL concentration. However, a pronounced levelling effect was obvious above 6 nM (Figure 4b) when the $\Delta T/20$ min value had reached about 200 and, thereafter, the increase in $\Delta T/20$ min with increasing GEWL concentration was small. When the data obtained at 35 nM GEWL concentration were converted into absorbance (Figure 5a), they could be fitted[15] to 2 consecutive second order processes. The amplitude of the faster process (which ended after 30 seconds) was 43% of the total change in 20 minutes (Figure 5a). The rate constant for the faster process (Table 1) was $5.5 \times 10^{-2}\ s^{-1}$, which gave a specific activity 4.9-fold greater than that of HEWL. The fast and slow rate constants were linear functions of the GEWL concentration up to the highest final assay concentration used, of 72 nM, and the fast phase was almost 5 times the magnitude of the slower process.

Lysis by Turkey Egg White Lysozyme

When the clearing curve in absorbance on the Cary spectrophotometer was determined for TEWL (63 nM) at an initial substrate concentration of

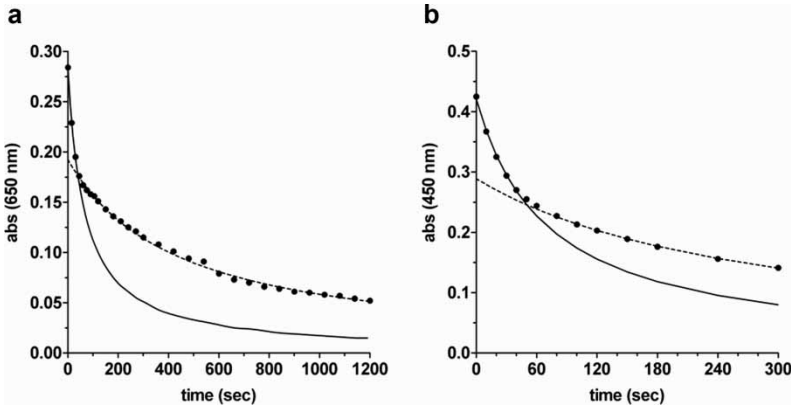

Figure 5. Panel a. The GEWL clearing curve (Figure 4a) after conversion to absorbance and fitting to two consecutive second order processes. Fast phase (——), slow phase (– – –); Panel b. The TEWL (63 nM) absorbance clearing curve fitted to two consecutive second order processes. Data obtained on the Cary spectrophotometer and at a *M. lysodeikticus* concentration of 113 mg/L. Fast phase (——), slow phase (– – –).

113 mg/L, it was also biphasic (Figure 5b), departing from simple second order behaviour after only 40 seconds, at which time the absorbance had been reduced by 48% from the initial value. Overall, TEWL had a decrease (ΔAbs) of only 0.285 in 5 minutes compared with 0.383 at the same concentration of HEWL (63 nM) over the same time period (Table 1). The clearing curve for TEWL fitted a single second order process for the first 40 seconds of clearing but, for a fit of the complete clearing curve, it was necessary again to assume two consecutive second order processes. The first of these gave an empirical second order rate constant of $3.4 \times 10^{-2}\,s^{-1}$ which gave a specific activity 1.31 times that of HEWL (Table 1). The second phase fitted from 80 seconds to the end of the full 5 minute clearing curve and gave a ratio of the rate constants for the fast and slow processes of 2.8.

Lysis by Human Milk Lysozyme

Figure 6a shows a comparison of the rate of lysis measured by change in absorbance (ΔAbs) at 450 nm on the Cary spectrophotometer as a function of time obtained with HuL and HEWL at a substrate concentration of 113 mg/L and an enzyme concentration of 21 nM. At these concentrations, the initial rate of the clearing reaction catalysed by HuL was approximately twice as fast (0.262 ΔAbs/min) as that obtained with HEWL (0.124 ΔAbs/min). Again, the clearing curve for HuL was biphasic with the fast phase constituting 43% of the total clearing over 5 minutes and much

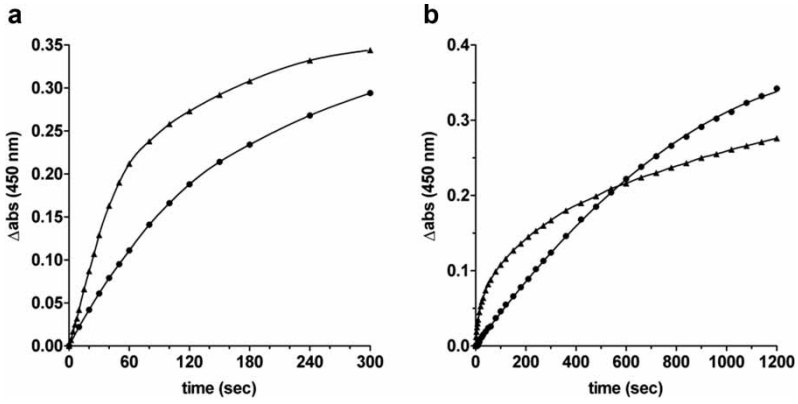

Figure 6. Panel a. Comparision of the HuL (▲) and HEWL (●) absorbance clearing curves. Data obtained on the Cary spectrophotometer and at a *M. lysodeikticus* concentration of 113 mg/L and an enzyme concentration of 21 nM; Panel b. Comparision of the T4L (▲) and HEWL (●) absorbance clearing curves. Data obtained on the Cary spectrophotometer and at a *M. lysodeikticus* concentration of 113 mg/L and an enzyme concentration of 7 nM.

of the initial advantage in lysis rate over HEWL was lost with ΔAbs/5 min being 0.344 for HuL and 0.294 for HEWL. The experimental HuL clearing curve was reproduced when each of the two phases was fitted to a second order rate equation as for GEWL and TEWL. The first phase followed second order kinetics for 60 seconds (Table 1) and had a k_2' value of $2.8 \times 10^{-2}\,s^{-1}$ at this enzyme concentration and a specific activity 4.11 times that of HEWL. The ratio of the k_2' values for the fast and slow phases was 3.1

Lysis by T4 Lysozyme

The rate of decrease of the change in absorbance of a solution of *M. lysodeikticus* catalysed by T4L (7 nM) was exceptionally fast but, with a rapidly declining rate. The clearing curve was multiphasic, having an initial rate of 0.190 ΔAbs/min (Figure 6b), which was approximately 7 times faster than that obtained with the same molar concentration of HEWL. However, over the 20 minute period of an end-point assay, the total absorbance change for T4L (ΔAbs 0.275) was low (Figure 6b), compared to that observed for HEWL (ΔAbs 0.342 units) over the same time period. The clearing curve could not be fitted to two second order processes, as the rate changed continuously over the 20 minute period. The continuously changing rate of lysis also meant that the specific activity could not be determined accurately.

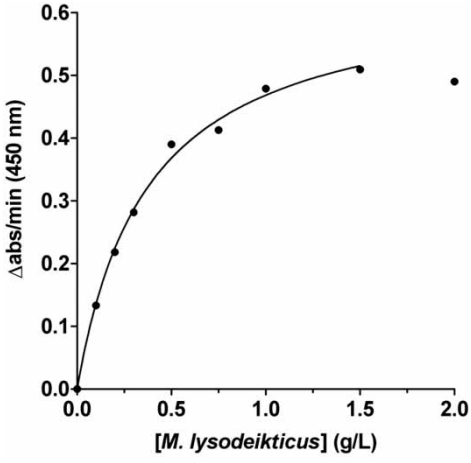

Figure 7. Dependence of initial rate on substrate concentration for HEWL (70 nM). Data obtained on the Cary spectrophotometer.

Michaelis Menten Parameters

Because T4L was found not to obey second order kinetics, the K_M for T4L could not be measured in terms of the second order fits. Thus, for convenience, the Michaelis Menten constants for the different enzymes were all calculated from absorbance initial rate data rather than with the second order rate constants. An example of the fitting of the initial rate data to the Michaelis Menten equation is given in Figure 7 for HEWL at an enzyme concentration of 70 nM. The lysis rate passed through a maximum and then decreased at the higher substrate concentrations, with a maximum value being observed at all enzyme concentrations and with all the different lysozymes examined including with the HEWL conjugates. Thus, the Michaelis Menten parameters were calculated for the range of substrate concentrations where the initial rate was still increasing with an increase in substrate concentration, as shown in Figure 7. A summary of the results obtained for the various lysozymes and for HEWL at several enzyme concentrations are shown in Table 2.

DISCUSSION

The Ovarian Monitor Lytic Assay

The Ovarian Monitor has proven to be a useful device[1–3] for the daily measurement of E1G and PdG excretion rates for monitoring fertility and has been validated independently against radioimmunoassay.[1] The Monitor measures the rate of lysis of a suspension of *M. lysodeikticus* by a lysozyme

Table 2. Michaelis menten parameters obtained for a variety of lysozymes and several Hen egg white lysozyme concentrations

Lysoyzme	[Enzyme] (nM)	K_M (mg L^{-1})	V_{max} (450 nm) (ΔAbs min^{-1})	Relative k_{cat} value (cf. HEWL)
TEWL	35	71	0.59	1.79
HuL	7	39	0.20	3.33
T4L	7	112	0.35	5.83
HEWL	7	15	0.06	—
HEWL	35	29	0.33	—
HEWL	70	38	0.65	—
HEWL	105	40	0.95	—

conjugate using an end-point assay in transmission (as shown in Figure 1a) but, in principle, the rate of lysis could also be measured in absorbance, by initial rates or by a second order fit to the absorbance data (Figure 3b). Despite the fact that the most rigorous procedure is the second order fit to the data,[15] in establishing the Monitor, a decision was taken to utilise the end-point assay in transmission. This allowed for simplification of Monitor design with reductions in cost. However, a consequence of this for the current Monitor assay is that, since a Beer's law plot in transmission is non-linear, the magnitude of the lytic rate ($\Delta T/20$ min) depends on the value chosen for the initial substrate concentration (Figure 1b) and the optics of the instrument. The larger $\Delta T/20$ min can be made, the greater the difference between the lysis rates for the free and antibody-bound enzyme and, hence, the more accurate the derived excretion rates (Figure 3a). However, although initially $\Delta T/20$ min increased as the substrate concentration increased (Figure 1b), once a value of 200 mg/L was exceeded, $\Delta T/20$ min decreased again. Thus, there was a practical upper limit to the substrate concentration that could be used. Experimentally, the optimum value was chosen as 214 mg/L, which gave a T_0 value of about 200 and a maximum value for $\Delta T/20$ min of about 350 at an enzyme concentration of 24 nM. Importantly, this transmission range corresponds to the region of greatest photometric accuracy for the optics of the Ovarian Monitor.

The homogeneous enzyme immunoassay works because the lytic activity of antibody-inhibited HEWL conjugates is very low ($<8\%$ of the control). A simple rate measurement, therefore, gives a measure of the concentration of free conjugate, and this is, in turn, determined by the amount of free E1G in the system. However, as is apparent from Figure 2b, above a free conjugate concentration of about 8 nM, $\Delta T/20$ min was not linearly related to the free conjugate concentration in the assay. The practical result of this is that the standard curve is most accurate (Figure 3a) at free conjugate concentrations below 8 nM in the assay. This corresponds to $\Delta T/20$ min values between 50 and 250; thus, the results are most accurate at E1G excretion rates below

300 nmol/24 h (Figure 3a). The range covered makes the Ovarian Monitor ideal for detecting the beginning of fertility, which can be defined physiologically as the selection of a dominant follicle and can be observed biochemically as an increase in E1G excretion rates from a baseline of 50–100 nmol/24 h.

As can be seen from the standard curve, as the E1G excretion rate increased from 300 nmol/24 h to 600 nmol/24 h, $\Delta T/20$ min changed only from 250 to 300. Over this range of E1G excretion rates, the amount of free conjugate increases from about 8 nM to approach the total conjugate concentration of around 20 nM (Figure 2b) with little apparent increase in the lytic rate. If accurate high E1G excretion rates are required (equivalent to $\Delta T/20$ min values >250), it is necessary to dilute such urine samples further to allow measurement in the most accurate range of the standard curve. No improvement in the accuracy of the standard curve at high excretion rates was obtained by converting the data into absorbance and calculating $\Delta Abs/20$ min, since this parameter was also a non-linear function of the E1 concentration. Equally, there was no extension of the accurate range of the standard curve by calculating $\Delta S/20$ min. Transmission for the Monitor therefore gives the simplest and most accurate procedure for measurement of the first E1G rises, as reported previously.[8]

The curvature of the HEWL-E1G conjugate (E1) generated clearing curve as a function of time is clearly an important factor in determining the accuracy of the Ovarian Monitor results. According to McKenzie and White,[15] the kinetics of the clearing reaction are second order; this is confirmed here (Figure 3b). Hence, any part of the clearing curve can be used to calculate the second order rate constants and the E1G concentration, and it is not essential to begin the measurement at time zero. For a different lysozyme to be a replacement for HEWL as a signal generator in an E1G homogeneous enzyme immunoassay, it must produce conjugates after acylation with E1G that are highly inhibitable by an anti-E1G antibody ($>90\%$). They must also have specific activities that are 3 to 4 times greater than HEWL conjugates under the same assay conditions using the end-point assay and, ideally, the clearing curves generated by such conjugates should remain second order as for HEWL and HEWL-E1G.

A three- to four-fold increase in specific activity is necessary to reduce the assay time for E1G measurement to about 5 minutes, which is near optimum for a home test. Unfortunately, it is not possible to merely increase the concentration of the current HEWL-E1G conjugate to obtain a faster rate of clearing and a shorter assay time. This strategy would have the concomitant effect of also increasing the amount of antibody that must be added to inhibit the lysis rate by 90%, or more which, in turn, would lead to a less sensitive homogeneous enzyme immunoassay.

Biphasic Clearing of *M. lysodeikticus* Catalysed by GEWL

Since the literature suggests that the initial rate of lysis of *M. lysodeikticus* catalysed by GEWL is greater than for HEWL,[19,20] a sample of native

GEWL was prepared from goose eggs and the clearing curve was examined. Although the GEWL initial rate of clearing was, indeed, faster than HEWL, the clearing curve was markedly biphasic (Figure 4a). Hence, the total change in 20 minutes was much less than expected on the basis of the first 30 seconds over the tested range (6–72 nM), with the initial fast rate finishing at approximately the same absolute $\Delta T/20$ min value, irrespective of the enzyme concentration. Once about 40% of the *M. lysodeikticus* cells had collapsed, there was a switch to the slower phase of lysis. Thus, the maximum ΔT value obtainable with GEWL was only 200–300 units in 20 minutes, even up to a GEWL concentration of 72 nM in the assay which was clearly inferior to the rate of lysis obtained with HEWL at 24 nM. This levelling effect (Figure 4b) renders GEWL unsatisfactory as a signal generator in an end-point assay.

On the other hand, the k_2' value for GEWL (35 nM) could give a potential reduction in assay time, since the specific activity calculated from it was nearly 5 times that of HEWL. However, at this concentration of GEWL, which was selected to give a reasonable $\Delta T/20$ min value, the fast initial rate was coupled with the very early turnover to a five-fold slower rate (Figure 5a) that made accurate analysis of the data difficult. If the GEWL concentration was lowered, for example, to 24 nM, the potential reduction in the Monitor assay time would be about four-fold, as required (based on a k_2' value of around $3 \times 10^{-2}\,s^{-1}$ extrapolated from the GEWL concentration dependence and compared with the value of $0.75 \times 10^{-2}\,s^{-1}$ for HEWL in Table 1). However, since there is no sequence homology and only poor structural homology between HEWL and GEWL,[21,22] the conjugation conditions and purification procedures already optimised for the conjugation of HEWL to steroid glucuronides would not necessarily apply.[3–5,7,23] Hence, much reworking of the conjugation and purification procedures would be expected; this was not considered justified by the present results. For this reason, no further work was carried out with GEWL.

Biphasic Clearing of *M. lysodeikticus* Suspensions Produced by Catalysis with TEWL, HuL and T4L

For these comparisons, the concentration of HEWL was increased to 63 nM to achieve the same amount of clearing as in the Monitor assay, but in 5 minutes. Also, the clearing curves were monitored at 450 nm rather than 650 nm, which enabled direct comparisons with the kinetic analysis of McKenzie and White.[15] The greater distance of the cuvette from the detector in the Cary spectrophotometer, relative to the Ovarian Monitor and the shorter wavelength of the measurements, resulted in an increase in the observed light scattering from the cellular substrate. Thus, the substrate concentration had to be reduced to 113 mg/L to remain in the region of greatest photometric accuracy. However, these changes had little discernible effect on the kinetics of lysis.

Second order kinetics fit both the absorbance clearing curve for HEWL at 450 nm (63 nM) and also the transformed Ovarian Monitor transmission data at 650 nm (24 nM). This shows that the equations and underlying assumptions apply at both wavelengths, on both instruments, and at both enzyme concentrations. The two clearing curves obeyed clean second order kinetics for a similar percentage of their initial absorbance values and gave similar, but not identical, values for the specific activity of HEWL (Table 1).

The absolute value of k_2' for clearing of a *M. lysodeikticus* suspension obtained on the Cary spectrophotometer with HEWL (63 nM) as the catalyst could be used as a control value for purposes of comparison of the different lysozymes on this instrument. However, in practice, it was preferable to vary the TEWL, HuL, and T4L concentrations to give a similar extent of clearing of the *M. lysodeikticus* suspension as obtained with HEWL over 5 minutes. A comparison of the different lysozymes was made always, therefore, with reference to an appropriate clearing curve for HEWL at comparable enzyme and substrate concentrations (see Table 1).

TEWL, HuL, and T4L all had specific activities greater than for HEWL for the first phase of the clearing reaction (Table 1), as expected. However, the fact that the clearing curves were again biphasic (Figures 5b, 6a and 6b) negated the greater specific activities if an end-point lytic assay was used utilising either absorbance or transmission. Only in the case of HuL (Figure 6a) did the ΔAbs/5 min value exceed 0.3 as obtained with the HEWL control rate of lysis (24 nM) and, then, only by 16%, despite the specific activity based on the fast phase of lysis being more than four-fold greater for HuL. Although the usual substrate for T4L is the bacterium *Escherichia coli* (*E. coli*), the kinetics of T4L were examined using *M. lysodeikticus* as a substrate, as the bacterium *E. coli* is a potential pathogen and, thus, cannot be utilised in any assay designed for home use. T4L had a relative initial rate (Table 1) 6.7-fold greater than that observed with the same molar amount of HEWL. However, despite this fast initial rate, T4L also failed to generate an advantage in ΔAbs even over 20minutes because of the multiphasic nature of the clearing curve (Figure 6b) giving only about 50% of the change caused by HEWL at a concentration of 7 nM. The concentration of T4L had to be reduced to 7 nM so that the initial rate could be measured, but then the later stages of clearing were so slow that the reaction had to be monitored over 20 minutes rather than 5. Thus, despite the greater specific activities based on data obtained from the first phase of the clearing reaction, none of these lysozymes can be used to replace HEWL in the Monitor if an end-point assay is to be utilised.

Biphasic Clearing of *M. lysodeikticus*

The change in light scattering of a suspension of *M. lysodeikticus* is used as a simple measure of the free enzyme concentration and, hence, the

concentration of E1G. However, the macroscopic changes resulting in light scattering are only indirectly related to the molecular events that take place at the cleavage sites on the cell wall, as it is possible in hypertonic solutions,[24] to have enzymatic cleavage without cell collapse and clearing. It is also important to note that, once a cell wall has collapsed, further enzyme activity will have no effect on the observed clearing. Thus, the relationship between catalysis and light scattering is not a simple one. The cleavage sites are believed to be heterogeneous and the *M. lysodeikticus* cell wall is believed to contain four different classes of NAM-NAG linkages,[20,25–27] which differ in the nature of the peptidoglycan side chain and on the degree of cross-linking.[20] These have been designated A, B, C, and D by Matsumura and Kirsch.[20]

It is known that electrostatic effects between the positively charged lysozyme molecule and the negatively charged cell wall are very important in controlling the rate of lysis and binding to the cell wall, but the precise details of the interactions are not known.[28] The ratio of negative charge in the subcategories is 1:2:3:4 for A:B:C:D; therefore, it would be expected that a positively charged lysozyme molecule would bind preferentially at subsite D, but pH and ionic strength effects are important factors as well.[29] Also unknown is how many acts of cleavage are necessary before rupture of the cell occurs, the effects of spatial relationships between the cleaved sites on the surface of the cell (clustered or dispersed), and the minimum number of lysozyme molecules which must bind per cell. Thus, the fact that HEWL catalyses lysis in a clean second order manner, but GEWL, TEWL, and HuL catalyse lysis in a biphasic manner (with two second order processes) in the present work is difficult to explain simply in terms of mechanistic principles at a molecular level.

It is known that all chicken type lysozymes have an aspartic acid residue at position 52 and, in the classical and widely accepted Vernon-Phillips mechanism[9,30] published in 1967, it is proposed that this residue stabilises an oxocarbenium ion intermediate. Since then, Strynadka and James[31] have proposed a second role for D52. A crystal structure of HEWL co-crystallised with NAM-NAG-NAM shows that D52 interacts with the active site NAM residue to help strain the pyranose ring into a more active half chair conformation. Biphasic clearing by GEWL, which lacks an equivalent residue to D52, has been explained, therefore, in terms of a need for extra stabilisation in the transition state in the absence of D52.[20] According to the substrate assistance model, the extra stabilisation is provided by interactions with a carboxylate group in the peptidoglycan network. Such substrate assistance was proposed as being maximal for subclass B, partly on the basis that the amplitude of the fast phase of lysis, or burst, is 2.7% of the total absorbance change which is consistent with the level of 5% calculated for the NAM-NAG subclass B.[20] Another factor is that subclass B did not suffer from restriction of conformational mobility as did sub classes C and D which were cross-linked, and models showed that the carboxylate group in

subclass B could be situated in the active site in place of D52. This explanation seemed to be confirmed by mutation of D52 into A52 in HEWL, in which case, biphasic clearing behaviour was also observed with an amplitude of 2.7% relative to the wildtype.[20] The substrate-assistance model[20] cannot be the full explanation of the current results, however, since the clearing of *M. lysodeikticus* catalysed by TEWL, HuL, and T4L, which all bear an equivalent of D52, was also markedly biphasic and with considerably greater amplitudes for the first phase (40–50% of the measured absorbance change) than reported by Matsumura and Kirsch.[20]

The greater amplitude observed in the present work cannot be explained by a model in which there is 40–50% of a hyper-labile class of linkages which is hydrolysed first, if the linkage heterogeneity is the same, or nearly so, for every cell. If sufficient bonds are cleaved by enzymatic action at the hyper-labile set of linkages to cause collapse of the cell, no further kinetic behaviour can be observed. Thus, a single phase of clearing would be expected, which is contrary to experiment, except for HEWL. To explain the results observed with GEWL, TEWL, and HuL, it is logical to assume that two kinetically distinct populations of cell-lysozyme assemblies must be present. These two populations must undergo lysis at different overall rates. If the *M. lysodiekticus* cells are basically identical, then this implies an unknown mechanism for producing the distinct kinetic populations in an environment where there are about 100,000 molecules per *M. lysodeikticus* cell (unpublished results) under the optimum kinetic conditions used here. If the individuals in the population of cells are heterogeneous in the distribution of their peptidoglycan linkages, then the biphasic clearing is easier to understand. However, since the two major classes of NAM linkage are those containing no peptide on the NAM-NAG polymer (50%) and those containing a bridged peptidoglycan network (30%) to account for the present results, it is necessary to assume that HEWL is unusual in that it does not differentiate between them catalytically. With the current state of knowledge, there is no obvious structural reason why this should be so. It was intriguing that GEWL, TEWL, and HuL all showed a change in the rate of the clearing reaction after approximately the same extent of lysis (40–50%) had occurred and, also, that the degree of biphasicity (rate constant of fast phase/rate constant for slow phase) correlated with the specific activity calculated from the fast phase of the clearing reaction. The greater the specific activity, the greater was the levelling effect of the biphasic clearing curve. It has been suggested by Dao-Pin et al.[32] that electrostatic interactions between the negatively charged cell wall[28] and the positively charged lysozyme molecule play an important role in the enzyme mechanism. For example, it was suggested that a higher potential difference between the positive and negative lobes of the lysozymes across their active site clefts enhanced the rate of catalysis. However, the order of the specific activities for HEWL, HuL, and T4L (Table 1), determined by the k_2' values, was not the same as the order of the electrostatic fields. On the other hand,

the specific activities for the different species of lysozyme do correlate with the total charge on the lysozyme molecule, as calculated using the Delphi model described by Dao-Pin et al.,[32] suggesting that it is the total charge which is the important determinant of k_2' specific activities.

Apparent K_M Value

Despite the levelling effect on the end-point rates for all of the alternative lysozymes, caused by their biphasic clearing curves, the possibility still exists of using one or other of these enzymes to give a shorter assay time. This could be achieved either by fitting the data to a second order equation or simply by calculating the initial rate. Before a decision on whether it was worthwhile developing procedures for production and characterisation of new lysozyme-steroid conjugate signal generators, it was decided to determine the steady state parameters for each lysozyme. During lysis, the substrate concentration is decreasing and, if the condition $[S] > K_M$ is not maintained, the term $(K_M/[S] + 1)$ will change throughout the standard curve in a non-linear fashion. Hence, for lysozyme catalysed homogeneous enzyme immunoassays, it is desirable to use enzymes with K_M values < 20 mg/L. The Michaelis Menten constant (K_M) is a kinetic parameter that is expressed in terms of substrate concentration (e.g., mg *M. lysodeikticus* cells/L).[17] It has been observed that the sensitivity of the commercial preparations of *M. lysodeikticus* cell walls to lysis has increased over the years and also differs for different suppliers.[17] Hence, the values for K_M reported in the literature for any lysozyme will vary depending on the batch of *M. lysodeikticus* used by the researcher and will generally decrease with time for the more recent batches. This is consistent with our finding where the range of K_M values (15–112 mg/L) obtained with a *M. lysodeikticus* batch purchased in the late eighties was lower (Table 2) than that reported in 1968 by Locquet et al.[33] (range 100–400 mg/L). However, our range (Table 2) encompasses the K_M value of 67 mg/L reported in 1987 by Muraki et al.[34] for HuL (enzyme concentration 69 nM) and the value of 24 mg/L for HEWL at an enzyme concentration of 10 nM reported in 1996 by Matsumura and Kirsch.[20] For meaningful comparisons of V_{max} and K_M, different lysozymes must be examined with the same batch of *M. lysodeikticus* and under the same conditions. This comparison of our lysozymes of interest is not available in the literature.

The surprising result was that the apparent K_M value for HEWL increased with increasing enzyme concentration, which is in apparent contradiction to one of the basic tenets of enzyme kinetics. It is usually assumed that the K_M value is independent of the enzyme concentration. For this tenet to be valid for a lysozyme catalysed clearing reaction, it would require the clearing to be the result of a 1:1 lysozyme:*M. lysodeikticus* complex, which is unlikely to be the case. The *M. lysodeikticus* molecule possesses a large number of cleavage sites for lysozyme[28] and, presumably, there is an

optimum number of sites that must be occupied and cleaved to give the maximum rate of lysis. This would be expected to depend on the ratio of the total enzyme concentration to the total substrate concentration. Clearly, there may be a limit beyond which any increase in the number of enzyme molecules bound per cell has no further observed lytic effect. On the other hand, addition of extra substrate, at a current optimum ratio, would lead to a decrease in lysis rates, as observed experimentally, as the number of functional lysozyme molecules per cell is reduced below the optimum ratio. Such an observation could be interpreted as substrate inhibition. A reduction in lytic rate at high substrate concentrations, which has been interpreted as substrate inhibition, has been reported previously.[29] This decline in lytic activity at high substrate concentrations depends on pH and ionic strength[29] and has been explained in terms of electrostatic interactions[35] with equations being derived to fit the data.[29] However, it should be noted that the K_M results were obtained with a single enzyme concentration. The magnitude of the derived K_M value agreed with the present results. However, substrate inhibition with *M. lysodeikticus* as the substrate appears unlikely because of its comparatively large size compared to the enzyme, as two substrate molecules will be precluded from simultaneous binding to HEWL. The apparent conformity of lysozyme-catalysed lysis of *M. lysodeikticus* to Michaelis Menten behaviour may be artefactual in the sense that the apparent hyperbolic relationship could be the net result of two effects. A maximum substrate concentration will be reached for each enzyme concentration where further increases are associated with a decrease in the observed rate of lysis, and the substrate concentration producing a maximum rate of lysis will increase as the enzyme concentration is increased. Hence, the apparent K_M value will also appear to increase as a function of enzyme concentration, since K_M is just the substrate concentration that gives half the maximal rate.

CONCLUSIONS

The apparent K_M values for HEWL were such that, at the *M. lysodeikticus* concentration of 214 mg/L used in the Ovarian Monitor, the lysis reaction was proceeding near its V_{max} value. Thus, the assay is not very sensitive to small variations in the initial substrate concentration, which is an advantage for obtaining accurate data.[1] The same situation is likely to apply for HuL (Table 2) although, at an assay concentration of 21 nM, the K_M value would be expected to increase above the value determined at 7 nM ($K_M = 39$ mg/L). On the other hand, the K_M values obtained for T4L and TEWL were both large enough to make the relative rates of lysis for T4L and TEWL dependent on the initial substrate concentration used and on its decrease during lysis (Table 2). The fact that the K_M value was high, combined with the difficulty in measuring initial rates (Figure 6b), rules T4L out as a candidate for use as a signal generator in immunoassays. Also,

the structural similarity of T4L with HEWL is relatively low and it would, therefore, also require much re-working of the reported conjugation and purification procedures.[4,5,7,23] The small increase in lytic rate obtained with TEWL (Table 2) was insufficient to justify the work required to prepare and test TEWL-E1G conjugates for use as signal generators, even though it has high structural similarity with HEWL.

The specific activity advantage of HuL offers a significant potential reduction in assay time to about 5 minutes or less, as desired. Its K_M value is low enough to ensure maximum lysis rates throughout most of the clearing reaction at 214 mg/L substrate, the biphasicity is moderate, the amplitude and duration of the fast phase is sufficient (Table 2) to give an accurate reading of initial rates and its close structural similarity with HEWL[36] potentially simplifies the conjugation procedures. Therefore, HuL is an attractive possibility for E1G conjugate formation as a signal generator in homogeneous enzyme immunoassays for E1G if the lysis rate is determined from the first phase of the clearing reaction. The production and testing of HuL-E1G conjugates in homogeneous enzyme immunoassays for E1G is the subject of a second paper.

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