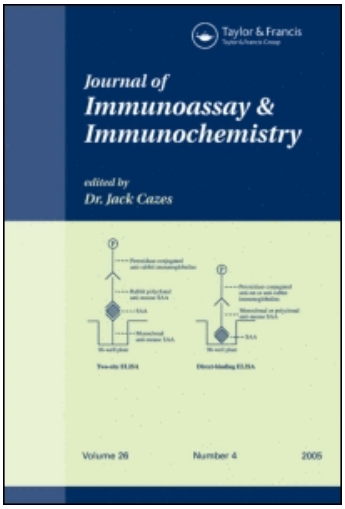

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Use of Defined Estrone Glucuronide–Hen Egg White Lysozyme Conjugates as Signal Generators in Homogeneous Enzyme Immunoassays for Urinary Estrone Glucuronide

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ABSTRACT

Three structurally characterized estrone glucuronide–lysozyme conjugates, E1 (a 60:40 mixture acylated at K3 and K97), E3 (acylated at K33), and E5 (acylated at both K33 and K97) were isolated and purified using a combination of cation-exchange chromatography on *S*-sepaharose in 7 M urea and hydrophobic interaction chromatography on butyl sepharose. Urea was essential to separate

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the conjugates into six chromatographically homogeneous fractions. In the absence of urea, complex mixtures of lysozyme and the six conjugate fractions were always encountered. The E1, E3, and E5 conjugates were highly inhibited by a sheep polyclonal anti-estrone glucuronide antibody only after the hydrophobic interaction chromatography step. The high level of inhibition enabled all three conjugates to be utilized as signal generators in homogenous enzyme immunoassays for urinary estrone glucuronide. Despite the apparently higher affinity of E3 for the antibody, both E1 and E3 gave standard curves that were indistinguishable provided that 1.7-fold more antiserum was used for E1. Both E1 and E3 yielded menstrual cycle urinary data that agreed with that provided by the Ovarian Monitor pre-coated assay tubes. Although, the menstrual cycle pattern was similar for the three signal generators, the E1G excretion rates yielded by E5 as the signal generator were only 60% of the reference values. Despite structural differences, there was no advantage gained in separating E1 and E3, but higher substituted conjugates such as E5 need removal for best assay performance.

Key Words: Estrone glucuronide; Hen egg white lysozyme; Urinary estrone glucuronide; Activity assay; Purification.

INTRODUCTION

The Ovarian Monitor assay for identifying the fertile period of the human menstrual cycle[1–5] is a classic example of an homogeneous enzyme immunoassay (HEIA).[6] The basis of the assay is that the lytic activity of hen egg white lysozyme (HEWL)–estrone glucuronide (E1G) and pregnanediol glucuronide (PdG) conjugates, which are used as signal generators in these assays, is extensively inhibited (over 95%) by binding to the appropriate anti-hapten antibody. This binding presumably causes inhibition by physically blocking access of the large bacterial substrate to the active site cleft.[2,7,8] The inhibition can be prevented in the presence of free hapten with the level of protection being determined by the hapten concentration; hence, a simple rate measurement allows determination of the hapten concentration. Since the bound signal generator has a very low rate of lysis, no separation of bound from free signal generator is required and all the essential steps can be carried out in a single assay tube as in the Ovarian Monitor. The extensive inhibition of lysozyme conjugates in the presence of an appropriate anti-hapten antibody was first exploited by Rubenstein et al.[6] for the measurement of morphine and has the potential to be used to assay for any hapten of low molecular

weight. A potential problem for such assays is that any free lysozyme left in the HEWL–hapten conjugate samples will show lytic activity which is not subject to inhibition by the anti-hapten antibodies. This factor reduces the assay sensitivity[8]; thus, one of the most important considerations in the preparation of a signal generator for use in a HEIA is the removal of unreacted native enzyme from the hapten-labelled enzyme conjugate. It is also thought that structural aspects of the signal generator can influence the performance of an immunoassay.[9] For example, the location of the hapten on the enzyme surface, and the length and chemical nature of any linker combine to determine the strength of the interaction between the signal generator and the anti-hapten antibody which in turn determines the sensitivity of the immunoassay standard curve.

To investigate the importance of these factors in assay development, it is necessary to purify the conjugate fraction and separate it into characterized populations. Efficient purification procedures for separating different enzyme conjugate families from a complex conjugation mixture are not generally available and, hence, these factors have not received much detailed study. Although affinity chromatography can be used to separate conjugated and unreacted native enzyme fractions, it is not usually possible to separate the different isomers in this way. Lysozyme, however, is unique in that the different conjugate families can be analyzed by cation-exchange chromatography in 7 M urea,[8] simply on the basis of charge differences. These charge differences also may be utilized in their isolation. We now report the details of a large scale purification procedure for three structurally characterized hen egg white lysozyme–estrone glucuronide conjugates and their behavior as signal generators in homogeneous enzyme immunoassays for estrone glucuronide.[2]

EXPERIMENTAL

Apparatus

All protein chromatography was performed at room temperature with a Pharmacia Fast Protein Liquid Chromatography (FPLC) system as described previously.[8] Large scale cation exchange separations were performed using either an *S*-sepharose fast flow or CM-sepharose fast flow resin packed into an HR 16/5 column (500×16 mm i.d.). Hydrophobic interaction chromatography was performed using a butyl sepharose resin packed into a 16/15 column

(150 × 16 mm i.d.), or with a Pharmacia Alkyl Superose (5/5) column (50 × 5 mm i.d.).

Samples for electrospray mass spectrometry analyses were desalted and introduced into the electrospray source by reverse phase high pressure liquid chromatography (HPLC) using a Hewlett Packard 1100 series HPLC and a Jupiter C4 column (50 × 2 mm i.d.).

Mass spectra were recorded in the positive ion mode on a Finnigan MAT LCQ ion trap mass spectrometer. The Ovarian Monitor equipment, including meter, pre-coated assay tubes, empty assay tubes, multi-assay heating block, and reference standard curves for the pre-coated tubes were supplied by St. Michael Natural Family Planning (NFP) Services Pty (2/380 Riversdale Road, East Hawthorn, Victoria, 3123, Australia).

All curve fitting was performed using the PRISM data analysis and graphing program Graphpad™ Prism.

Chemicals and Reagents

17-Oxoestra-1,3,5(10)-triene-3-yl-β-D-glucopyranosiduronic acid (estrone glucuronide or E1G) was synthesized essentially according to Conrow and Bernstein.[10] Hen egg white lysozyme (grade VI, three times recrystallized, dialyzed, and lyophilized), and lyophilized *Micrococcus lysodeikticus* were obtained from the Sigma Chemical Company (St. Louis, MO, USA). All other chemicals were of analytical or reagent grade, and water was of Milli-Q quality.

All buffers and samples prepared for FPLC were prefiltered through 0.2 μm GVWP Millipore filters, and the Tris–maleate stock buffer (1 M) for the activity assays was prepared as described previously.[8] All polyclonal E1G antisera were raised in sheep by Dr. Keith Henderson, AgResearch, Wallaceville Animal Research Centre, Upper Hutt, New Zealand, using a thyroglobulin–E1G conjugate prepared by the active ester method.[11] The lysozyme conjugate, as used in the pre-coated E1G assay tubes, was a gift from Professor Emeritus James Brown in Melbourne. Conjugation of E1G to lysozyme using the *N*-hydroxysuccinimide/dicyclohexyl carbodiimide active ester coupling method,[12] was carried out essentially as described previously[8,13] using a molar ratio of E1G (4.6 mg, 10.4 μmoles) to lysozyme (100 mg, 6.99 μmoles) of 1.5:1. The reaction was terminated after 1 h by dialysis of the mixture against Milli-Q water (3 × 1 L).

KINETIC METHODS

Activity Assays

All assay reagents were prepared fresh daily. Conjugate was diluted with 0.35 M Tris–maleate buffer (pH 7.0) into a plastic vial and the polyclonal anti-E1G antiserum was diluted with 75 mM Tris–maleate buffer (pH 7.0) one part in 50 into a small glass vial. The assays were carried out in plastic Ovarian Monitor plastic assay tubes essentially as described previously.[8] For the inhibition studies, lysozyme conjugate was diluted such that a small aliquot (1–20 μL) gave a change in transmission (ΔT) at 650 nm of approximately 350 arbitrary units over 20 min for the standard activity assay.[8] The diluted antiserum (1–50 μL) and conjugate were added to opposite corners at the bottom of an empty Ovarian Monitor assay tube, the total volume in the assay tube made up to 100 μL with 40 mM Tris–maleate buffer (pH 7.0), and the resulting mixture shaken for a count of three. A further 240 μL of 40 mM Tris–maleate buffer was then added and the assay mixture was incubated for 5 min at 40°C. The assay was initiated by the addition of 10 μL of the bacterial suspension (15 mg 2 mL^{-1}) with vortexing (2–3 s) and the assay tube placed in the Ovarian Monitor. The first transmission value ($T1$) was recorded upon shutting the lid, followed by the second transmission ($T2$) value 5 or 20 min later, depending on the assay. The rate was recorded as the change in transmission ($\Delta T = T2 - T1$) at 650 nm over the selected time period. All assays were performed in duplicate. The data were corrected for the blank rate (i.e., spontaneous rate of lysis without enzyme).

Estrone Glucuronide Standard Curves

Estrone glucuronide (E1G(H) acid form) was dried under high vacuum for a minimum of 2 h before weighing and E1G standards then prepared by serial dilution from a stock solution (500,000 nmol E1G 24 h^{-1} or 138,889 nM) using 10 mM Tris–maleate buffer as the diluent. Two different sources of urine (one from an ovariectomized woman at The Royal Woman's Hospital, Melbourne, Australia, diluted to an excretion rate equivalent to 150 mL 24 h^{-1}, and the second from a young girl aged 24 months) were used as urine blanks (low in endogenous steroids) throughout the course of this work. All E1G cycle profiles were obtained using daily urine samples diluted to 150 mL h^{-1} of collection supplied by a 26 year-old woman over cycle days 2–29 of a 29 day cycle. For construction of the E1G standard curves, both the antiserum and

conjugate stock solutions were diluted such that the required amounts could be added in a volume between 1–10 μL. Assays in the presence of a urinary control were performed using the general protocol described above except that an aliquot of blank urine (50 μL) was added with the E1G standards. The final reaction volume was maintained at 350 μL by reducing the volume of assay buffer. Urine samples were assayed using the same general assay protocol, the only difference being that the E1G standards were replaced with a 50 μL aliquot of time diluted urine. Standard curves and antiserum titration curves were fitted to a Boltzman sigmoidal dose response curve with a variable slope. The reading of the unknowns from the standard curve was performed using the fitted standard curve calculation of unknown's option on the PRISM data analysis and graphing program. The fit of the titration curve data was used to calculate the inhibition midpoint of the different antisera (where the inhibition mid-point was defined as the volume required for 50% inhibition of conjugate activity for a given conjugate concentration).

Statistical Analyses

The E1G excretion rate data calculated for the same cycle using different methods were compared using the Deming Regression Model[14,15] as calculated by the Best Fit program written by Mr. Andrew Rowsall of the Professorial Unit, Royal Women's Hospital, Melbourne. This model is similar to the standard linear regression model, except that it places an equal weighting on the two sets of data, i.e., it recognises that variations between the two sets of data may be due to inaccuracies in both sets of data, not just the Y data set.

Analysis of Clearing Curves

For analysis of the clearing curves, the transmission data were converted to apparent A_{650} values and then fitted to a second order equation as described by McKenzie and White.[16] For a second order decay, a plot of $(A_{650})_0/(A_{650})_t$ against time is linear with a slope of $k'(A_{650})_0$. The true second order rate constant for the enzyme (k_{cat}) is given by the relationship $k_{cat} = (k'/[E])/(\mathrm{Km}/S + 1)$, where Km is the Michaelis constant and S is the *Micrococcus lysodeikticus* concentration. When $S \gg \mathrm{Km}$, the equation reduces to $k_{cat} = k'/[E]$.

RESULTS

Mono-S Cation Exchange Chromatography at pH 4.3 in the Absence of Urea

Figure 1 shows a typical mono-S trace obtained with the reaction mixture of an active ester conjugation of E1G to lysozyme (molar ratio of 1.5:1 steroid:enzyme). The last peak eluted (labelled L) is unreacted lysozyme while all of the earlier eluting peaks are conjugate peaks and are labelled E1–E6 with the conjugate peak closest to lysozyme designated E1.[8] The conjugate yield based on peak areas was 50% and the two largest conjugate fractions, E1 and E3, which were present in 52 and 24% yield, respectively, together comprised 76% of the total conjugate yield. E5 constituted 7% of the total conjugate yield. Aliquots of the dialyzed active ester conjugation supernatant were injected onto a mono-S column equilibrated with 50 mM phosphate buffer (pH 4.3) as described previously[8] and elution of the sample was effected with a two-step sodium chloride gradient (see Fig. 2A). One large broad peak was

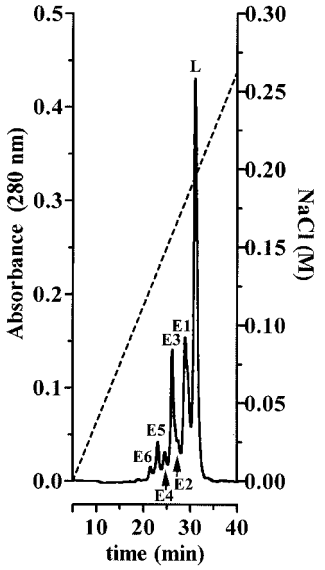

Figure 1. Mono-S FPLC trace of the reaction mixture from a conjugation of E1G with lysozyme (at a ratio of 1.5:1) in 7 M urea and pH 6.0 phosphate buffer. Lysozyme is marked as L and the conjugates in the reverse order of elution as E1–E6. The flow rate was 0.5 mL per minute.

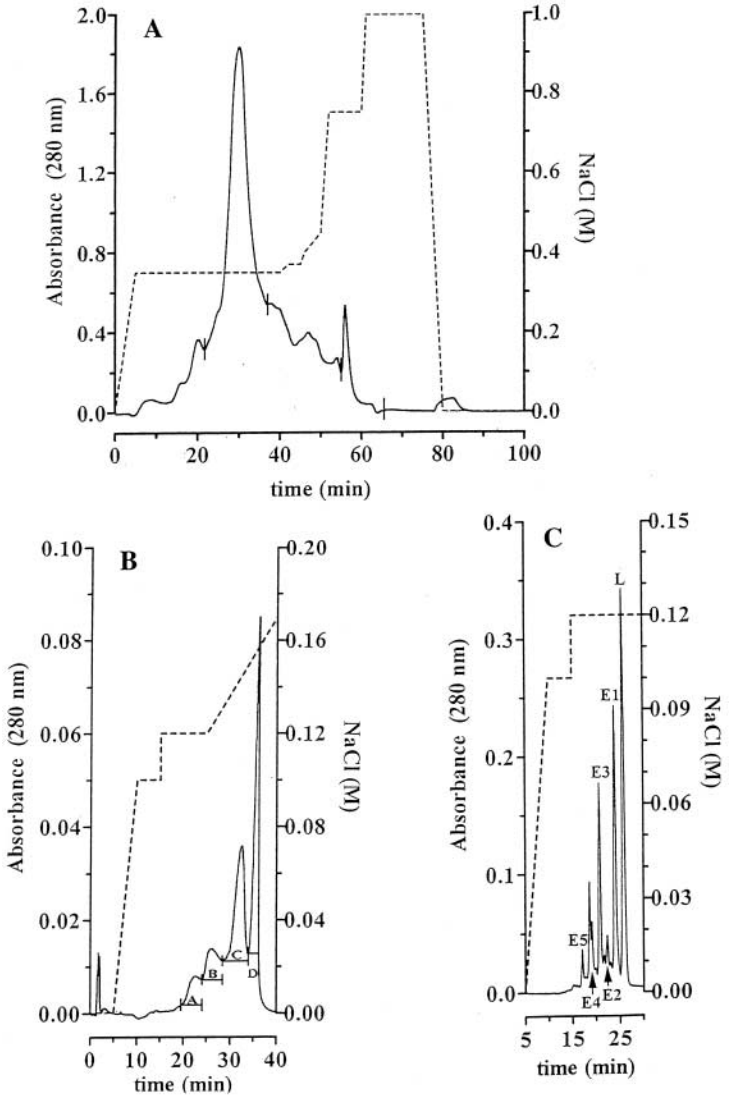

Figure 2. Panel A: The elution profile for the reaction mixture prepared from a 1.5:1 mixture of the active ester of E1G and lysozyme at pH 4.3 on a Pharmacia mono-S cation exchange column in the absence of urea. Panel B: The same reaction mixture on a Pharmacia mono-S cation exchange column at pH 9.5. Panel C: The same reaction mixture on a Pharmacia mono-S cation exchange column at pH 9.5 in 7 M urea. The nomenclature is as in Fig. 1. The gradients were individually optimized.

eluted at a sodium chloride concentration of 0.35 M followed by a broad band and a small sharp peak at the end as the gradient was stepped to 0.75 M salt. Analysis of the fractions using standard denaturing conditions on the analytical mono-S column[8,17] showed that the major peak consisted almost entirely of unreacted lysozyme while the late eluting band contained a mixture of conjugates (E1–E6) and trace amounts of lysozyme.

Mono-S Cation Exchange Chromatography at pH 9.5

Attempts were undertaken to purify the lysozyme conjugates in the absence of urea at pH 9.5 on the mono-S column using 50 mM glycine buffers (equilibrating buffer—50 mM glycine, pH 9.5; eluting buffer-50 mM glycine, 1 M NaCl, pH 9.5). Dialyzed supernatant from an active ester conjugation (prepared at a molar ratio of 1:1) was diluted 1/10 with equilibrating buffer, before loading and elution was effected using a combination of step and linear sodium chloride gradients. The profile (Fig. 2B) consisted of one large peak (D) preceded by three broader peaks; one moderately sized peak (C), and two smaller early eluting peaks (A and B). All four peaks were analyzed using the standard 7 M urea (pH 6.0) analytical mono-S column[8,17] and were shown to contain a mixture of lysozyme and conjugates (E1–E6) with the three earlier eluting peaks (peaks A, B, and C) consisting largely of conjugates and the late eluting peak (peak D), consisting mainly of lysozyme and E1. The same conjugate mixture was also run at pH 9.5 in 7 M urea buffers with a gradient optimized for maximum separation. The resulting FPLC profile was similar to that obtained under the standard analytical conditions (7.0 M urea, pH 6.0) (compare Fig. 2C with Fig. 1) but with better resolution. E1 was now a single sharp peak and a smaller peak (labelled E2) was well separated from E3, while the peaks labelled E4 and E5 now appeared as a split pair.

Two-Step Purification of E1G–Lysozyme Conjugate Families on *S*-Sepharose Fast Flow in 7 M Urea

The lysozyme conjugates (E1–E6) were successfully purified by a two-step chromatographic procedure. In the first step, the conjugation mixture was separated into the seven different conjugate peaks by cation exchange chromatography on an *S*-sepharose fast flow column. Figure 3A

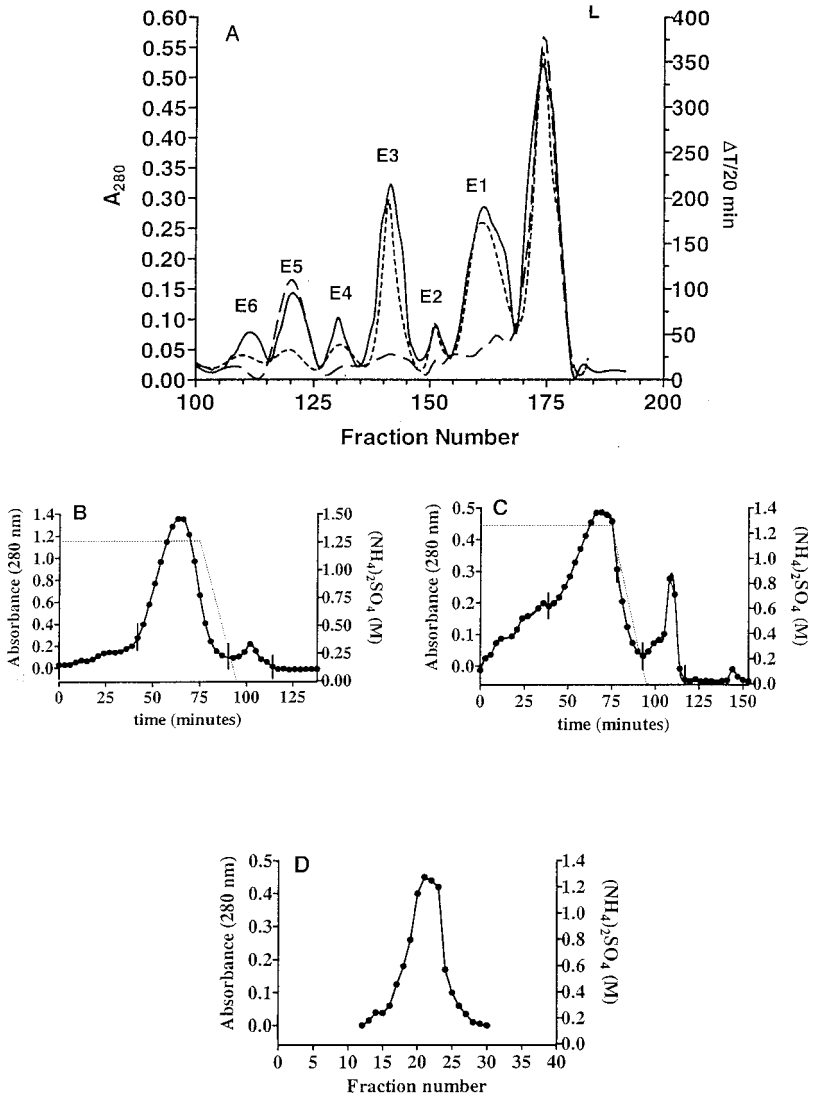

Figure 3. Panel A: The elution profile for the *S*-sepharose cation exchange column with the conjugation mixture from Fig. 1 in 50 mM pH 6 phosphate buffer in 7 M urea. The gradient was 0–0.21 M NaCl over 693 min at a flow rate of 1.5 mL per minute. The fraction size was 6 mL. Panel B: The butyl-sepharose column profile for peak E1. Panel C: The butyl-sepharose column profile for peak E3. Panel D: The butyl-sepharose column profile for peak E5. For Panels B–D the flow rate was 1.5 mL per minute and the fraction volume 4.5 mL.

shows the resulting elution profile. The total protein recovery was 88% and the three main conjugate peaks were E1, E3, and E5 (see Table 1). All of the conjugates had lytic activities that correlated with the absorbance profiles and all were inhibited (ca. 60%) when excess anti-E1G antiserum was present in the assay (Table 1). The main peak (L) was unaffected by the antiserum, and, from the absorbance and activity profiles, it was evident that E1 and E3 had specific activities similar to that of native lysozyme (Table 1). E5, on the other hand, had only 58% of the specific activity of lysozyme.

The second step in the purification of the separated conjugates (E1, E3, and E5) involved a single passage through a hydrophobic interaction column. Ammonium sulphate and sodium dihydrogen phosphate were added to the concentrated pooled samples from the cation exchange column to give a 1.26 M ammonium sulphate/50 mM phosphate solution that was adjusted to pH 6.6. The filtered sample was then loaded onto a butyl sepharose column pre-equilibrated with 1.26 M ammonium sulphate/50 mM phosphate buffer (pH 6.6). Elution of the column was effected using a negative ammonium sulphate gradient (1.26 M for 80 min and then from 1.26 to 0 M ammonium sulphate in 20 min). In each case, a single main peak was obtained (Fig. 3B) and the conjugate material pooled, dialyzed against water (3×1 L), and concentrated by ultra-filtration. The concentrated conjugates were transferred into 0.35 M Tris–maleate buffer (pH 7.0) before storage at -20°C. The samples were analyzed by mono-S

Table 1. Characteristics of hen egg white lysozyme–E1G conjugates.

Conjugate	% yield	Specific activity[a]	Mr[b]	% inhibition before HIC[c]	% inhibition after HIC[c]
E1	25.6	95.1	14,734.9	65	96
E3	19.1	94	14,734.7	59	99
E5	7.7	57.6	—	59	85
Fraction B[d]	—	92	—	60	—
E3[e]	—	96	—	76	91[f]

[a]Relative to lysozyme as 100%.
[b]Determined by electrospray mass spectroscopy.
[c]HIC = hydrophobic interaction chromatography on a butyl sepharose column.
[d]Purified on a mono-S column at pH 9.5 under non-denaturing conditions (see text for details).
[e]Purified on a mono-S column at pH 6.0 in 7 M urea (see text for details).
[f]Purified at pH 6.6 using an alkyl superose column.

chromatography under standard 7 M urea conditions.[8] All fractions were essentially pure (> 98%) and the electrospray mass spectra for the isomers E1 and E3 both gave a calculated mass of 14,735. Passage through the butyl sepharose column produced highly inhibitable (>90%) material for all three conjugates (Table 1). E3 was also isolated on a small scale from a mono-S cation exchange column using 7 M urea followed by passage through an Alkyl superose hydrophobic interaction column (Table 1). Again, subjection to hydrophobic interaction chromatography produced a conjugate with a high degree of inhibition. Fraction B, obtained from the mono-S column purification at pH 9.5 (Fig. 2A) in the absence of urea, also showed a low degree of inhibition before passage through a hydrophobic interaction column (Table 1).

Kinetic Characterization of Hen Egg White Lysozyme-Estrone Glucuronide Conjugates

The clearing curves produced in the presence of E1, E3, and E5 were all clearly second order for at least 2 half lives (14 min) and the relative k_{cat} values were 3.63×10^5, 3.42×10^5, and 1.82×10^5 L mol^{-1} s^{-1}, respectively (Fig. 4A). Figure 4B shows the dependence of the lysis rate (in units of $\Delta T/20$ min) on conjugate concentration as measured by the Ovarian Monitor (at 650 nm) for E1, E3, and E5. The curves for E1 and E3 were non-linear and fitted equally well, within experimental error, to a hyperbolic equation ($r > 0.994$). On the other hand, the E5 curve appeared to be nearly linear over this range, but the $\Delta T/20$ min values were considerably lower at all conjugate concentrations.

When anti-E1G antiserum was added to solutions of E1, E3, and E5, adjusted to give a control lysis rate of about 350 $\Delta T/20$ min, the lysis rate was decreased in each case. As the amount of antiserum in the assay increased, the lytic activity of E1, E3, and E5 decreased in a non-linear fashion until a minimum rate of lysis (< 10% of the control) was obtained (Fig. 5A). Although the enzyme concentration required to produce the control rate was similar for E1 and E3 (E1, 21 nM; E3, 19 nM), the E1 conjugate required approximately twice as much antiserum to achieve the same level of inhibition as the E3 conjugate. When the difference in conjugate concentration was taken into account, E5 required about four times as much antiserum to achieve a comparable level of inhibition to E3.

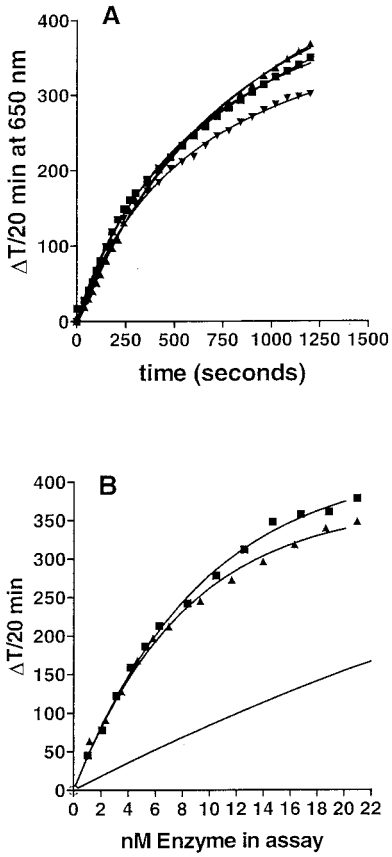

Figure 4. Panel A: Clearing curves for lysis of *Micrococcus lysodeikticus* catalyzed by conjugates E1 (▲), E3 (■), and E5 (▼). Panel B: Relationship of lysis rate with conjugate concentration in the assay tubes. The curves are for E1 (▲), E3 (■), and E5 (—).

Standard Curves for E1, E3, and E5

Based on the apparent end points derived from the antiserum titration curves (Fig. 5A), standard curves for E1, E3, and E5 were then constructed and optimized with respect to the conjugate/antiserum ratio. The optimum standard curves are shown in Fig. 5B. The standard curve for E1 (21 nM conjugate and 0.08 μL undiluted antiserum 243 bleed 4) had a low baseline with increase in the E1G excretion rates

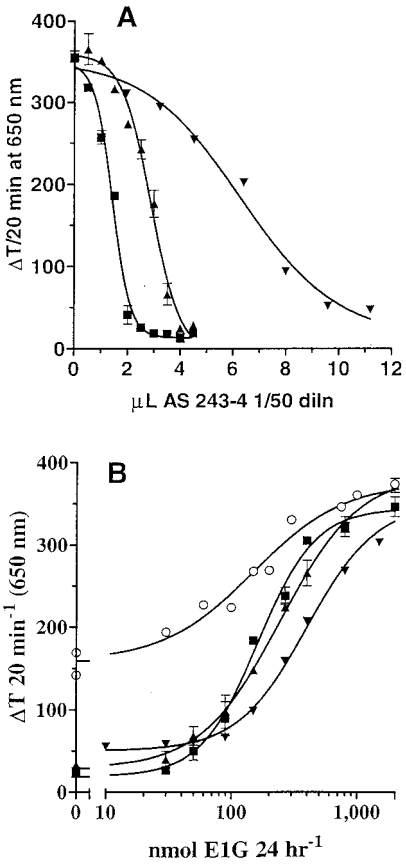

Figure 5. Panel A: Antiserum titration curves with sheep antiserum 243 bleed 4. Rates of lysis versus volume of diluted antiserum for E1 (▲), E3 (■), and E5 (▼). Panel B: Standard curves for the different signal generators. E1 (▲) under optimal conditions (see text), E3 (■) under optimal conditions (see text), E5 (▼) under optimal conditions (see text), and E1 (○) under the optimal conditions for E3 (see text for details).

to 50 nmol $24\,h^{-1}$ or higher being clearly distinguishable from the zero standard rate. The optimized standard curve generated with E3 (19 nM conjugate and 0.048 μL undiluted antiserum 243 bleed 4) was similar to E1 (Fig. 5B) even though considerably less antiserum was required. The E3 standard curve had a lower baseline rate, and a more rapid increase in lysis rates with increasing E1G excretion rates (a steeper, more sensitive curve) with a working range from 70–300 nmol E1G $24\,h^{-1}$. When E1

was used as the signal generator at the same conjugate and antiserum concentrations that were optimal for E3, the standard curve had a mid-point of 162 nmol E1G $24\,h^{-1}$ and a baseline rate (zero standard) of $160\,\Delta T/20$ min. The standard curve using E5 (49 nM conjugate and 0.22 μL undiluted antiserum 243 bleed 4) as the signal generator was markedly shifted towards higher E1G excretion rates compared to those obtained with E1 or E3. The optimum mid-point concentrations were 244, 149, and 403 nmol $24\,h^{-1}$, respectively, for E1, E3, and E5.

Application of the Standard Curves to Human Menstrual Cycle Urinary Hormone Data

Daily urinary E1G values were determined for a complete menstrual cycle using the Ovarian Monitor pre-coated assay tubes and reference standard curves. The cycle was also analyzed using the new standard curves generated with the E1 and E3 lysozyme conjugates. Figure 6 shows the three sets of menstrual cycle data after conversion into E1G excretion rates using the appropriate standard curve. The data for days 15–18 were obtained by further diluting the urines by a factor of 2, so that the lysis rates were in the accurate working range of the standard curves and multiplying the results by 2. When the menstrual cycle data obtained with E1 and E3 as the signal generators were correlated with the corresponding daily values obtained using the pre-coated assay tubes, straight lines were obtained with a correlation coefficient of > 0.93 in each case and a slope close to 1 (Table 2). For E5 as the signal generator, the E1G excretion rates were only 60% of those obtained with E3 as the signal generator (Table 2), but a good correlation was obtained with the reference data.

Table 2. Deming correlation between the precoated ovarian monitor assay tubes and the optimized E1 and E3 hen egg white lysozyme–estrone glucuronide conjugates for the measurement of urinary estrone glucuronide excretion rates throughout an endocrinologically normal menstrual cycle.

Correlation	Slope	*Y*-intercept nmol E1G $24\,h^{-1}$	*R*
Precoated vs. E1	1.023	10.9	0.930
Precoated vs. E3	0.968	35.4	0.974
E3 vs. E5	0.590	53.7	0.839

DISCUSSION

Hen egg white lysozyme (HEWL) is a relatively small (Mr 14,300), very basic protein (pI = 10.5)[18] with a roughly ellipsoidal shape and a well-defined enzymatic cleft.[19] Lysozyme's very basic nature is due to the presence of a relatively high proportion of lysine and arginine residues on the protein surface, these being essential for maintaining the kinetic integrity of the enzyme. Since substrate binding is highly dependent upon the electrostatic attraction between the positively charged enzyme and the negatively charged cleavage sites on the surface of the *Micrococcus lysodeikticus* cell wall,[20] chemical modification can alter the kinetic properties. Lysine residues occur at positions 1, 13, 33, 96, 97, and 116[21] in the primary structure, and all six residues including the N-terminal amino group are found near the surface of the molecule. It is thus theoretically possible to acylate all seven amino groups, but very high substitution levels result in the removal of all of the enzyme's specific activity.[22] Thus, for maximum lysozyme conjugate activity, it is essential to keep the number of substitutions per molecule low. For this reason, the acylation ratio when coupling E1G to lysozyme was kept below 1.6:1, although even at these ratios, six conjugate peaks were always produced as seen in Fig. 1. The same substitution pattern was observed for all conjugation reactions carried out under these conditions.

Large Scale Purification Procedure for Hen Egg White Lysozyme–Estrone Glucuronide Conjugates

Initially, attempts were made to isolate and purify the conjugate families in the absence of urea to avoid possible denaturing effects and associated loss of immunological integrity, with the aim of scaling up to a larger cation exchange *S*-sepharose fast flow column. At pH 4.3, a high salt concentration (0.35 M NaCl) was required to effect elution of the broad lysozyme peak (Fig. 2A) and even higher values were needed to elute the ill-defined band of conjugate material. However, there was no discrete peak of conjugate at this pH (Fig. 2A) as reported previously,[8] and attempts to re-optimize the gradient to facilitate separation of the conjugates were unsuccessful. On the other hand, at pH 9.5, some separation of the conjugates from the unreacted lysozyme was evident. The mainly lysozyme peak (D) in the chromatogram was much narrower than at pH 4.3 (Fig. 2B) and eluted last. This behavior is expected if the hydrophobic interactions with the column matrix reported previously[8] were less than the electrostatic interactions at the lower salt

concentration now required for elution (0.2 M) (compare Figs. 1 and 2B). However, the fact that peaks A, B, C, and D (Fig. 2A) were mixtures of the various conjugate families and lysozyme suggested that protein–protein interactions were nevertheless important in these separations. Complex mixtures were not found in the presence of urea (as in Fig. 1). Thus, to obtain pure lysozyme–steroid glucuronide conjugate families, it was necessary to use urea in the cation exchange step. Evidence from many studies indicates that the effect of urea on the tertiary structure of lysozyme is minimal.[23–25] This was confirmed in the present work by the fact that highly inhibitable conjugates with high specific activity were isolated, suggesting that no permanent structural changes were caused. A preparation of E3, purified in this manner, has remained stable and immunologically active for over six years.

The first step of the purification scheme was a scale up of the standard analytical procedure.[8] The separations performed with the *S*-sepharose fast flow column gave essentially the same profile as observed with the small scale mono-S column, although the resolution of the peaks on the larger column was lower (Fig. 3A). Nevertheless, the resolution was sufficient to allow each conjugate fraction (E1–E6) to be isolated with a single pass through the column, as shown by subsequent FPLC analysis. In rare cases, a second passage through the column was required to obtain a chromatographically homogeneous fraction. A notable feature of the purification scheme was that the conjugates showed relatively low inhibition of the lysis rate in the presence of excess anti-E1G antiserum (Table 1), even though the fractions were free of lysozyme contamination as shown by the FPLC traces and the ESMS data. This poor inhibition was characteristic of conjugates purified both with and without urea using both the strong cation-exchange mono-S and *S*-sepharose columns (Table 1). However, it was not observed when using the weak ion-exchange CM-sepharose columns (J. Brown, personal communication). Thus, it was not the use of cation exchange chromatography, or urea, per se, which was responsible for the low inhibitions obtained, but contact with the strong ion-exchange matrices that was the important factor.

Fortunately, the low degree of inhibition was readily reversed upon a single passage through a butyl sepharose or alkyl sepharose hydrophobic interaction column. Although the mechanism for this restoration of immunological activity was not determined, it is possible that the binding of the hydrophobic steroid groups to the hydrophobic resin allows the protein to rearrange itself back into its native, immunologically competent, form. Whatever the explanation, the excellent separation of conjugates with highly inhibitory properties (Table 1) obtained after the butyl

sepharose column step raises the possibility that this procedure could be used as a one step purification procedure. However, in practice, this was not the case. When large amounts of unreacted lysozyme were still present (as in Fig. 1), a large proportion of the available lysozyme was retarded on the hydrophobic interaction columns co-eluting with the conjugate fractions. This was, presumably, again due to protein–protein interactions.

Thus, it was not possible to separate the different lysozyme–steroid conjugates from each other, or from lysozyme, by means of a single chromatographic step. If the extent of lysozyme contamination could be reduced to less than 20% (for example, by initial precipitation of the conjugate fraction with 1 M NaCl), then a single hydrophobic interaction column step could be used to give a complex mixture of highly inhibitable conjugates. However, a heterogeneous mixture was unsuitable for the present purposes. At present, therefore, the two-step isolation and purification procedure reported here is the preferred method for the large scale preparation of pure (lysozyme free), homogeneous, highly inhibitable hen egg white lysozyme–estrone glucuronide isomers.

Kinetic Characterization of the Estrone Glucuronide Conjugates for Use as Signal Generators

In a parallel study,[26] the acylation sites for the six main conjugate peaks (E1–E6) from the active ester reaction mixture (Fig. 1) have been determined. With the exception of the E1 peak, all the conjugate peaks are homogeneous, consisting of a single conjugate that is uniform with respect to the number and position of the acylations. The E1 peak consists of two mono-substituted conjugates; a conjugate acylated at K97 and a conjugate acylated at K116 in a 60:40 ratio.[26] Analysis of the other conjugates (E1–E6) showed that, under the near stoichiometric ratios of active ester to lysozyme (1.5:1) used here, only three of the six possible lysine residues (K33, K97, and K116) were conjugated, but all possible combinations were represented.[27] E3 is mono-acylated at K33, and E5, a di-substituted conjugate, is acylated at both K33 and K97. The fact that only these three lysine residues were acylated is in good agreement with their calculated ratios of solvent exposure of 1.0, 0.9, and 0.5, respectively,[28–30] obtained from the crystal structure.

Acylation of lysozyme residues K33, K97, and K116, singly or in combination, had no obvious effect on the mechanism of the bacterial cell clearing reaction. All three clearing curves were second order and compared favorably with that produced with native hen egg white lysozyme, which followed second order kinetics for almost the entire

20 min assay period.[17] The Ovarian Monitor assay for E1G depends on the difference in light transmission (ΔT) measured over a set time period of 20 min[2] for a solution of *Micrococcus lysodeikticus* with an initial concentration of intact cells of 214 mg L^{-1} in the assay tube. The maximum lysis rate of 350 ΔT/20 min used in the standard curves[2] is optimum and corresponds to 70% lysis and collapse of the initial concentration of intact cells. It also corresponds to an almost linear portion of the transmission vs. cell concentration curve. The relationship between the increase in transmission with a time-dependent decrease in the concentration of intact cells is logarithmic at 650 nm. Thus, the second order equation suggested by McKenzie and White[16] could be used to calculate k_{cat} values for E1, E3, and E5. The second-order dependence of the lysis and clearing reaction indicates that two molecules of *Micrococcus lysodeikticus* are involved in the rate-limiting step of the reaction[16] for the conjugates as well as for lysozyme itself. The relative (second-order) k_{cat} values obtained from the fits to the data were in the order of their relative specific activities (Table 1); thus, a single acylation with E1G at K33, or K97 had very little effect on the specific activity of the conjugates. When both these lysine residues were acylated, as in E5, the specific activity was reduced by almost 50%. This result is consistent with previous observations with lysozyme where the specific activity of the conjugates decreased with the extent of acylation.[22] The major kinetic difference was that the conjugate had a weaker second order dependence, only following it for 14 min as opposed to 20 min as for native lysozyme. Practically, this meant there was a loss in signal for the conjugate as the assay neared its 20 min endpoint. This was reflected in a flattening of the calibration curves at high conjugate concentrations (a hyperbolic relationship, as shown in Fig. 4B) and in the standard curves using E1, E3, and E5 at high levels of E1G (Fig. 5B). The loss of signal is also accentuated by the fact that a ΔT value of around 350 also brings the final transmission value of the assay tube close to the value for pure water. For this reason, whenever the ΔT/20 min (E1G) or ΔT/5 min (PdG) value exceeds 300, great uncertainty exists in the derived value for the E1G excretion rate extrapolated from the standard curve. In practice, therefore, all lysis rates greater than 300 require that a further dilution of the urine sample be made before an accurate measurement can be carried out. This was done for the peri-peak E1G excretion rates on days 15–18 for the menstrual cycle analyses shown in Fig. 6. Without correction, the E1G peak was truncated and there was little apparent difference for the E1G excretion rates on days 15–17. The assay results are intrinsically more accurate at lower conjugate concentrations, which is ideal for detection of the first rise in E1G levels,

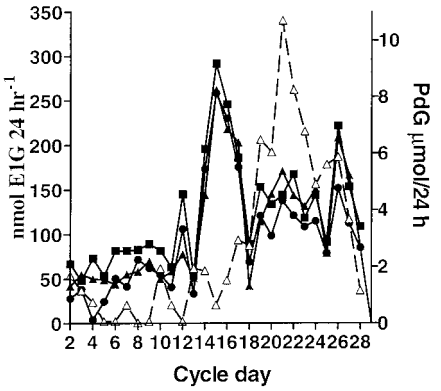

Figure 6. Menstrual cycle profile for 26 year old woman (see text). The E1G profiles were determined with the different signal generators E1 (▲) and E3 (■) and with the Ovarian Monitor assay tubes (●). The PdG data were obtained with the Ovarian Monitor PdG assay tubes (△).

an event which corresponds with the beginning of ovarian estrogen production and the beginning of the woman's potentially fertile period.[4]

Effect of Signal Generator Structure on Urinary E1G Data

For the first time, it has been possible to use structurally characterized, pure conjugates as signal generators to investigate whether there are any advantages in sensitivity or detection limit of the resulting immunoassays that depend on the conjugate structure. Structural differences between the three signal generators were shown by titration with a common antiserum sample as described by Rubenstein et al.[6] Since the titration curves for the E1, E3, and E5 conjugates (Fig. 5A) were performed using the same antiserum (243 bleed 4), any differences must be due to the different affinities of the E1, E3, and E5 conjugates for the E1G antibodies in the serum. For example, relative to the E3 titration curve, the titration curves for the E1 and E5 conjugates were shallower and required a much higher volume of the neat antiserum to produce the maximum degree of inhibition. This presumably indicates that the E1 and E5 conjugate-antiserum systems have higher mean K_d values. The values could not be determined from the data. For E1 and E5, the first additions of small volumes of antiserum resulted in little decrease in the lysis rates.

However, these apparent lag phases are an artefact of the relatively lower affinities for these two conjugates, combined with the poor discrimination of the end point assay procedure at high conjugate concentrations (Fig. 4B). The differences between the mean K_d values for E1, E3, and E5 presumably lie in the balance of favorable and unfavorable interactions between the antibody-binding surface[31] and the microenvironment of the lysozyme surface surrounding the E1G moieties at residues K33, K97, and K116. Computer models of the E1G–lysozyme conjugate structures show that the almost planar steroid moieties[7] are, more or less, parallel to the lysozyme surface. A three-dimensional structure is available for an E1G-Fab immune complex.[31] This shows that the antibody binding site for E1G lies in a cleft in the centre of the antibody binding surface with the carboxyl group of the glucuronide ring exposed to the solvent[31] and with the steroid sitting more or less at right angles to the antibody binding surface. Hence, the lysozyme and antibody surfaces must lie in close proximity in the conjugate-antibody immune complex. However, in the absence of a crystal structure for the immune complex, a detailed explanation for the apparent differences in K_d values cannot be given.

Whatever the correct explanation for the different binding behavior, it suggests that a more sensitive immunoassay might result by using E3 as the signal generator because less antiserum should be required to maximally inhibit E3 in the absence of any exogenous E1G. Since E1G is added first in the assay protocol,[2] less of it should be required to bind to the antibody before the subsequent binding of E3 is affected. Thus, there should be a rise in the rate of lysis at lower E1G concentrations (a more sensitive assay) for the E3 conjugate than for the other two signal generators. When E3 was used as the signal generator, it was possible to construct a standard curve identical to the pre-coated assay tube control within experimental error. The mid-points were indistinguishable (149 nmol E1G $24\,h^{-1}$) and the working ranges and assay discrimination were identical (as shown in Fig. 5B for E3). However, when the same concentration of conjugate (21 nM) and antiserum (0.048 μL) were used for both E1 and E3, although the mid-point of the standard curve generated with E1 was similar, the assay discrimination was reduced by a third due to the high baseline rate of lysis (Fig. 5B). Surprisingly, however, the E1 conjugate-antiserum 243 bleed 4 combination could be used, also, to obtain a quality standard curve simply by increasing the amount of antiserum by a factor of 1.7–0.08 μL (see Fig. 5B). Under these conditions, the standard curve, using E1 as the signal generator, was almost indistinguishable, experimentally, from the standard curves obtained with the pre-coated assay tubes and the E3 conjugate (Fig. 5B), and

met all the criteria set for an E1G fertility detection assay. The baseline rate of lysis was low, the increase from this baseline was clearly apparent at an E1G concentration equivalent to 50 nmol E1G $24\,h^{-1}$, the assay was maximally sensitive over the critical physiological range (50–400 nmol E1G $24\,h^{-1}$), and the within-run assay variability was low. On the other hand, it was not possible to generate a standard curve with E5 as the signal generator that overlapped with those generated with E1 and E3, or the pre-coated assay tubes (Fig. 5B).

The apparent levels of urinary hormone indicated by the lysis rates in the immunoassay system serve as the ultimate test of suitability of a conjugate for use as a signal generator. The pre-coated assay tubes have been validated against the chemical methods of Brown et al.[32] and Barrett and Brown[33] that are of known specificity and reliability, as well as a World Health Organization radioimmunoassay.[34] Thus, the E1G values obtained with them can be taken as the reference values. The data obtained with the standard curves generated with E3 correlated almost perfectly with corresponding data obtained using the pre-coated assay tubes (Table 2). Thus, the values delivered by this immunoassay were used as control values. Since these methods are direct assays with no separation steps, matrix effects on the standard curves needed to be considered. However, there was virtually no effect on the standard curves when the ''blank'' urine was added to the assay tube. Thus, the data reported in Fig. 6 were calculated from the reference standard curves shown in Fig. 5B.

The E1G excretion rates obtained with E1 and E3 as the signal generators were generally indistinguishable from the values obtained with the pre-coated assay tubes with the mid-cycle E1G peak day being identical in all three cases. The first rise days (as previously defined by Blackwell and Brown in Ref.[4]), which are used to define the beginning of the potentially fertile period, were the same for the E3 assay data and the data obtained with the pre-coated assay tubes (day 13 for this cycle). In contrast, use of E1 as the signal generator gave a delayed signal for the beginning of fertility (day 15), but the reason for this is unclear since the E1 standard curve had the lowest rates of lysis at low E1G excretion rates, thus minimizing any ''iceberg'' effects. The fertile period was clearly defined by all the assay systems lasting 7 days from day 13–19. The last day of fertility was the day before the PdG threshold (as previously defined by Blackwell et al. in Ref.[5]) that was reached on day 20 (when the PdG excretion rates first equalled, or exceeded, 6.3 μmol $24\,h^{-1}$). The cycle was ovulatory, as judged by both the rise in PdG to exceed the threshold value on day 20 and the length of the luteal phase which was calculated from the day of peak E1G excretion to the day prior to the next menstrual bleed (inclusive)

and was 13 days. Also, the luteal phase was adequate for supporting a possible pregnancy as shown by the fact that at least one PdG value exceeded a PdG excretion rate of 10 μmol $24\,h^{-1}$.

As expected, there was a high correlation (Table 2) between the data (slopes of 1.0) obtained from the E1 and E3 assay systems, and also from the pre-coated assay tubes, but with a slightly higher intercept for the E3 data. The E5 assay system also gave the same relative daily profile for the menstrual cycle when compared with the E3 assay system (Table 2), despite the standard curve being apparently less sensitive. The same fertile period was indicated by the data obtained using E5 as the signal generator, but the E1G excretion rates were, on average, only 60% of the values given by the other signal generators. This is a serious discrepancy and disqualifies E5 as a suitable signal generator for measurement of urinary E1G levels.

At first sight, it is surprising that the structural differences between E1 and E3 do not lead to greater differences in assay sensitivities. However, computer simulations based on an equilibrium model for the assay[35] confirm that the standard curves for E1 and E3 can be overlayed by increasing the antibody concentration for E1 and keeping all other concentrations the same. To simulate the experimental data, K_d values in the nanomolar range were required for both E1 and E3. As for the experimental data, when the concentration of antibody sufficient to give extensive (>90%) inhibition of the lysis rates for E3 was used to simulate the E1 standard curve, the zero standard rate was too high for practical use. However, the standard curves could be made to approach each other simply by increasing the antibody concentration for the lower affinity conjugate (E1 in the present case). In the simulations, there needed to be a compromise between making the zero standard rates identical for E1 and E3 and making the midpoints of the curves comparable. As with the experimental data, the overlapping was not perfect and the zero standard rate for E1 had to be left higher than for E3 to achieve a midpoint for the E1 standard curve which was not much higher than for E3. This overlapping effect only seems to be possible for low values of the dissociation constants (< 1 nM) and, thus, may not be a general result since the simulations indicated that this superposition was not possible for K_d values in the micromolar, or higher ranges. The E1 and E3 assay systems could both be used to give accurate menstrual cycle profiles; hence, their separation cannot be justified by the results. However, a partial separation is clearly desirable to eliminate E5 from the conjugate mixture and by analogy all of the higher substituted lysozyme–E1G conjugates. This can be achieved simply by the isolation and purification scheme presented here.

REFERENCES

1. Brown, J.B.; Blackwell, L.F.; Cox, R.I.; Holmes, J.M.; Smith, M.A. Chemical and homogeneous enzyme immunoassay methods for the measurement of estrogens and pregnanediol and their glucuronides in urine. Progr. Clin. Biol. Res. **1988**, *285*, 119–138.
2. Brown, J.B.; Blackwell, L.F.; Holmes, J.; Smyth, K. New assays for identifying the fertile period. Int. J. Gyn. Obstet.—Suppl. **1989**, *1*, 111–122.
3. Brown, J.B.; Holmes, J.; Barker, G. Use of the Home Ovarian Monitor in pregnancy avoidance. Am. J. Obstet. Gyn. **1991**, *165* (6 Pt 2), 2008–2011.
4. Blackwell, L.F.; Brown, J.B. Application of time-series analysis for the recognition of increases in urinary estrogens as markers for the beginning of the potentially fertile period. Steroids **1992**, *57* (11), 554–562.
5. Blackwell, L.F.; Brown, J.B.; Cooke, D.G. Definition of the potentially fertile period from urinary steroid excretion rates. Part II. A threshold value for pregnanediol glucuronide as a marker for the end of the potentially fertile period in the human menstrual cycle. Steroids **1998**, *63*, 5–13.
6. Rubenstein, K.; Schneider, R.; Ullman, E. Homogeneous enzyme immunoassay. Biochem. Biophys. Res. Comm. **1972**, *47*, 846–851.
7. Smales, C.M.; Blackwell, L.F.; Waters, J.M.; Burrell, A.K. An estrone–glucuronide conjugate. Acta Cryst., Sect. C: Cryst. Struct. Commun. **1997**, *C53* (8), 1082–1084.
8. Smales, C.M.; Cooke, D.; Blackwell, L.F. Use of ion-exchange and hydrophobic-interaction chromatography for the rapid purification of lysozyme–estrone glucuronide conjugates. J. Chromatogr. B: Biomed. Applic. **1994**, *662*, 3–14.
9. Paek, S.-H.; Bachas, L.G.; Schramm, W. Defined analyte–enzyme conjugates as signal generators in immunoassays. Anal. Biochem. **1993**, *210* (1), 145–154.
10. Conrow, R.B.; Bernstein, S. Steroid conjugates. VI. An improved Koenigs-Knorr synthesis of aryl glucuronides using cadmium carbonate, a new and effective catalyst. J. Org. Chem. **1971**, *36* (7), 863–870.
11. Mattox, V.R.; Goodrich, J.E.; Vrieze, W.D. Synthesis of C-21 glucosiduronates of cortisone and related corticosteroids. Biochemistry **1969**, *8* (3), 1188–1199.
12. Rajkowski, K.M.; Cittanova, N. The efficiency of different coupling procedures for the linkage of oestriol-16α-glucuronide,

oestrone-3-glucuronide and pregnanediol-3α-glucuronide to four different enzymes. J. Steroid Biochem. **1981**, *14*, 861–866.
13. Henderson, K.M.; Camberis, M.; Hardie, A.H. Evaluation of antibody- and antigen-coated enzyme immunoassays for measuring oestrone-3-glucuronide concentrations in urine. Clin. Chim. Acta **1995**, *243* (2), 191–203.
14. Martin, R.F. General deming regression for estimating systematic bias and its confidence interval in method-comparison studies. Clin. Chem. **2000**, *46* (1), 100–104.
15. Deming, W.E. *Statistical Adjustment of Data*; John Wiley and Sons: New York, 1943; 184pp.
16. McKenzie, H.A.; White, F.H.J. Determination of lysozyme activity at low levels with emphasis on the milk enzyme. Anal. Biochem. **1986**, *157*, 367–374.
17. Cooke, D. *Studies Relating to the Ovarian Monitor*; Massey University: Palmerston North, 1993.
18. Verhamme, I.A.; Van Dedem, G.W.K.; Lauwers, A.R. Ionic-strength-dependent substrate inhibition of the lysis of *Micrococcus luteus* by hen egg-white lysozyme. Eur. J. Biochem. **1988**, *172* (3), 615–620.
19. Phillips, D.C. The hen egg-white lysozyme molecule. Proc. Natl. Acad. Sci. **1967**, *57*, 484–495.
20. Price, J.A.R.; Pethig, R. Surface charge measurements on *Micrococcus lysodeikticus* and the catalytic implications for lysozyme. Biochim. Biophys. Acta **1986**, *889* (2), 128–135.
21. Canfield, R.E. Peptides derived from tryptic digestion of egg white lysozyme. J. Biol. Chem. **1963**, *238* (8), 2691–2697.
22. Habeeb, A.F.S.A.; Atassi, M.Z. Enzymic and immunochemical properties of lysozyme. V. Derivatives modified at lysine residues by guanidination, acetylation, succinylation or maleylation. Immunochem. **1971**, *8*, 1047–1059.
23. Warren, J.R.; Gordon, J.A. Denaturation of globular proteins. II. The interaction of urea with lysozyme. J. Biol. Chem. **1970**, *245* (16), 4097–4104.
24. Parente, E.S.; Wetlaufer, D.B. Influence of urea on the high-performance cation-exchange chromatography of hen egg white lysozyme. J. Chromatogr. **1984**, *288* (2), 389–398.
25. Pike, A.C.W.; Acharya, K.R. A structural basis for the interaction of urea with lysozyme. Protein Sci. **1994**, *3*, 706–710.
26. Smales, C.M.; Moore, C.H.; Blackwell, L.F. Characterization of lysozyme–estrone glucuronide conjugates. The effect of the coupling reagent on the substitution level and sites of acylation. Bioconj. Chem. **1999**, *10* (4), 693–700.

27. Smales, C.M.; Elgar, D.F.; Moore, C.H.; Blackwell, L.F. Acid-polyacrylamide gel Electrophoresis of lysozyme–estrone glucuronide conjugates. Bioconj. Chem. **1998**, *9* (6), 838–841.
28. Gerken, T.A.; Jentoft, J.E.; Jentoft, N.; Dearborn, D.G. Intramolecular interactions of amino groups in ^{13}C reductively methylated hen egg-white lysozyme. J. Biol. Chem. **1982**, *257* (6), 2894–2900.
29. Imoto, T.; Johnson, L.N.; North, A.C.T.; Phillips, D.C.; Rupley, J.A. Vertebrate Lysozymes. In *The Enzymes*; Boyer, P.D., Ed.; Academic Press: New York, 1972; 866–868.
30. Lee, B.; Richards, F.M. The interpretation of protein structures: estimation of static accessibility. J. Molec. Biol. **1971**, *55* (3), 379–400.
31. Vargas-Madrazo, E.; Lara-Ochoa, F.; Almagro, J.C. Canonical structure repertoire of the antigen-binding site of immunoglobulins suggests strong geometrical restrictions associated to the mechanism of immune recognition [published erratum appears in J. Molec. Biol. May 24, 1996; *258* (5), 893]. J. Molec. Biol. **1995**, *254* (3), 497–504.
32. Brown, J.B.; Macnaughtan, C.; Smith, M.A.; Smyth, B. Further observations on the Kober color and Ittrich fluorescence reactions in the measurement of oestriol, oestrone and oestradiol. J. Endocrinol. **1968**, *40* (2), 175–188.
33. Barrett, S.A.; Brown, J.B. An evaluation of the method of Cox for the rapid analysis of pregnanediol in urine by gas–liquid chromatography. J. Endocrinol. **1970**, *47* (4), 471–480.
34. Sufi, S.; Ahan, R.; Crocombe, K.J.; Shapton, S.B. Laboratory method manual for radioimmunoassay of pregnanediol-3α-glucuronide and estrone-3-glucuronide **1988**.
35. Cummock, B.P. *Studies Towards the Development of a Urinary Steroid Immunoassay*; Massey University: Palmerston North, 1998.

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