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STEROID IMMUNOASSAY IN CLINICAL CHEMISTRY

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Abstract - **Immunochemical methods for the assay of steroid hormones first commenced in the form of radioimmunoassay in 1969. This assay has been extensively used during the last ten years, transforming the field of clinical chemistry. Steroid radioimmunoassay can no longer be regarded today as a pure research tool, it is emerging as an indispensible practical aid to clinical chemistry, the assay of steroid hormones being of great value in both routine clinical diagnosis and treatment. The present communication describes research recently pursued in our laboratory, made in order to obtain a clearer understanding of the fundamental principles involved in steroid immunoassay, and to cut down the cost of the assays. A new approach for the correct assessment of specificity is given in detail, and the question of homology and heterology in these types of assay discussed. Our latest research in the development of non-isotope immunoassays is presented with details of conjugation, purification of enzyme labels for enzyme-immunoassay. Immuno-enzymatic assay, fluoro-immunoassay and immunofluorimetric assays developed in London are described, together with a new competitive protein-binding technique for the measurement of femtogram [10^{-15}g] amounts of steroids.**

INTRODUCTION

Steroid radioimmunoassay has been a revolutionary development which has been with us for the last ten years. It is now becoming an indispensible aid to the clinical chemist. Unfortunately the many advantages of radioimmunoassay afforded by the use of the all-important highly specific and highly avid immunochemical agent - the antiserum, are being overshadowed by the ever-increasing cost of counting the radiolabel. This has been an impetus for the development of non-isotopic steroid immunoassays. The present communication describes the development of such non-isotopic methods at Queen Elizabeth College, University of London. Particular emphasis is placed on enzyme-immunoassay and on a new approach for the correct assessment of the specificity of immunoassay.

STEROID RADIOIMMUNOASSAY

History of development

In the early fifties, Yalow and Berson in the U.S.A., whilst studying ^{131}I-labelled proteins, made the discovery that insulin-treated diabetics possessed insulin-binding antibodies. After initial rejection by their scientific peers with comments such as 'everyone knows that insulin does not make antibodies', these workers went on to develop, in 1959, a protein-binding technique for insulin using these (as then questionable) antibodies as the protein-binding agents (1). Almost simultaneously, Ekins in Britain, working independently reported a similar method for thyroxine (2). In the early sixties, it was soon recognized that the general simplicity, the potential sensitivity and the wide applicability of this type of assay, made it the undoubted method of choice for most protein and polypeptide hormones. No single method, particularly in clinical chemistry and endocrinology, was adopted so rapidly and widely as this radioimmunoassay method for proteins. Unfortunately, this valuable technique was not developed for steroids until many years later. Steroid biochemists in the early and mix-sixties did not appreciate that steroids, being haptens, are not antigenic and needed to be coupled to proteins in order to elicit antisteroid antibodies. Undoubtedly it was an irony of fate that despite the work of the great immunologist Landsteiner (who, using this principle had raised antihapten antibodies as early as 1936) and despite the elegant chemical studies in 1957 by Erlanger *et al.* (3) (who synthesized antigenic steroid-protein conjugates), a decade elapsed between protein and steroid immunoassay development. It was not until 1969 that steroid radioimmunoassay actually commenced when Abraham (4) used an oestradiol-17β-17 hemisuccinyl-bovine serum albumin conjugate to elicit antibodies to oestradiol-17β. During the next 3 years (1969-1972) investigators

raised antisera against many steroids, but they invariably conjugated the steroid to the protein carrier via the steroids' functional groups. The specificities of the elicited antisera were generally poor with the result that workers had to use tedious chromatographic separation prior to the actual radioimmunoassay step. During this time, often referred to as the first generation antisera period, it appeared that radioimmunoassay had little to offer above the receptor competitive protein-binding techniques of the day. Consequently, radioimmunoassay of steroids commenced slowly, particularly in Britain. An hypothesis was developed that conjugation of the steroid to protein carriers via a position distal to its functional groups would elicit antibodies of greater specificity than those antibodies raised by conjugation through their functional groups. The first indication that this hypothesis was correct was made in our laboratory in 1971 (5), when my colleagues and I reported on antisera raised by oestradiol-17β-6(O-carboxymethyl)-oxime bovine serum albumin, showing it to be highly specific for oestradiol-17β. This was the birth of what has been termed the second generation antisera period. Use of this type of antisera led to simplified types of assay since it obviated the need for tedious chromatographic separations prior to radioimmunoassay. Reasonably specific competitive protein-binding techniques had now commenced. Since 1972 the use of high affinity and specific antisteroid sera has enabled simple specific radioimmunoassays to be made in the lower picogram [10^{-12}g] range for most steroids. The main advantages of present-day haptenic (steroid) radioimmunoassay using high specific activity tritium or iodine radioactive labels, are its sensitivity, specificity, precision, practicality and wide applicability. These advantages are well known. Radioimmunoassay is undoubtedly the popular technique of the day in all fields, it is certainly a great success in the steroid field.

Sensitivity

The potential sensitivity of all competitive protein binding methods (of which radioimmunoassay is but one example) is strictly governed by the factor E/K_a (6), where E represents the total error involved in the assay and K_a, in the case of radioimmunoassay, represents the apparent effective equilibrium constant of the heterogeneous collection of antibody sites comprising the antiserum. Let the protein binding agent (or in our case an antibody site) = A, and the hapten or antigen (ligand) i.e. steroid = H. Then:

$$[A] + [H] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [AH], \qquad K_a = k_1/k_2$$

Acquisition of the total potential sensitivity depends on the specific activity of the labelled steroid. Low specific activity labels will present insensitive assays, despite the fact that K_a may be high. High specific activity labels are usually required for radioimmunoassay to take full advantage of the potential sensitivity offered by the reasonably high effective K_a of the antiserum used. Immunoassay systems can occur in which the very high specific activity afforded by the label is too sensitive for the K_a presented by the antiserum (see below - enzyme-immunoassay). Thus the higher the K_a of the antiserum used, the higher the sensitivity it affords. Attempts to obtain higher K_a fractions of an antiserum (which may lead to slightly less specificity (7)) by affinity chromatography have invariably failed. The highest recorded K_a for an antiserum against steroids is that for oestradiol-17β at 2×10^{10} litres/mole and this means that with minimum error of assay the limit set for steroids is about 2 picograms [2×10^{-12}g].

Specificity

Recent studies in this laboratory regarding the relationship of specificity to affinity of anti-hapten sera (7) have led to an appreciation of a need for a standardized procedure for the assessment of the specificity of antisera. Present-day radioimmunoassay assessment of specificity is unfortunately made under different conditions of radiolabelled tracer binding and of antibody concentration. Data regarding these parameters is invariably not published. Due to this, varying results are obtained for the same antisera in different laboratories. A proposal has therefore been made (8,9) for the standardization of specificity assessment which enables investigators to verify other workers' results and permit reproducible authentic specificity data to be obtained.

Proposed conditions for specificity assessment

The specificity of an antiserum is normally assessed by comparing the ability of various cross-reacting ligands against a standard ligand to displace labelled tracer from antibody sites. The relative ability of labelled tracer displacement (relative potency = P) has been calculated for a collection of univalent homogeneous receptor molecules, all of which have the same equilibrium constant (10). The analytical solution for this idealized case, calculated in terms of the equilibrium constants of labelled tracer (K_*) of standard ligand (K_s) or cross-reacting ligand (K_c) and the function $R = B/F$. $R = B/F = B/(1 - B)$, (where B = antibody-bound labelled tracer = bound/total tracer added; $F = (1 - B)$ = free labelled tracer) is:

$$P = [M_c]/[M_s] = (1 + (K_c/K_*)R)K_s \,/\, (1 + (K_s/K_*)R)K_c \qquad \text{(i)}$$

$[M_c]$ and $[M_s]$ are the respective molarities (moles/litre) of the cross-reacting and standard ligands. When the equilibrium constant $K_* = K_s$ (an assumption usually made in radioimmunoassay), then:

$$P = [M_c]/[M_s] = (1 + (K_c/K_s)R)K_s \,/\, (1 + R)K_c \qquad \text{(ii)}$$

substituting $R = B/(1 - B)$, $(1 - B) + B = 1.0$, and rearranging K_c, then

$$P = [M_c]/[M_s] = (1 - B)\, K_s/K_c + B \qquad \text{(iii)}$$

Thus the latter equation indicates that P varies according to B and the ratio of the equilibrium constants when estimates are made for a collection of antibody sites possessing the same equilibrium constant.

Notwithstanding allosteric effects, one can assume that the effective apparent equilibrium constant of the total heterogeneous collection of antibody sites present in an antiserum can be equated to the summation of all the respective equilibrium constants divided by their number. This however only holds at saturation of the antibody sites, so provided one works at saturation (or near saturation), equation (ii) above should apply for a typical antiserum. The relative potency of cross-reaction (P) is then related to the ratio of the affinity (the apparent equilibrium constant) of a cross-reacting ligand (K_c) to the apparent equilibrium constant of the standard ligand (K_s) produced by the heterogeneous collection of antibody sites present in an antiserum, and the amount of bound/unbound labelled tracer as expressed in (iii).

Relative potency estimates must be assessed under standardized conditions, since the apparent equilibrium constants are a function of both temperature and the concentration of antibody sites (11). Of particular importance, equation (iii) shows that the relative potency depends on B, so it is very necessary to know the value of B when the non-radioactive concentration is zero, i.e. B_o. Valid assessment of P for a particular antiserum and cross-reacting ligand and standard ligand means B_o must be the same for both ligands. Unfortunately this requirement leads to difficulty. Different antisera cannot usually be maintained at the same concentration of antibody sites and still satisfy the condition of having the same B_o value (due to having different apparent equilibrium constants). This dilemma can, however, be solved by working at fixed antibody site concentrations and eliminating the binding values by setting B = 0 (i.e. zero binding). The relative potency equation (iii) then becomes:

$$P = [M_c]/[M_s] = K_s/K_c) \qquad \text{(iv)}$$

i.e. it is independent of all binding values. Unfortunately, zero binding is an hypothetical condition not achievable by practical immunoassay. However, an approximation, i.e. near zero binding, is readily obtained by extrapolation of logit transform data. Transforming the variable $y = B/B_o$ to the response metameter logit $(y) = \log_e y/1 - y$ and plotting against $\log_{10}$ ligand concentration, under conditions of saturation of antibody sites, yields straight line plots. Provided assays are performed at near saturation, these logit-log plots give a linear response with a slope of circa $-\log_e 10$ (12). Major departures from saturation still tend to give straight line plots, but this condition affects the slope only. Thus, at near saturation, the usual sigmoidal standard curve constructed from B/B_o versus $\log_{10}$ ligand concentration is linearized when $K_* = K_s$.

Linear logit-log plots are, however, only obtained when $K_s = K_c = K_*$. This is a very special case of relative potency when $K_s/K_c = 1.0$, and thus $P = 1.0$ for all values of B. The cross-reactivity (reciprocal of P) of any particular cross-reacting ligand is conveniently assessed as the ratio of the molarity of this ligand $[M_c]$, to that of the standard ligand $[M_s]$, which will displace the same amount of labelled tracer. Linear logit-log plots are thus only obtained when $[M_c]/[M_s] = 1.0$, i.e. $P = 1.0$. Since $[M_c] = [M_s]$ in this case construction of linear logit-log plots enables $[M_s]$ at approximate zero binding to be obtained. In practice it has been found useful to extrapolate these linear data plots to cut the $\log_{10}$ ligand concentration ordinate at a molar concentration of 1 mole/litre. At this point the binding value is very low, approximating to zero, and log $[M_s]$ is 0, and this enables the constant C of the equation of the straight line $\log_e B/B_o - B = a \log [M_s] + C$ to be calculated, since $C = \log_e B/B_o - B$ at this point.

All cross-reacting ligands have a $K_c \neq K_s$ and this causes the relative potency to vary according to B, since from equation (iii) when $B = 1.0$, $P = 1.0$, but when $B = 0$, $P = K_s/K_c = [M_{c_o}]/[M_{s_o}]$ where $[M_{c_o}]$ and $[M_{s_o}]$ are the respective molar concentrations of cross reactant and standard ligand at zero binding. Thus, plots for cross-reacting ligands to not produce linear logit-log relationships because the $[M_c]/[M_s]$ values vary according to the relative potency equation (iii). Despite the fact that logit-log plots for $[M_c]$ appear to be parallel, particularly at low binding values, these respective plots are only really parallel at B = 0. Rearranging equation (iii):

$$K_c = (1 - B)K_s/[M_c]/[M_s] - B \qquad \text{(v)}$$

Since in the construction of the logit-log plots for the cross-reactant, the values of B and of $[M_c]$ are known, setting $K_s = 1.0$ leaves only $[M_s]$ for this value of B in equation (v) unknown. $[M_s]$ however, can be calculated since $\log_e B/B_o = a \log [M_s] + C$. Rearranging for $[M_s]$ and incorporating in equation (v), then:

$$K_c = 1 - B/[M_c] \sqrt[a]{(B_o - B)\, C^{2.303}/B) - B} \qquad \text{(vi)}$$

The value of B used in this equation is the same for $[M_s]$ and $[M_c]$. At saturation, the slope a should approximate to -2.303 hence equation (vi) is then simplified. This approach enables a value of K_c to be obtained for every value of B plotted. Thus if six triplicate values are determined for the sigmoidal (B/B_o versus $\log_{10}$ ligand concentration) curve in an immunoassay, the standard deviation of these plots can be assessed to obtain the best sigmoidal curve before transformation to logit-log plots, and then six K_c values obtained, thus enabling the mean K_c and its standard deviation to be assessed. Assessment of cross-reactivities of an individual antiserum need only involve graphical or mathematical interpretation by calculation on a restricted scale so the operator can satisfactorily process the data manually. Calculation of the immunoassay results, statistical evaluation of their reliability prior to logit transformation to both standard (linear) plot and the cross-reactant (potency weighted) plots; assessment of parameters a, c and K_c can easily be made with the aid of a simple hand calculator. A simple computer programme has been devised in our laboratory to provide the K_c values. Setting $K_s = 1.0$, then the ratio K_s/K_c is equal to the reciprocal of K_c. Thus once the value of this ratio is known assessment of potency for any practical starting B_o in the actual radioimmunoassay can be determined from equation (iii).

Standardized conditions of assessment

The above method for assessment must be totally standardized. Unfortunately the popular dextran-coated charcoal technique causes dissociation of the antibody-hapten complex, hence disturbing the equilibrium (11), it is therefore proposed that the double antibody precipitation technique be used for this determination of specificity. The amount of antiserum used for the assessment, i.e. concentration of antibody sites, and the amount of radioactive or non-isotopic label must also be standardized. It is essential to work at near saturation of antibody sites, and a proposal has been made (8) that determinations be made using $3/K_a$ moles/litre antibody sites, and the label concentration be $4/K_a$ moles/litre where K_a is the effective apparent equilibrium constant of standard or label, i.e. $K_s = K_*$ (determined by a saturation curve).

Disadvantages of radioimmunoassay

The use of radio-labelled steroids (or any labelled haptens) has unfortunately, however, a number of disadvantages, not the least of which is expense. Some millions of radioimmunoassays are performed with increasing expense each year. The ever-increasing cost of petroleum based products has seriously increased the cost of liquid scintillant cocktails for counting tritium and also the cost of scintillation vials, and this together with the ever-rising expense of just maintaining radioactive counters and the cost of isotopes, makes present-day radioimmunoassay rather expensive. Radioimmunoassay also presents an extra health hazard, particularly when β-emitters are used. In some countries (including France) legislation has been so framed as to prohibit all but a few centres from performing radioimmunoassay. The assay is difficult to automate due to the isotopic counting involved. It is not surprising that alternative analytical procedures have been considered which take full advantage of the specificity and sensitivity afforded by present-day antisera, but do not employ a radioactive isotope.

ENZYME IMMUNOASSAY

The most popular alternative label used to date has been an enzyme. Commencing in the early seventies, this enzyme approach pioneered by Schuurs and van Weemen (see their review (13)) has unfortunately been slow in offering any real competition to radioimmunoassay. Steroid methods using this technique have been developed for testosterone, progesterone, and for oestradiol-17β; they are probably cheaper methods than radioimmunoassay. Whilst comparable sensitivity to radioimmunoassay has been achieved, the problem of specificity has been particularly perplexing, since up to recently the sensitivity has usually been obtained at the expense of specificity. Recently using judicious heterology (see below) we have been able to overcome this problem enabling us to produce both a sensitive and specific enzyme-immunoassay for oestradiol-17β. Full details of enzyme requirement, steroid-enzyme conjugate preparation and purification, and the method are presented below.

Criteria for choice of enzyme

Reasons for the emphasis given to enzyme labelling are its prior application in histochemistry and cytochemistry, the wide availability and relative inexpensiveness of many enzymes, the prolonged shelf-life of labelled products, the ready existence of manual and automated systems for their assay, freedom from radiation hazards, and a potential sensitivity and specificity as well as an applicability, similar to radioimmunoassay. Many

considerations, however, appear to limit the suitability of most enzymes as potential labels. Considering the rather stringent list of properties required for an ideal enzyme for enzyme-immunoassay we have; the enzyme should (i) be freely available in a highly purified form at reasonable cost, (ii) have a high specific activity to substrate used which is retained after conjugation to steroid and during storage of the enzyme-steroid complex, (iii) be soluble at pH 6.0 - 8.0 for antibody-hapten interaction, (iv) contain several conjugable R groups (usually lysyl), (v) differ from enzymes present in significant concentrations in biological fluids, (vi) the enzyme substrate should be stable and inexpensive, (vii) the enzyme substrate reaction should have an optimum pH that does not impair steroid-antibody binding, (viii) it should afford a simple, rapid, extremely sensitive, reproducible enzyme activity determination using inexpensive freely available equipment.

No enzyme entirely satisfies all these criteria. Two enzymes, more than others, appear to satisfy most of the conditions: E.coli β-D-galactoside-galactohydrolyase (EC 3.2.1.23) and horse-radish peroxidase (EC 1.11.1.7). Both enzymes have been tried in the laboratory, the former galactosidase however has been mainly used, because despite expense it has a higher specific activity to its substrates than the peroxidase.

Enzyme-Steroid conjugation

After treatment of the steroid (in the form of a derivative, usually a carboxyl group) with either a carbodiimide such as 1-ethyl-3-(3)-dimethyl-amino-propylcarbodiimide-HCl (EDC) or alternatively with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide-metho-p-toluene sulphonate (CMC) or by the mixed anhydride method using isobutylchloroformate and tri-N-butylamine, an intermediate is formed which is incubated with the enzyme. This forms a peptide link between the reactive lysyl groups of the enzyme and the carboxyl group of the steroid derivative. After conjugation, the resultant reaction mixture contains several moieties, these are (i) the enzymatically and antigenically active enzyme-steroid conjugate (required product), (ii) free unconjugated steroid, (iii) free unconjugated enzyme, (iv) antigenically active enzyme-steroid conjugate without enzyme activity, (v) extraneous protein-steroid conjugates (impure enzyme) which make no contribution to enzyme activity, (vi) carbodiimide or mixed anhydride reaction breakdown products. Until recently most enzyme conjugates prepared by investigators have not been rigorously purified prior to their use as enzyme labels. In particular moieties (iii), (iv), and (v) above have not been removed. Investigators using these impure labels have justified their action because automatic purification occurs at the separation stage (i.e. double antibody precipitation, Sepharose, or Microcellulose solid phase systems). Vigorous washing of the precipitate after removal of the supernatant certainly removes free unconjugated enzyme (the greatest hazard). Moieties (iv) and (v), which make no contribution to the enzyme activity are not removed, however they lower the sensitivity of the assay. Consideration, however, shows that in order to take full advantage of subsequent ease of automation, it is preferable to take an aliquot of the supernatant for measurement. Impure labels, particularly the presence of large amounts of free unconjugated enzyme, seriously jeopardize this approach since they produce high blanks.

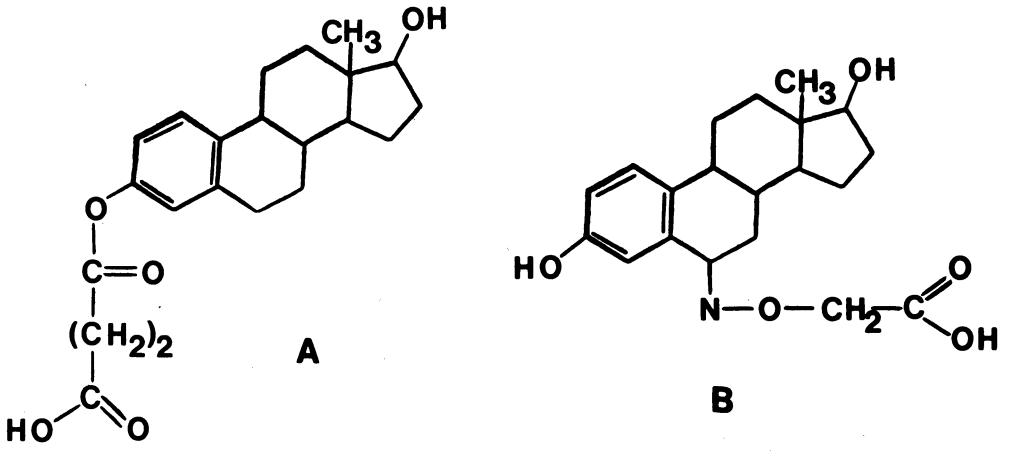

Figure I: Structure of oestradiol-17β-3 hemisuccinate ($E_2$3HS) = A, and oestradiol-17β-6-(O-carboxymethyl)oxime ($E_2$6CMO) = B.

Development of enzyme-immunoassay

Recently a highly purified conjugate of E.coli β-D-galactosidase-oestradiol-17β has been prepared in our laboratory (14). This has been used for the enzyme-immunoassay of oestradiol-17β (15). The preparation and purification steps used for obtaining this pure conjugate are outlined below. The hapten used for conjugation was oestradiol-17β-6-(O-carboxymethyl)oxime ($E_2$6CMO) (Fig.I): it is synthesized according to (16). Immunochemical consideration of the antibody-steroid interaction involved in the enzyme-immunoassay shows that maximum sensitivity is achieved by using a 1 : 1 enzyme/steroid ratio in the conjugate.

Preparation of the conjugate

Figure II shows the steps involved in the preparation and purification of the conjugate. The first step was to prepare and extensively purify the enzyme. E.coli mutant K12 3300, cells were cultured and harvested in the normal way and then the cells ruptured in a French press machine. The resultant suspension was centrifuged to remove insoluble material before subjection to 40% w/v ammonium sulphate precipitation. The crude enzyme was extensively purified by gel filtration on Sepharose 6B.

As stated, the conjugate was prepared using $E_2$6CMO. Condensation of this compound with lysyl groups of the enzyme to form a peptide link was effected using the carbodiimide CMC. An intermediate of $E_2$6CMO and CMC was first formed in weak (0.01 M) acetate buffer, pH 4.7, by reacting 20 mg enzyme with 0.4 mg of $E_2$6CMO at 10-15° C for 30 min. This intermediate was added slowly over 10 min. to a solution of the enzyme in (0.1 M) acetate buffer, pH 5.8. The stoichiometry of the reaction was arranged to give a minimal steroid/enzyme ratio (usually a 20-50 M ratio of steroid to enzyme was required to give about 30% yield of such conjugate). The mixture was then stirred at 4° C overnight and the reaction stopped by adding phosphate buffer, pH 7.2.

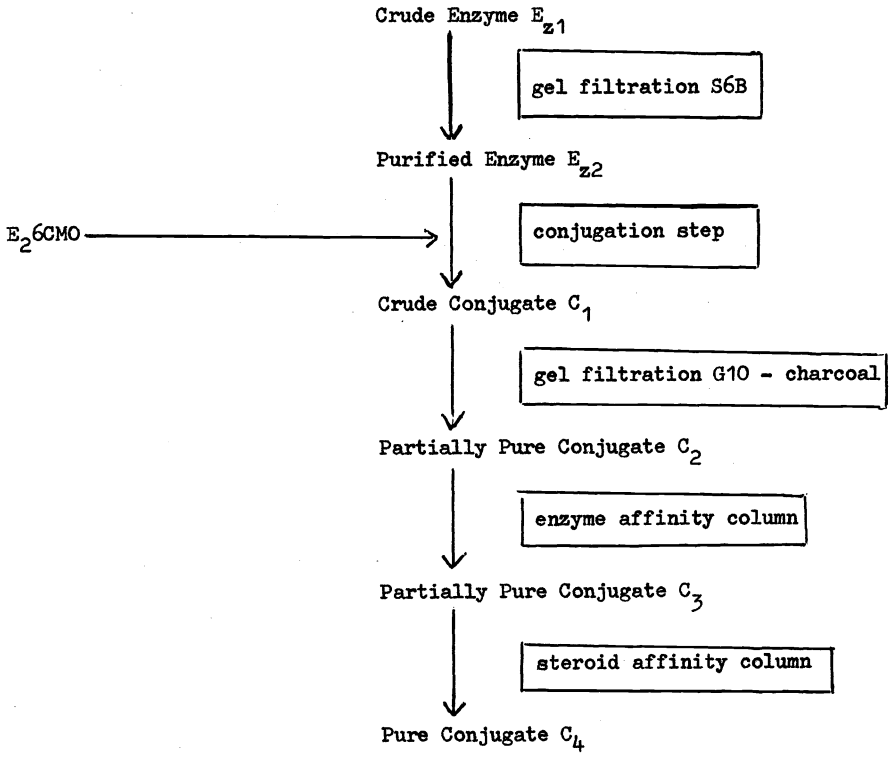

Figure II: Purification steps of the enzyme-steroid conjugate.

Purification of the conjugate

As shown in Fig.II, three steps are involved in the purification of the crude conjugate (C_1). First, the majority of the unconjugated steroid, and carbodiimide by-products were removed by dialysis, and then last traces by use of a Sephadex G10 column in series with a small charcoal column. After this treatment fractions containing the enzyme were collected and concentrated. This was the partially purified conjugate (C_2). The next purification step involved affinity chromatography to remove non-enzymically active molecules (whether conjugated to steroid or not) from C_2. This conjugate was poured down a column of CH-Sepharose 4B-galactosylamine which was washed with phosphate-azide salt (PAS) buffer, pH 7.0, until no further protein was detected in the eluate. The affinity column bound active enzyme was then eluted with 100 mM galactose. Fractions containing the active enzyme were concentrated and dialysed and provided the partially purified conjugate (C_3).

The last step in the purification, one that removes all unconjugated enzyme is a focal one. This again involved affinity chromatography. Antiserum raised against oestrone-6(O-carboxymethyl)oxime ($E_1$6CMO-BSA) which is heterologous to the hapten ($E_2$6CMO) conjugated to the enzyme was coupled to activated Sepharose 4B. In this heterologous affinity chromatography technique the affinity of the bound antiserum for the $E_2$6CMO moiety of the conjugates was only 10% of that for its homologous hapten ($E_1$6CMO), thus excess antibodies were bound to ensure efficient immunoadsorption. This affinity column attracted the enzyme-$E_2$6CMO conjugate only. After immunoadsorption and elution of the free enzyme by PAS buffer, the bound conjugate was eluted from the column by the stronger affinity homologous hapten $E_1$6CMO. The column fractions containing the conjugate were then concentrated and dialysed to remove most of the $E_1$6CMO eluant, then subjected to Sephadex G10 and charcoal treatment before finally concentrating with polyethylene glycol. This was the final purified conjugate, C_4. The enzyme/steroid ratio of the purified conjugate was assessed by acid hydrolysis to be 1 : 1.7. The conjugate had an enzyme specific activity of 320 uM/mg/min. towards the synthetic substrate O-nitrophenyl-β-D-galactoside (ONP-G) used for assessing its activity. An antiserum elicited by oestradiol-17β-3-hemisuccinate (E_2-3HS-BSA) (Fig.I) was used in the immunoassay: it was prepared according to (17). The reason for the choice of this antiserum and the general principles of heterology and homology are given below.

The enzyme-immunoassay method

E_2-3HS antiserum 0.1 ml (dilution 1 : 3,200; 3.6×10^{-10} mol/litre anti-E_2 activity), 0.1 ml enzyme-steroid conjugate (82 ng/ml) and 0.1 ml various doses of oestradiol-17β standard, were incubated at 37° C for 1 hr. The mixture was then allowed to cool for 20 min. then 0.1 ml donkey anti-rabbit antiserum (dilution 1 : 25) added. Incubation was continued for 16 hr at 4° C. The immune precipitate was washed twice by the addition of 1.0 ml assay buffer then centrifuged at 3,000 x g for 20 min. in a refrigerated centrifuge. The supernatant was then decanted. The enzyme activity in the immune precipitates was measured by adding 0.2 ml ONP-G substrate (0.7 mg/ml in assay buffer containing 0.1 M mercaptoethanol) and incubating for 20 min. at 37° C in a thermostatically-controlled waterbath. The reaction was stopped by the addition of 1 ml 1.0 M sodium carbonate solution. The amount of hydrolysed substrate was estimated by measuring A_{405} nm.

A radioimmunoassay method was set up using exactly the same conditions as above, except that the enzyme label was replaced by its calculated equivalent of 12.7 pg (2,4,6,7-^{3}H)-oestradiol-17β and the performance of both immunoassays compared (Fig.III). Various doses of oestradiol-17β, ranging from 50 to 500 pg were added to human male plasma extracted with ether and assayed by both immunoassay procedures. Almost identical results were obtained from these methods indicating that the enzyme-immunoassay technique could be used to assay human plasma samples.

The described oestradiol-17β method involves probably the simplest, most sensitive and specific enzyme-immunoassay technique yet produced. The high sensitivity of the enzyme-immunoassay method can be mainly attributed to the use of the high specific activity enzyme and the preparation and purification of the low molar ratio enzyme-steroid conjugate used as immunoassay label. Factors involved in the preparation and purification procedures which helped to preserve the enzyme activity of the conjugate and therefore the sensitivity of the immunoassay, are the mild enzyme-steroid conjugation technique which did not cause any loss of enzyme activity, and the affinity chromatography purification step which still preserved this activity. Again the affinity chromatography step removed all unconjugated enzyme and thus eliminated any background enzyme activity, which if not removed would interfere with enzyme immunoassay by increasing the blank, hence lowering the sensitivity. Another factor in the enzyme-immunoassay method affecting sensitivity is the use of the double antibody precipitation separation procedure, which unlike solid phase methods is not only simple, but the immune precipitate does not interfere with enzyme activity.

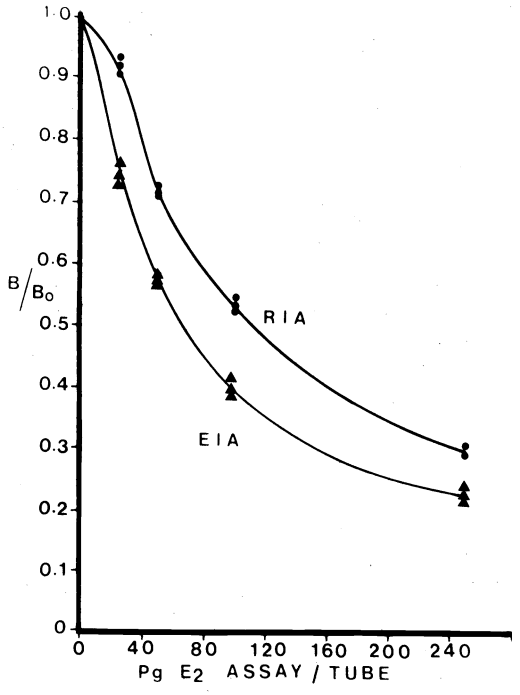

Figure III: Standard curves showing % of bound enzyme activity for various oestradiol-17β doses (B) as a ratio with that bound using enzyme-labelled conjugate alone (B_o); compared with similar results for radioimmunoassay (●), enzymeimmunoassay(▲).

Alternative assessment of the E.coli β-D-galactosidase activity by its more sensitive synthetic fluorogenic substrate 4-methylumbelliferyl-β-D-galactoside was found to be too sensitive for the enzyme-immunoassay endpoint determination. Since the limit of sensitivity of any immunoassay method is dictated by the apparent effective equilibrium constant (K_a) of the heterogenous antibody collection with the antigen or hapten (6) this means that use of higher K_a antiserum should enable determination of oestradiol-17β at sub-picogram levels. This is one of the rare occasions when the measurement of the labelled tracer has been recorded as being too sensitive for immunoassay at picogram levels. The simple automatable colorimetric measurement of enzyme activity using a standard laboratory spectrophotometer and an inexpensive readily available substrate, makes the enzyme-immunoassay method cheaper and possibly simpler than radioimmunoassay. These desirable advantages, coupled with the fact that the enzyme-immunoassay method is just as sensitive and specific as radioimmunoassay methods for oestradiol-17β, possessing freedom from radiation hazard and using an enzyme label superior to radioactive labels as regards shelf-life, all makes the enzyme-immunoassay method more than a serious rival to radioimmunoassay. This type of approach may well become a very popular technique of the future.

Homology and heterology in enzyme-immunoassay

As previously stated steroid radioimmunoassay is based on the fundamental principle that the apparent effective K_a of the antiserum used is identical for the radioactively-labelled steroid and the standard or endogenous steroid to be measured. Doubts are obviously cast that exact equivalence of K_a's can ever really be obtained. Tritiated labelled steroids, however, certainly have a very similar K_a towards an antiserum as unlabelled steroids. Unfortunately most other types of labelled steroid can seldom achieve this near equivalence and enzyme labels can be particularly affected. Antigenic recognition of the chemical

bridge used to conjugate the steroid to the enzyme and the amino acid (usually lysine) involved in the conjugation, will make the K_a between the antiserum used and the enzyme-conjugate label higher than the K_a between antiserum and unconjugated steroid. Matters become even more serious when the molar ratio of steroid/enzyme is >1.0, since then the K_a becomes greater the number of steroids conjugated. If the antiserum raised by an antigen in which the protein carrier is conjugated to the steroid by a similar chemical bridge at the same site and to the same amino acid, is used in enzyme-immunoassay to that used to conjugate steroid to enzyme, we have a condition which has been described by previous workers as homologous enzyme-immunoassay (18). When the antiserum used is reasonably specific for the steroid, this condition leads to specific, yet relatively insensitive enzyme-immunoassay. To overcome this lack of sensitivity, past workers have decreased the K_a of the steroid-enzyme conjugate to the antiserum. This was generally achieved by using a steroid derivative for the preparation of the enzyme-conjugate which differed from the derivative used for eliciting the antiserum. This has been termed heterologous enzyme-immunoassay.

As expected the sensitivity of the enzyme-immunoassay is increased by this approach, however in the past the methods have invariably been less specific, rendering them poor competitors to radioimmunoassay. The enzyme-conjugate label used in enzyme-immunoassay has an apparent effective equilibrium constant towards the antiserum used in the assay which has been defined as K_e (9), thus in this case $K_e \neq K_s \neq K_{ce}$, where the latter K_{ce} represents a cross-reactant in enzyme-immunoassay. Attempts to obtain reproducible specificity using the procedure presented above for radioimmunoassay thus leads to difficulty. Direct determination of relative potency using the previously described principles is not possible with enzyme-immunoassay since $K_s \neq K_e$ and no linear logit plots can be obtained for K_e. When the use of radioactive labels is permitted then this problem can be solved by radioimmunoassay (9), however without such isotopic labels, since the enzyme label K_e must be assessed using another moiety with the same equilibrium constant it is necessary to use an enzyme label, whose enzyme activity is permanently inhibited by active site directed conjugated inhibition. The inhibited enzyme-steroid complex is then allowed to compete with the active enzyme-steroid label as in normal immunoassay, using a limited amount of the active enzyme label. The immunoassay can now be constructed and transformed into a linear logit-log plot. Cross-reactions in enzyme-immunoassay (K_{ce}) can now be obtained using equation (vi) above.

The $E_2$6CMO-E.coli-β-D-galactosidase label used in our enzyme-immunoassay method for oestradiol-17β used a specific E_2-3HS-BSA elicited antiserum in the assay. This combination which involves both site and chemical bridge heterology, allows specificity to be preserved without loss of sensitivity, enabling one of the most specific and sensitive enzyme-immunoassay methods yet developed to be produced. Assessments made showed that the K_e of the enzyme label in this case was of a similar magnitude to K_s (presented by oestradiol-17β) for the antiserum. This suggests that the (O-carboxymethyl)oxime bridge was not antigenically recognized. Results therefore suggest that a judicious choice of either chemical bridge, site of attachment, or both types of heterology made to lower the K_e of the enzyme-immunoassay label to a similar magnitude to that presented by the antiserum of the steroid to be measured, produces specific and sensitive assay provided the antiserum used is specific for the steroid. Specificity is always dictated by the antiserum used, no amount of heterology will produce specificity from a non-specific antiserum. This approach now enables enzyme-immunoassays to be developed which can be serious rivals to the established costly radioimmunoassay techniques of the day. Provided with the enzyme label, these techniques can certainly be much cheaper than radioimmunoassay. The problem of obtaining authentic reproducible specificity data has undoubtedly been with us ever since Berson and Yalow's first radioimmunoassay method, and it is probably equally true that unbeknown to us we have had to live with heterology and homology for the same period of time. Certainly this latter problem became much more serious at the beginning of the present decade, slowing down the rapid development of specific and sensitive enzyme-immunoassay. Labelling of haptens by any means automatically introduces heterology, probably very small in the case of tritium labelling, larger with iodine labelling, and often very large indeed in the case of enzyme labelling.

FLUOROIMMUNOASSAY

Another label used in our laboratory has been the fluorogenic label. Fluoroimmunoassay methods based on the detection of fluorescently-labelled haptens, by fluorescent polarization and fluorescent enhancement techniques have been confined to the drug field; they are not very sensitive compared with radioimmunoassay. The development of fluorimmunoassay for steroids demands the use of an extremely highly sensitive fluorogenic label, especially since measurements are usually made in the picogram range. A single fluorogenic label, even with the use of the most powerful fluorimeter, hardly reaches the required sensitivity, so we have used multi-labelling. This approach has been achieved by the use of various carrier molecules which allow not only the attachment of the steroid, but also many molecules of fluorescent label. Applying this idea, fluoroimmunoassay techniques have been developed for 5α-dihydrotestosterone (5αDHT) (19,20), and oestradiol-17β (19), using 4-methylumbelliferyl-3-acetic acid as fluorogenic label. Methods involved the synthesis of 3-amino-5αDHT and 6-amino-oestradiol-17β. These amino steroids were conjugated to the lone terminal carboxyl

group of poly-L-lysine which was subsequently fluorescently labelled. The polylysine carrier held about 25 molecules of the fluorogenic compound. The double antibody precipitation technique was used in the separation of free from antibody-bound label. Methods have a sensitivity and specificity comparable to radioimmunoassay. The advantages of fluoroimmunoassay are that the technique is cheap, the lyophilized conjugates have a long shelf-life and there is no radiation hazard. The main disadvantage is that despite using 25 molecules of one of the most fluorogenic substances known (it is on a par with fluorescein over which it has several analytical advantages), there is still a need for an expensive and powerful fluorimeter. This is the first report of fluoroimmunoassay in the steroid field.

IMMUNOFLUOROMETRIC ASSAY

Another approach, that of fluorescently labelling the antibodies has been made (21). Antibodies to oestradiol-17β-6-(O-carboxymethyl)oxime ($E_2$6CMO)-bovine serum albumin have been fluorescently labelled with 4-methylumbelliferyl-3-acetic acid. As previously indicated this particular fluorescent label offers several analytical advantages over fluorescein and its derivatives. This is due to the fact that the Stokes shift (shift between activation and emitting fluorescence spectra) is greater and gives less light scattering effects. Antiserum raised in a goat against $E_2$6CMO-BSA was fractionated to give its respective IgG fractions. Oestradiol-17β specific antibodies were isolated and their IgG_1 fraction fluorescently labelled with 4-methylumbelliferyl-3-acetic acid. An immunofluorometric assay for oestradiol-17β was developed in which endogenous steroid competes with AH-Sepharose 4B immobilized $E_2$6CMO, for these fluorescently labelled antibodies. The method developed was slightly less sensitive than radioimmunoassay (due to homology). This method was the first time an immunofluorometric assay had been used for steroid estimation. Results showed that some 16 molecules of fluorogen were conjugated per molecule of antibody. The commercial production of fluorescently labelled antisera using this type of label or fluorescein appears an attractive possibility. The use of immobilized hapten ($E_2$6CMO) as a solid-phase technique for separating free from antibody-bound complex, obviates the need for second antibody separation, hence preserving costs.

IMMUNOENZYMATIC ASSAY

Assay of steroids by labelled antibody techniques such as by the fluorogenic label above, or by labelling with enzymes, has not attracted much attention due to difficulties encountered in purifying large amounts of specific steroid antibodies and the fact that labelling the hapten offers similar advantages and less work is involved in preparing the label. A study made in this laboratory has indicated that in general the anti-oestradiol-17β antibodies in a good antiserum raised by $E_2$6CMO-BSA is less than 3% when expressed as a percentage of the total immunoglobulin present. This specific fraction was isolated by affinity chromatography on AH-Sepharose 4B (hexamethylenediamine spacer) conjugated to $E_2$6CMO. The fraction was then labelled with E.coli-β-D-galactosidase (unfortunately being such a large enzyme only one could be incorporated). This anti-steroid enzyme complex was then used in an immunoassay system in which competition of free steroid and steroid bound to Sepharose 4B occurs. The distribution of the anti-steroid enzyme complex between the solid phase-linked hapten and the free hapten depends on the concentration of the latter.

Another approach recently used has been to enzyme label donkey (anti-rabbit) antibodies. This antiserum is the second antibody precipitant of the first antibody in the normal double antibody precipitation technique. Immunoaffinity chromatography, using rabbit IgG coupled to Sepharose 4B, was employed in the purification of these donkey (anti-rabbit) antibodies. In this immunoenzymatic assay it is the second antibody-enzyme complex in the bound fraction which corresponds to the level of the first antibody in that fraction. The level of sensitivity achieved using O-nitrophenol galactoside as enzyme substrate approached 50 pg (50×10^{-12} g) in both of these immunoenzymatic methods, so they were not quite as sensitive as radioimmunoassay. As far as steroid immunoassay techniques are concerned it is at present difficult to see any practical advantages of immunoenzymatic methods over hapten-labelled enzyme-immunoassay, particularly due to the difficulty of isolating reasonable amounts of antibody. The double antibody immunoenzymatic method has, however, the advantage over immunoenzymatic techniques (where the individual steroid antibody is labelled), in that only one species of antiserum needs to be labelled, so it can be used for all steroids and even polypeptide antigens, so this approach could probably have some impact on the steroid field in the future.

Consideration shows that it is better to use a smaller molecular weight enzyme (such as horse-radish peroxidase) for labelling antibodies when probably six or more enzymes may be conjugated per antibody molecule. Enzyme labelling is preferable to fluorogenic labelling. Consider the case of fluorescent labelling with 4-methylumbelliferyl-3-acetic acid mentioned above. Purified oestradiol-17β IgG antibodies possessed an average of 16 fluorescent molecules per IgG molecule. Calculations show that just one molecule of E.coli-β-D-galactosidase per IgG molecule would potentially give 3.58×10^5 molecules of 4-methylumbelliferone per min. If, as usual, a 10 min. incubation time was used, then 3.58×10^6 molecules of

4-methylumbelliferone would be produced, i.e. 2.23 x 10^5 times more sensitive than the fluoroimmunoassay end point. Labelling of the antibody (immunoradiometric, immunoenzymatic, immunofluorometric assays) allows equivalence of K_a of label and endogenous steroid or standard since all heterology and homology is obviated. Problems arise however, when one has of necessity for assay to distinguish free unoccupied antibodies from steroid-bound antibodies. The only solution at the moment for rapid assay is to immobilize on a solid phase, either antibody or steroid, and this introduces the problem of heterology. More sophisticated approaches such as antibody spin-labelling or use of antibody compliment may overcome this problem, however they lead to complicated methods.

ENZYME INHIBITION IMMUNOASSAY

A variant of enzyme-immunoassay now used for drugs, known as enzyme inhibition immunoassay, which is trade-named EMIT (enzyme multiplied immunoassay technique) by its pioneers at SYVA Pharmaceutical Company in California, probably offers a new approach for steroid immunoassay. Present-day techniques using this approach for drug determinations are far too insensitive for steroid determinations, but research to improve the sensitivity (at present suitable for micrograms and not picograms) is now being actively pursued in our department. This approach invariably operates by allosteric modification of the active site of an enzyme when an antibody binds to haptens conjugated on this enzyme. The separation stage necessary in all the above previous immunoassay systems described that of separating antibody-bound hapten from free hapten is obviated. This enables direct assays to be performed in just a few minutes.

SUB-PICOGRAM STEROID ASSAY

Serious problems beset the veterinary clinical chemists of the day regarding levels of residual anabolic steroids in animal meat tissues after growth promoter treatments. Steroid levels have to be measured at the sub-picogram level. The highest K_a known to man is that produced when the protein Avidin binds its natural ligand Biotin. This K_a is 10^{15} litres/mole and is 50,000 times higher than that recorded for anti-steroid serum. As stated radioimmunoassay has a limit of 2 pg [6 x 10^{-15} M oestrogen]. Thus the Avidin-Biotin has a potential sensitivity capable of measuring 1.2 x 10^{-19} M (20,000 molecules). Work has recently commenced in this laboratory to utilize this system, since up to the date of starting, the only method reported is that using ^{125}I-Biotin for measuring Biotin in plasma over the same range as radioimmunoassay. The very high potential sensitivity afforded by the Avidin-Biotin system has never been harnessed for assay. The binding of Biotin to Avidin has, however, been extensively examined, and findings strongly suggest that chemical modification or conjugation to the terminal carboxyl group of Biotin does not effect its binding to Avidin. This is indeed fortuitous since it means that any chemical moiety can be conjugated to Biotin and the resultant complex will have the same high K_a of 10^{15} L/M to Avidin as its natural ligand Biotin. Thus Biotin-steroid conjugates have the same high K_a; and competitive protein binding systems are being developed in the department for such steroid conjugates. A system using labelled biocytin has already been developed (22) which allows measurements of steroid down to 40 x 10^{-15} g (i.e. 50 times more sensitive than radioimmunoassay) and at present we are attempting to perfect an enzyme-labelled competitive protein binding system using E.coli-β-D-galactosidase, which will enable measurement in the 100-1000 attogram (10^{-18} G) range.

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