Competitive Binding in Reproductive Physiology

The Scientific Basis of Medicine
With Professor AE Kellie, Middlesex Hospital Medical School, University of London.

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<**Opening titles**>

<**Gilliland to camera** >

Professor Kellie works at the Courtauld Institute and the Middlesex Hospital. He has done considerable work on the application of competitive binding in reproductive physiology. This is a comparatively new field, particularly in the steroids, and it is bound to give rise to increasing interest in the succeeding years. Professor Kellie.

<**Kellie to camera**>

It is to be hoped that everyone present will be familiar with the juvenile party game which is usually called musical chairs. If some are so old that they have forgotten about such things, judicious enquiries among the very young generation will soon put this matter right. In practice the game is played by placing a small number of chairs

in the middle of the room and arranging for someone to play a piano while children cavort around the chairs.

<**Kellie stands and turns to a series of drawings on boards behind him. He narrates over these, using an indication stick**>

The chairs are usually placed in a straight line with alternative chairs facing in opposite directions. While the music plays the children dance round the chairs, when the music is suddenly stopped each partaker must endeavour to find an empty chair and to sit down on the chair. Those who succeed in finding an empty chair continue in the game and those who are less fortunate drop out of the game and the game continues with a reduced number of chairs until there is only 1 survivor, the winner.

<**Kellie to camera**>

There is a close analogy between this childish game and the assay of specific molecules by competitive binding. An impresario could organise a gigantic game of musical chairs, supplying 100 vacant chairs and issuing 3 tickets to 100 girls of party age laying down as the sole condition of admission, that they should wear red frocks. Under these circumstances, when the music was interrupted all the guests would find seats and everyone would be happy, i.e. 100% of the chairs would be occupied by red-frocked girls.

But, if unknown to the organiser there were some gatecrashers present who wore green frocks then, even if he were colour blind to green, he would very quickly discover that something had gone wrong with the arrangements, as something less than 100% of the chairs would be occupied by his official guests.

<**Camera returns to earlier drawings**>

The larger the number of gatecrashers, the smaller the percentage of chairs occupied by official guests wearing red frocks.

<**Kellie to camera**>

But it is not difficult to conceive that there is a strict mathematical relationship between the number of gatecrashers and the percentage of seats occupied by official guests.

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In the molecular analogy, which can be readily illustrated by the assay of the steroid compound oestradiol by competitive binding, the chairs are represented by molecules with specific oestradiol binding sites. Such a molecule is found in the cytosol prepared from rabbit uterine homogenates. And the role of the red-frocked girls is played by radioactive oestradiol molecules of very high specific radioactivity. Conditions are then arranged so that all the original binding sites on the receptor molecule are occupied by radioactive oestradiol molecules, i.e. there is 100% binding of the radioactivity present.

If into this system we now introduce non-radioactive oestradiol molecules, these will compete with the radioactive molecules for the available sites and something less than 100% of the radioactivity in the system will be bound. In practical terms, whether one studies the numerology of the party game or the change in the percentage of radioactive oestradiol molecules bound in relation to the non-radioactive oestradiol added, the mathematical relationship is the same and can be seen in the following diagram.

< **Kellie stands and turns to a series of diagrams on boards behind him. He narrates over these, using an indication stick** >

If we plot, on the vertical axis, either the percentage of sites occupied by red-frocked girls or the percentage of sites occupied by radioactive oestradiol, and on the abscissa<*points to horizontal axis*> the increasing number of competitors either gatecrashers wearing green frocks or non-radioactive oestradiol, the type of graph that one gets is a curve representing the hyperbolic function.

A more practical method of presenting the same data is to plot not the percentage of sites occupied but the reciprocal of the sites occupied against the increasing number of competitors. Under these circumstances one gets a linear calibration graph, the same type of graph is obtained from both the party game and from the experimental determination of oestradiol.

< **Kellie to camera** >

How sensitive is this method of measuring non-radioactive oestradiol? It is dependent on several factors which include the number of oestradiol binding sites which are present. It is also dependent on the ease with which one can separate the radioactive bound oestradiol from the free radioactive oestradiol. And it is also dependent upon the ease with which the radioactivity may be counted. This in turn is dependent upon the specific radioactivity of the isotopically labelled oestradiol. But, subject to these limitations, the assay of oestradiol by competitive binding to receptor molecules present in rabbit uterine cytosol, covers the picogram range where 1 picogram is equivalent to 10^{-12} grams. The method is at least as sensitive as methods based on double isotope derivative dilution and gas liquid chromatography even when using an electron capture detector. It is infinitely easier and faster to carry out in practice. It has sufficient sensitivity to measure plasma oestradiol levels in the follicular and luteal phases of the human menstrual cycle.

A similar competitive method, based upon the binding of progesterone to corticosteroid binding globulin will measure the plasma progesterone levels in the luteal phase of the cycle.

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The analogy of the party game can be stretched to explain two important problems inherent in competitive binding methods which relate specifically to the specificity of the binding sites or receptor molecules.

The red-frocked girls may cheat by sitting on window ledges or on cushions on the floor and so upset arrangements. This means that unless the receptor molecule is pure, non-specific binding of radioactive oestradiol will occur on other molecules which may be present.

In the second instance, red-shirted boys may be present who will compete with the girls for the available chairs. And this corresponds to a situation where, unless the molecule has absolute specificity, other molecules resembling oestradiol, for example testosterone will occupy some of the sites and so disturb the true position.

No receptor molecule has yet been characterised which has absolute specificity. The uterine cytosol receptor which binds the oestradiol will also bind oestrone, oestriol and stilboesterol. And the receptor molecule which binds progesterone binds a wide variety of corticosteroids and related pregnane derivatives.

Because of this lack of absolute specificity, when oestradiol is measured, oestrone and oestriol must be removed. When progesterone is measured, cortisol, cortisone, 17-hydroxyprogesterone, 20-alpha and 20-beta-dihydroprogesterone etc. etc. must be removed by purification. So that the principle steps in the competitive binding assay of steroids in biological samples consist of the extraction of the steroid from the biological sample, secondly the purification of the sample and finally the competitive assay.

The ultimate aim of this type of work is to find a binding receptor molecule of absolute specificity and if this were achieved, the purification step could be omitted and the biological fluid assayed simply by diluting it until it came within the calibration range of the assay method. No such absolute specificity has been found to date and virtually all competitive binding assays in the steroid field include a purification step.

The search for absolute specificity of binding has led to the exploitation of the immunological binding of antigen with antibody as the basis of competitive binding. The gonadotropic hormones, follicle-stimulating hormone (FSH) and luteinising hormone (LH) are sufficiently large proteins to be antigenic in many species. And

anti-FHS serum and anti-LH serum have been successfully prepared and have been used for the determination of these gonadotropins by radioimmunoassay.

In this kind of assay, then antigenic protein hormone is usually labelled with isotopically labelled iodine. Either iodine 125 or iodine 131. Although variants of this procedure have been described in which the antibodies have been iodine labelled and synthetic iodine labelled lyogens have been prepared.

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Steroid molecules with a molecular weight from 200-300 are too small to be antigenic. But they can be linked covalently as haptens to micromolecules such as bovine serum albumen, molecular weight 70,000, or to polylysine and the resulting complexes can be used as antigens to raise antisera which show some specificity towards the serum hapten used. The antibodies thus obtained can be used as the binding protein in radioimmunoassay.

This field of application of the technique for the measurement of small molecules of biological interest is not confined to the steroid field and is expanding rapidly at the present time.

Whether the immunological techniques are used to measure gonadotropins, FSH and LH or peripheral steroid hormones, oestrogens and progesterones, they are of considerable value in the field of obstetrics. The radioimmunoassay method is in fact a special form of competitive binding assay.

Having introduced the subject I would like to continue by applying the techniques that have been described. In the first case I would like to describe the effect of the application of the determination of plasma oestradiol and progesterone during the menstrual cycle.

The competitive binding assays for oestradiol, when applied to such plasma samples taken at short intervals throughout the menstrual cycle, revealed the cyclic variations of the ovarian follicular and luteal phases.

< **Kellie stands and turns to a series of graphs on boards behind him. He narrates over these, using an indication stick** >

In the figures shown, samples have been taken from two healthy women of pre-menopausal age. This graph represents the measurement of plasma oestradiol and the dotted line represents the subsequent measurement of progesterone during the luteal phase. In each case there is a sharp peak of oestradiol towards the end of the follicular phase, this is believed to coincide with ovulation, and following ovulation there is a rise in the level of progesterone in both cases. In these two examples shown there is a secondary rise in oestradiol which occurs fairly frequently but not invariably.

Such clear indications of the changes during the cycle are not always obtained. But a high concentration of oestradiol at the mid-period followed by raised progesterone levels may be interpreted clearly as an indication that ovulation has occurred and a corpus luteum has been formed.

These changes in oestradiol and progesterone are accompanied by contemporary changes in the level of follicular stimulating hormone and luteinising hormone as determined by radioimmunoassay using I 13 y, I-131[?] labelling techniques.

This figure shows changes which have been recorded. First of all in the level of luteinising hormone and in the level of follicular stimulating hormone throughout a menstrual cycle. The actual peak of oestradiol would occur at this point so that this particular cycle is somewhat later than the one we saw in the previous diagram. The peak of oestradiol concentration occurs almost simultaneously with that of the luteinising hormone.

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<**Kellie moves away from boards and back to camera**>

This type of demonstration is a useful academic exercise, but the application of these techniques are more important in screening women with a history of infertility, particularly when such women are treated with an ovulatory drug. We have examined a large number of such women after treatment with Clomid which is a proprietary drug containing clomiphene citrate. And we have recorded plasma oestradiol concentrations and progesterone concentrations after such treatment.

< **Kellie stands and turns to a series of graphs on boards behind him. He narrates over these, using an indication stick** >

The first diagram relates to the response of a normal woman to this type of treatment. In this diagram here again, one sees the administration of the clomiphene on 5 successive days at the beginning of the cycle and this is followed by a sharp oestradiol response which is very much higher than the response that one gets from a normal ovulation. This particular point representing the normal maximum oestradiol concentration. This high response of oestradiol during the luteal phase is followed by an equally large increase in the plasma progesterone levels, again this point represents the maximum of the normal ovulatory response. So that one can in fact induce ovulation by clomiphene treatment in a normal woman.

When this type of stimulatory treatment is administered to infertile women we have recorded very large increases in the oestradiol levels. There are 4 cases illustrated here, it was impossible to get more cases onto the figure without destroying the clarity, and in each case there is a subsequent increase in the plasma progesterone level. The normal level of progesterone for normal ovulation is shown at this particular point here.

<**Kellie moves away from boards and back to camera**>

In a recent study of 92 patients with a history of infertility treated with clomiphene, 39 responded showing raised oestradiol and progesterone levels. The dosage with infertile women began at 5 x 50mg per day and was raised in subsequent courses of treatment to a maximum of 5 x 200mg per day or until ovulation was observed. The representative cases, four shown, illustrate the typical picture encountered after this form of stimulation.

The incidence of ovulation which is difficult to establish by other clinical methods is unmistakable when studied by these assay procedures and ovulation can be detected by the determination of progesterone alone provided it is carried out at 3 day intervals.

Over a period of 16 months we have examined 65 women in this way and studied their behaviour through a total of 146 cycles or 4-weekly periods. Of these patients, 23 women, 40 cycles, were amenorrheic, i.e. there was no menstrual period for the previous 12 months. 30 women through 62 cycles were algomenorrheic, i.e. they had 1 or more periods during the previous 12 months. And 12 women had irregular cycles varying from 20-40 days. All of these women had a history of infertility. No patients with primary amenorrhea were included in the study, and other recognisable causative factors including aspermia and tubular inclusion had been excluded. The plasma concentrations of both oestradiol and progesterone encountered after clomiphene treatment are very high and much higher than in normal women ovulating spontaneously.

A clear-cut decision on the incidence of ovulation after clomiphene treatment was possible in 83 out of 92 cycles studied. Ovulation after clomiphene treatment was observed in infertile women in each group but was more frequent in subjects with irregular cycles, 27 out of a total of 28 cycles, than in those with algomenorrhea, 30 out of 48 cycles, or amenorrhea, 5 out of 16 cycles.

Not only have we used these methods of competitive binding to establish ovulation, but several women during this series of experiments became pregnant and we have

followed the levels of oestrogen, of oestradiol and progesterone during the first 2 months of gestation.

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< **Kellie stands and turns to a series of graphs on boards behind him. He narrates over these, using an indication stick** >

Two such pregnancies which terminated in the delivery of a normal child are illustrated in these 2 figures here. Here is the first rise of oestradiol, the inception, the conception, and subsequently an increase in oestradiol and a steady rise in progesterone levels. This period corresponds to the first 2 months of pregnancy. And here again is a case in which a healthy child was subsequently delivered.

This one has some interest because it resulted in a twin birth and although the progesterone levels are not very high, the oestradiol level started quite high and was high throughout the subsequent period of gestation.

The fourth case which is illustrated in the diagram relates to an abortion which occurred on the 50th day of pregnancy.

Within this group of normal pregnancies, I believe there was a total of 19 pregnancies, a very wide range of oestradiol, plasma oestradiol level, and progesterone levels were encountered, and it is quite clear that individual determinations of hormone levels have little clinical value.

<**Kellie moves away from boards and back to camera**>

In addition to applying the standard methods of competitive binding to infertility treated with clomiphene, we have also applied the methods to the treatment of infertile women with gonadotropins and have compared this with measurements of total urinary oestrogens. The customary method of monitoring treatment of infertility with gonadotropins is based upon the measurement of total urinary oestrogens and

urinary pregnandiol, and these procedures are both very time consuming. They cannot be considered to be entirely satisfactory as a guide to the control of multiple pregnancies.

Because of these difficulties, a limited study has been made of the value of the determination of plasma oestradiol and progesterone to provide a more direct and rapid assessment of ovarian function during the follicular and luteal phases after gonadotropin therapy. [tape jumps] of 4 women have been recorded and the work must be considered to be incomplete at the present time. All 4 subjects studied had secondary amenorrhea and had failed to respond to clomiphene treatment at a level of 200mg per day over a period of 5 days. The ovaries were not cystic and had been shown at biopsy to contain primary follicles. There was no evidence of neoplastic or endocrine lesion.

In each course of treatment the subject received 3 injections of human menopausal gonadotropin on alternate days of the cycle. On day 1, 3 and 5.

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< **Kellie stands and turns to a series of graphs on boards behind him. He narrates over these, using an indication stick** >

On day 1, 3 and 5. Subsequently on day 8, the patient received a dose of human chorionic gonadotropin of 10,000 units. The does of menopausal gonadotropin began at 150-250 units per day and increased with each successive course of treatment to a maximum of 750 international units per day or until ovulation occurred. The plasma oestradiol measurements were made on days 1,3,5 and 7. And after injection of human chorionic gonadotropin on day 8, progesterone determinations were made.

It has been established on this regimen that when oestradiol levels fail to exceed a level of 30 nanograms per 100ml there was no subsequent luteal phase, i.e. no rise in plasma progesterone concentration, and subsequently when this occurred, no human chorionic gonadotropin was administered. When the oestradiol response on

day 7 exceeded a level of 30 nanograms per 100ml, human chorionic gonadotropin was administered and the progesterone levels rose during the subsequent luteal phase.

These levels of plasma oestradiol which were encountered under treatment, compare with values of 20-80 nanograms per 100ml in spontaneous cycles in normal women and with 50-200 nanograms in infertile women treated with clomiphene. There is clearly some relationship between the oestradiol concentrations occurring on day 7 and the progesterone concentrations occurring on day 15. But no close correlation could be established from the data so far accumulated.

Plasma oestradiol concentrations change rapidly in the 30 hours preceding day 8 and more frequent sampling is necessary during this critical period. The concentration of plasma oestradiol levels on day 7 has been compared with values for the total urinary oestrogens on the same day. Here we have plotted the plasma oestradiol levels and here the total oestrogen levels. A significant correlation was obtained. It seems likely that plasma oestradiol levels and oestrogen levels give a more direct assessment of ovarian function and these assays are definitely less time consuming than the corresponding total urinary oestrogen measurement.

<**Kellie, standing, reads from notes to camera**>

A more detailed and extensive study of the oestradiol response to human menopausal gonadotropin and the subsequent progesterone response to human chorionic gonadotropin may lead to an improvement in the control of infertile women receiving gonadotropin treatment.

Patients did not show a consistent response to successive courses of treatment. In some cases a satisfactory response was obtained during the first course of treatment, oestradiol levels greater than 30 nanograms per 100ml, and subsequent progesterone levels greater than 10 nanograms per ml, was followed by an unsatisfactory response to a second course of treatment.

In the present limited study, 2 of the women became pregnant after gonadotropin treatment, 1 aborted after developing hydramnion after 17 weeks of pregnancy – at post-mortem 5 foetuses were observed which were apparently normal. It is perhaps significant that the plasma oestradiol concentration, 18 nanograms per 100ml, and the progesterone concentration, 50 nanograms per ml, were high when compared with normal values at this stage of gestation.

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<**Kellie sits down again, narrates to camera**>

In the final application of competitive binding methods in the obstetric fields, I would like to refer to changes in oestrogen concentration in late pregnancy.

The concentrations of oestrogens in plasma, even in late pregnancy, is low, and the total value represents the sum of total oestrogens in the free and in the conjugated form. Because of this complexity, technical limitations have hitherto prevented a systematic study of changes in oestrogen levels during the terminal stages of pregnancy. Attempts to use fluorometric methods for this purpose have had some success, but although the sensitivity of this method is high, the specificity is low and the fluorescent response can be markedly influenced by adventitious quenching.

More sophisticated methods such as gas-liquid chromatography, used in conjunction with electron capture detection, and methods based on double isotope derivative dilution have precision and accuracy but they are time consuming methods and they are costly and they are unsuitable to the application of the multiple analyses which a dynamic study necessitates.

The development of competitive binding methods has now provided a simple method of adequate sensitivity and specificity for the measurement of oestrone, oestradiol and oestriol in plasma pregnancy.

< **Kellie stands and turns to a series of graphs on boards behind him. He narrates over these, using an indication stick** >

This figure shows the response, by competitive protein binding, of oestradiol, oestrone and oestriol and all 3 oestrogens can be measured by competitive protein binding.

Unfortunately the conjugated forms of the oestrogens which are present in the plasma in late pregnancy do not bind to the uterine oestrogen receptor and these conjugates can only be assayed after hydrolysis. The plasma concentrations of oestrone, oestradiol and oestriol, as free compounds in late pregnancy, have been measured, and the corresponding conjugates have been hydrolysed and the released oestrogens have been separated and measured.

<**Kellie, standing, reads from notes to camera**>

Separation of individual oestrogen was carried out by reversed phase gel filtration on Sephadex LH-20 and procedural losses throughout the entire assay have been off-set by the use of radioactive internal standards. Duplicate assays in this work have given good agreement.

The results of the application of these techniques to measure oestrogens and oestrogen conjugates in serial plasma samples withdrawn at intervals from pregnant women between […]

<**Kellie narrates over series of graphs**>

[…] the 13th and 40th week of pregnancy are shown on the following 3 slides which are presented in sequence.

In general terms the most salient feature in changes in plasma oestrogen concentration in advancing pregnancy, was the uniform pattern of circulating oestrogen conjugates which are shown in the dotted line of this portion of the figure.

In all subjects studied to date, the concentration of oestriol conjugates were substantially higher than that of any other conjugated form and rose progressively in each pregnancy as the period of gestation lengthened. In contrast, the conjugation of concentrated oestradiol remained at all times very low. There is at least some superficial resemblance between the conjugated oestrogens measured in plasma and the composition of the urinary oestrogen fraction.

The oestrogens present in the free form showed a very much greater variety in individual subjects with oestradiol occupying a predominant position in most pregnancies. Nevertheless, the levels of oestradiol encountered in this work in normal pregnancies was so wide that a single oestradiol determination can have little clinical value.

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The unconjugated oestriol level rose progressively throughout the period of gestation and was occasionally higher than the free oestradiol level. The total concentration of free oestrogens was at all times less than the concentration of oestriol conjugates and the pattern of circulating unconjugated oestrogens did not in any way represent the conjugation of the urinary oestrogen fraction.

The pregnancy recorded in the final figure is of interest because it had to be terminated artificially because of a delay in the first stage of labour. The patient whose levels are indicated in these diagrams was a 28-year-old woman in her second pregnancy and she delivered by caesarean section, after induced labour, a male child of 3.9 kg, associated with a placenta of .58 kg.

The last plasma sample was taken on the 30th April, about this period here, on the 40th week and delivery was induced a few days later on the 3rd May. As early as the 37th week there was a pronounced fall in free plasma oestradiol levels and the plasma free oestriol fell progressively during the subsequent week. The decrease in plasma oestradiol preceded the fall in conjugated oestriol by at least 1 week and all

plasma components had decreased substantially by the 38th week, 10 days before delivery.

<**Kellie, seated, to camera**>

There is little doubt that plasma oestrogen concentrations reflect changes more rapidly and faithfully than urinary analyses especially in relation to the important components, free oestradiol and conjugated oestriol. The former component which presumably indicates placental function is not recorded by urinary analysis. And the latter component which represents both foetal and placental function can be measured just as easily in plasma as in urine. While the plasma analysis gives a composition of the medium only at the time of sampling, the relative simplicity of the competitive binding method permits the analysis of serial samples which may reveal rapid changes in normal and abnormal gestation.

In summary, it is clear that methods of competitive binding, whether based upon naturally occurring specific receptors or on radioimmunoassay, now permit the presence of gonadotropins and peripheral steroid sex hormones at very low concentrations. Individual determination are, in most cases, of very little clinical value but the need for multiple assays is off-set by the relative simplicity of the competitive binding methods, and there is little doubt that they will shortly be automated. The methods are sufficiently sensitive to be applied to plasma samples, and may be found to be more satisfactory than the present urinary assays.

The value of these methods in the field of obstetrics and gynaecology has already been established.

<**End credits**>