STEROID IMMUNOASSAY

Proceedings of the 5th Tenovus Workshop

1975

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PREFACE

In recent years, the use of immunoassay techniques has revolutionised the measurement of steroid hormones since they afford the necessary simple, sensitive and specific methods fundamental to the advancement of Endocrinology in general and of certain aspects of Cancer Research in particular. The last major International Conference on Steroid immunoassay *per se* took place at the Worcester Foundation for Experimental Biology, U.S.A. in 1969. Many significant advances have been made since then, and this Workshop was organised to provide a forum to discuss and assess the value of the recent work in this field.

The committee are deeply indebted to the Tenovus Organisation for financing the Workshop. We are also grateful to Mr. G. Read for the work he undertook in helping to compile the book. We would like to thank Dr. Diane Fahmy, Dr. Meriel Golder, Dr. E. N. Cole, Dr. M. E. Harper and Mr. B. Joyce for their assistance in reading and checking the various proofs and to Miss Barbara Towler and Miss Delia Stephens who typed the transcripts of the discussion sessions. We would especially like to thank Mr. John Burke of Tenovus and the Alpha Omega Publishing Company, who plays a major role in ensuring a rapid publication of our Workshop series of books. We are indebted to Mrs. Joyce Morgan, Mr. Steven McAllister and Mr. Colin Smith of the Medical Illustration Unit, Tenovus Institute, who prepared many of the figures for the book and to Mr. Ralph Marshall, Medical Illustration, University Hospital of Wales, who kindly prepared photographs of some of the slides presented during the Workshop. We also appreciate the generosity of Dr. P. Leonard, Searle Diagnostic Services, who provided an excellent reception for the participants. Finally, we thank the participants themselves for their contribution to the success of this Workshop at both a social and academic level.

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SESSION 1: Chairman: P. K. Besch

STEROID IMMUNOASSAY — PROBLEMS AND POTENTIAL

V. H. T. James

Many people working on the problems of assaying steroid hormones will remember only too well the time the only practical analytical techniques for the quantitative assay of steroids in biological fluids were relatively insensitive colorimetric assays. Since then, there have been major steps forward, through fluorimetric and isotope derivative methods to the present time when competitive protein binding analyses and currently radioimmunoassay methods for steroid compounds, offer a degree of sensitivity which is sufficient to allow their measurement in relatively small volumes of fluid. These developments have opened up very considerable possibilities and lines of investigation which were closed before, but at the same time have produced their own specific problems. My brief was to review these possibilities and problems, but in a short time I can only do so in an introductory fashion and, in any case, all the subsequent papers will undoubtedly be touching again in detail on the various aspects which I shall mention.

The main potential which one can see in the steroid field relates not to any major or immediate improvement in immunoassay techniques, but to the exploitation of the particular virtue of the assay itself. Thus, although one would be wrong to pretend that current methodology is ideal and unlikely to undergo significant improvement, it is already capable of offering, in practical terms, good sensitivity and a relatively high degree of specificity, conferred by the immunological process. Instead of seeking, usually empirically, for a suitable analytical reagent, we can now tailor these reagents as required and already immunoassays exist for every known steroid hormone. Thus we can assay the three major oestrogens, and the progestogens, testosterone, 5α-dihydrotestosterone and androstenedione, together with the adrenal corticosteroids and the adrenal androgens. All these assays can be made on relatively very small volumes of plasma and so, apart from single analyses for diagnostic purposes, this opens up the possibility of repeated sequential blood sampling. Since the pulsatile secretion of many hormones, including steroids, is now a well recognized phenomenon, this type of study is now feasible and enables the investigation of steroid hormone patterns through the 24-hour period, or even longer. Not long ago, this type of study would have been precluded because of the unacceptably large volumes of blood required, whereas now the problems are of sampling, not of analysis. The clinical potential of studies of this type remain to be explored but, for example, mapping of the nocturnal pattern of plasma cortisol levels probably represents the most sensitive test of hypothalamic-pituitary-adrenal function currently available. Studies of this type also make it possible to study details of control mechanisms. For example, by following the detailed pattern of cortisol and androstenedione, it is clear that they follow very similar if not identical patterns of change, with the clear inference that secretion of both hormones is subordinate to ACTH. Plasma aldosterone levels also reveal a strong ACTH dependence. Further detailed study of steroid hormone patterns is likely to provide more information of this type.

It has become apparent over the last few years that the binding to proteins of many steroid hormones profoundly affects their biological activity and metabolism, and several techniques exist for the measurement of free plasma steroid levels. Of considerable diagnostic value, though, is the level of free or unmetabolized steroid in urine, since it reflects this binding to plasma protein. Urinary free cortisol is a good example of this. Since the amounts of free steroid are small, the analytical problem is not inconsiderable, but with immunoassay techniques the possibility to determine free steroid levels in urine

exists and should certainly repay exploration. Some time ago, West and his colleagues explored the use of parotid fluid for a similar purpose and again, immunoassay methods make this much more practicable. Determination of steroid levels in sweat is also possible, and this represents a further unexplored area of steroid metabolism.

Whilst discussing the usefulness of urinary steroid measurements, we might consider the problem of assaying urinary steroid metabolites. Although some small part of most steroid hormones appears to be excreted unchanged, as I have just mentioned, the larger part of the hormone is metabolized and conjugated prior to excretion. Virtually all analytical methods have therefore started with a hydrolysis stage to liberate the steroid fraction which is then extracted with organic solvent prior to analysis. It would be more logical to assay the conjugate itself, and the only reason we do not do this is because we lack the necessary analytical methods. To take aldosterone as an example, about 12% of the total amount produced is excreted in the form of the 18-glucuronide. The urinary aldosterone glucuronide is hydrolysed, usually at pH 1, and the freed aldosterone is then extracted, and purified chromatographically before being subjected to radioimmunoassay. Ideally though, we would choose to avoid this work-up, and to assay the conjugate itself. This requires antisera against steroid conjugates. Professor Kellie has already demonstrated that a glucuronide is a suitable hapten for coupling directly to protein, as there is a ready-made means of attaching the conjugate to protein through the carboxyl group. However, it is also possible that other types of linkage might prove more advantageous. Using this methodology, a radioimmunoassay for testosterone glucuronide in urine has already been published [1]. Steroid sulphates can also be assayed directly, and dehydroepiandrosterone sulphate is an example of this. Thus direct assay of steroid conjugates in biological fluids is likely to be a future area of development, offering simplification of the assay procedure.

Steroid immunoassay techniques also have some disadvantages and problems. Perhaps one of the most serious at present is the need for the analyst to carry out, in many cases, a fairly substantial degree of preliminary purification before using immunoassay as an endpoint. This is because of the relatively low concentrations of steroid which are usually involved, and contrasts markedly with the situation with most peptide hormones where direct immunoassay has almost invariably been carried out without any initial purification. This has led to problems in some cases where other materials, such as biological fragments of the peptide may cross-react with the antiserum. Whilst this initial purification does improve the specificity of the assay and, indeed, sometimes makes it possible at all, it is time-consuming and adds technical problems and direct immunoassay would be advantageous. This requires, basically, better antisera with good specificity and high affinity and progress towards this end has already been made. Thus, a simple, direct immunoassay for dehydroepiandrosterone sulphate in plasma has been described by Buster and Abraham [2], and others have reported methods for testosterone and aldosterone. It is obviously important with methods of this type to ensure specificity, especially when they are applied in abnormal clinical conditions.

This brings us back to the general problem of pre-assay fractionation to which I alluded earlier. There are several procedures which have been used for the separation of steroids prior to immunoassay. Extraction with organic solvents usually forms the initial purification step and careful choice of the appropriate polarity solvent is sensible. Some steroids are labile compounds, and when handling small amounts in solvents, care is needed to avoid losses or transformations which may invalidate the analysis. For example, aged dichloromethane can effectively destroy aldosterone completely.

The problem of contamination should not be overlooked, and several authors have reported on the hazards of employing pregnant ladies in laboratories attempting to assay small amounts of oestradiol! As the methods we use become increasingly sensitive, the problems of our own environmental contamination become increasingly significant.

Further fractionation of the steroid extract can be made with a variety of techniques, including column methods using Sephadex, silica gel, alumina or Celite. Abraham and his colleagues have used Celite extensively to fractionate a number of steroids prior to immunoassay [3]. Alternatives are thin-layer chromatography and paper chromatography. None of these methods is ideal since they are all time-consuming and not easily mechanised. Our preference has been for paper chromatography, since it has good discrimination and by using a β-ray detector (Panax Betagraph), location of the exact region on the chromatogram is possible. Here again, with more specific antisera the need for high resolution will become less and very simple fractionation procedures are likely to be adequate.

The immunoassay itself still presents some difficulties. Some of the main problems lie in the separation of the bound and free fractions, the relatively long equilibration times and the need for expensive radioisotope counting equipment.

The various separation methods in use at present include adsorbents such as charcoal and Florisil, double-antibody, Sephadex, solid phase methods and ammonium sulphate. Adsorption methods using charcoal have been very widely employed and with considerable success, although they may not be very successful with some antisera and careful attention to timing is important. This tends to limit the batch size. Double-antibody methods often work nicely, but are lengthy procedures and we have generally preferred to use ammonium sulphate precipitation. This is usually very reliable, although we have had considerable problems on occasions with some batches of ammonium sulphate, or with ageing solutions which cause precipitation of free steroid as well as the bound fraction.

There may well be other advantages in employing particular separation techniques. Thus the specificity of the antiserum can relate to the method of separation used and Tyler and his colleagues [4] found that there was very substantially less cross-reaction of an anti-testosterone antiserum with 5α-dihydrotestosterone if they used ammonium sulphate rather than dextran coated charcoal.

There are still some problems associated with the "radio" part of the immunoassay. Unlike peptide analysis, steroid immunoassayists have been able, in many cases, to draw upon commercially available radio-labelled steroids of a specific activity high enough to be satisfactory for analytical purposes. These steroids, labelled with tritium, are sufficiently similar in their physical properties to the natural steroids, to present no real problems from this point of view, and can be used as tracers in the method both for recovery purposes and also for the immunoassay step. Tritium has a very long half-life, effectively eliminating any problems of storage. Thus, unlike non-steroid antigens, there is often no need to carry out any labelling procedures, the problems of radiation damage are small and consistency of labelled material is easily achieved. As against this, not all steroids are commercially available labelled with tritium and, compared to γ-emitters, tritium is somewhat more difficult and expensive to count, and counting times need to be longer.

Quite a lot of progress towards the development of γ-labelled steroid antigens has been made [5], although the current methods are not ideal and other approaches to the problem are needed. However, radio-labelling is not the only possible end-point for immunoassay, and other techniques have been investigated and described for other compounds, notably drugs [6]. There is no obvious reason why these methods cannot also be applied to steroids, with the advantage of simplifying the methodology and making the development of automated assay methods easier.

One of the major outstanding problems of steroid immunoassay is that it is a repetitious, tedious and boring technique and requires careful and precise technical application to maintain high quality results. With the increasing demand for large numbers of analyses, there is an obvious requirement for the development of automated methods of analysis. Precise and repetitive pipetting is better done by machines than by people, and they can

maintain the same standard of work indefinitely. Complete automation of a steroid immunoassay, in the proper sense of the term, is a considerable task, and requiring a lot of complex and expensive equipment, and I am not aware of any published description of such a piece of apparatus.

The various purification stages, and the discontinuous nature of the chemistry do not lend themselves ideally to continuous flow systems. However, mechanization of the various stages of the assay process is entirely feasible and practicable, and several commercially available pieces of equipment can be employed for this purpose, such as the LKB, Micromedic and Analmatic.

We have been using the latter because, compared with the other two, it is a relatively inexpensive piece of equipment. Our attempts have been largely towards mechanising the method for measuring testosterone in female plasma, an assay for which there is considerable clinical demand.

The problem with the assay is that it requires initial purification of the plasma sample, and so mechanization of this stage, and of the immunoassay step, is needed. The Analmatic machine has proved satisfactory for both these purposes.

Major advantages of using mechanized techniques are the increased number of samples which any one technician can handle, and the improvement in analytical precision. Even with the problem of extraction to cope with, up to 80 or 100 assays per day can be carried out. The only real problem of non-specificity lies in the fact that the antiserum cross-reacts with 5α -dihydrotestosterone, and until an antiserum with superior characteristics in this respect becomes available, separation of this steroid remains unavoidable if complete specificity is essential. In practise, this is rarely needed for clinical purposes, but may be essential for some research investigations.

There is no reason why exactly the same mechanised procedures cannot be used for other steroid assays and, given an appropriately specific antiserum, direct immunoassay of serum can be carried out without difficulty.

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CHEMICAL AND MICROBIOLOGICAL TRANSFORMATION OF STEROIDS

G. F. Woods

It is not possible in a short presentation to cover such a general subject comprehensively. I intend therefore to review the developments in the field of microbiological steroid transformations since its inception in 1937 and show how the combination of microbiological and chemical reactions can lead to some interesting new steroid haptens. Two books by Capek *et al* [1] and Charney and Herzog [2], with essentially the same title, provide interested readers with a comprehensive coverage of the subject up to 1966-1967. They each contain more than 1200 references and although they have different approaches to the subject, both stress the practical problems associated with microbiology as a tool in steroid chemistry.

The use of fermentation processes for the conversion of steroids has developed in parallel with the discovery of the steroid hormones and the demand created by the realisation that they had great clinical importance. Because of limitations in steroids available from natural sources and the knowledge of their chemistry at the time, resort was made to micro-organisms to provide commercial syntheses in advance of the more economical chemical methods which often, though by no means always, were developed later. This was a direct consequence of the extensive knowledge of the chemistry of yeasts that had been built up since the middle of the 19th century.

Since 1937 when it was first demonstrated that steroids provide suitable substrates for microbiological transformations, development of the processes and techniques fall into four phases covering the periods 1937-1940, 1940-1949, 1949 onwards and 1966 onwards.

During the first period Mamoli and Vercellone [3, 4] demonstrated the reduction of 17-oxo steroids to 17β-hydroxy steroids by means of yeasts. The process found limited use for the manufacture of testosterone and oestradiol. This period also saw the introduction of bacteria for steroid conversions.

The second period 1940-1949, covering the war years was relatively quiet partly because of the absence of commercial pressures and partly because manpower was diverted away from the field. However, two important discoveries set the stage for the most important applications of microbiology to steroid chemistry which were to follow in a few years. These were the conversion of cholesterol to 7-dehydrocholesterol with *Azotobacter species* [5] and 7-hydroxycholesterol with *Proactinomyces species* [6, 7].

The most important developments have come since 1949 as a result of the search for improved methods for making anti-inflammatory corticosteroids. Cortisone and hydrocortisone were established as the primary glucocorticoids of the adrenal cortex. After Hench and his collaborators [8] demonstrated the dramatic relief of the symptoms of rheumatoid arthritis with cortisone acetate there followed one of the greatest bursts of excitement and enthusiasm ever known in medical research. The demand for corticosteroids was high, but no ready source of 11-oxygenated precursors was known and chemical syntheses were very long and laborious.

Encouraged by the earlier successes of Mamoli and Horvath and the knowledge that deoxycorticosterone could be hydroxylated to corticosterone by adrenal perfusion [9], an Upjohn group looked for a microbiological method for the direct introduction of oxygen, hopefully at C-11, in ring C unsubstituted steroids.

Peterson and Murray [10] soon reported the successful 11α-hydroxylation of progesterone with a fungus. The process of 11α-hydroxylation was then improved and

extensively covered in a patent [11]. Soon afterwards another group at Upjohn [12] reported the microbial 11β-hydroxylation of Reichstein's Compound S. Although potentially of greater commercial interest for the manufacture of hydrocortisone the latter process was much less efficient than the former which soon became the key step in a commercial synthesis.

The consequences of the Peterson-Murray discovery were manifold. It brought about an upsurge of interest in microbiological conversions mainly as a consequence of the discovery that fungi could be used effectively. Also it founded a new technology for the manufacture of steroids and led indirectly to the ultrapotent corticosteroids which are in extensive clinical use today.

As a result of their attempts to convert 11-epicortisol to cortisol, Fried and Sabo [13] discovered the highly potent 9α -halogeno-steroids. Meanwhile, a Schering group had developed a new synthetic route to cortisol-11, 21-diacetate which they were unable to hydrolyse. Hopeful of repeating Mamoli's earlier success with the removal of an ester group with a bacterium, they tried *Corynebacterium simplex*. Instead of the expected hydrolysis, 1,2-dehydrogenation occurred. This heralded the discovery of the clinically important 1-dehydro steroids the first of which were prednisolone and prednisone. Subsequent studies [14, 15] showed that the conversion applied generally to a variety of substrates and soon led to improved methods for their production.

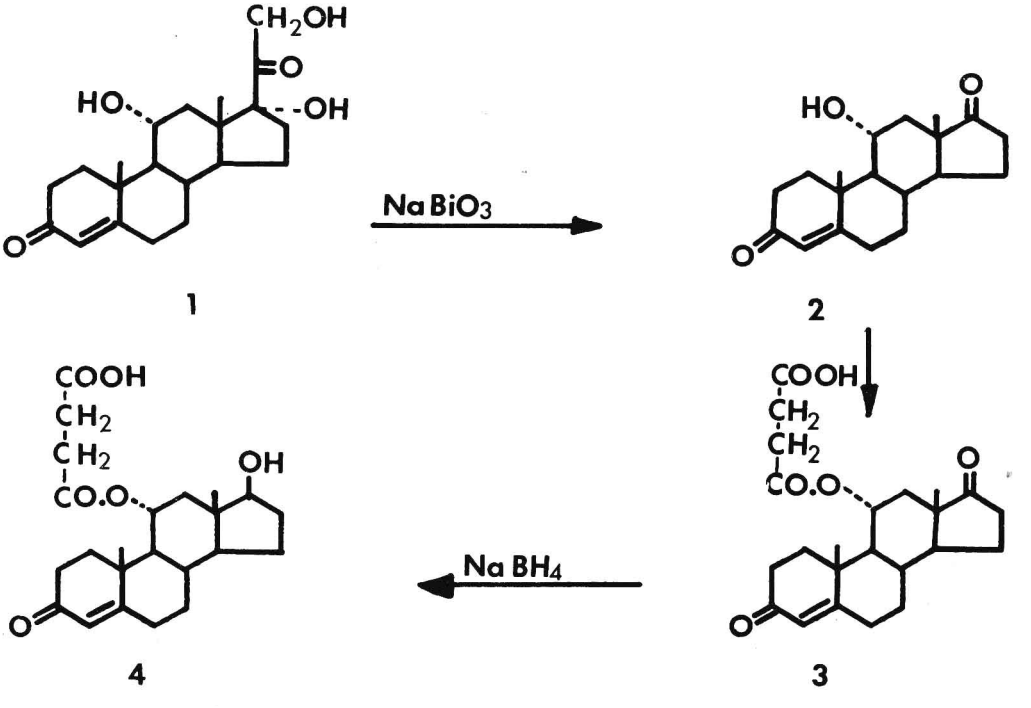

Fig. 1. Synthesis of testosterone-11α-hemisuccinate.

Another microbial conversion with interesting potential for making intermediates for hapten formation is the aromatisation of 19-hydroxyandrostanes [16, 17]. New synthetic methods have made 19-hydroxyandrostanes fairly readily available and microbial aromatisation could provide a route to otherwise inaccessible substances.

Fermentation processes continued to play a vital part in the manufacture of corticosteroids both natural and synthetic.

Since about 1966 Jones and his group at Oxford have been studying the action of some yeasts on steroid substrates with the object of rationalising the processes involved [18-24]. Using mono- and diketo-androstanes as substrates they have established a relationship between the position of the substituent and the position of hydroxylation, but the extent to which the substituent directs the point of attack by the organism is not yet certain. In 3- and 17-oxo 5α-androstanes, *Calonectria decora* introduces two equatorial hydroxyl groups at positions 12 and 15, and 1 and 6 respectively. In both cases the points of attack are about equidistant from the oxo-group. In polyfunctional molecules the preferred sites of attack may be blocked leading either to less favourable points of attack or to lower conversions.

Since microbiological transformations are mediated by enzymes they come within the general class of enzymatically catalysed chemical reactions. Thus in theory at least any reaction that can be brought about by an enzyme should find an illustration in the microbial transformation of a steroid. The known transformations in steroids include a wide range of oxidation and reduction reactions; esterification, hydrolysis and amide formation; as well as a number of miscellaneous reactions such as aromatisation, isomerisation and rearrangement, addition and elimination, and asymmetric synthesis.

The first generation of steroid haptens were fashioned by utilising one of the primary functional groups to form a derivative containing a carboxylic acid or amino group which was then conjugated with a protein to form the antigen. Antisera raised from these antigens mostly suffered from the disadvantage of low specificity and tended to crossreact substantially with related steroids. It was then suggested [25] that if the carboxylic acid (or amine) function was introduced at a remote site in the steroid the antigen formed by conjugation with protein would yield antisera with increased specificity. To test this hypothesis we utilised a number of 11α-hydroxysteroids prepared and kindly supplied by Mr. J. E. Ribbers of our Microbiological R&D Laboratory in the Netherlands. From them we have prepared the 11α-hemisuccinoxy derivatives of a variety of steroid hormones. Conditions for succinoylation of the 11α -hydroxy group were first established with 11α -hydroxyprogesterone. Because of the relatively hindered position of the 11α-hydroxyl group moderately forcing conditions are required and the steroid has to be heated for several days with succinic anhydride at 60-70°C in pyridine solution. The reaction tends to be rather dirty making the product difficult to purify. Fortuitously however once formed the 11α-hemisuccinoxy ester is stable enough to allow other reactions to be carried out under mildly alkaline conditions without substantial hydrolysis.

Having prepared 11α-hemisuccinoxy progesterone we turned our attention to the androgens. 11-Epicortisol (1) was oxidised with sodium bismuthate (Fig. 1) to 11α-hydroxyandrostenedione (2) which was succinoylated by the method already described. Selective reduction of the 17-oxo group in 11α-hemisuccinoxy androstenedione (3) with sodium borohydride under essentially the same conditions as described for androstenedione by Norymberski and Woods [26], furnished 11α-hemisuccinoxy testosterone (4).

The corresponding derivatives of the oestrogens were made from 11α-hydroxyoestrone (5) Fig. 2. Succinoylation under the usual conditions gave the di-hemisuccinate which was then selectively hydrolysed at C-3 with very little loss of the 11-ester. 11α-Hemisuccinoxy oestrone thus formed was reduced with sodium borohydride to give 11α-hemisuccinoxy oestradiol (8).

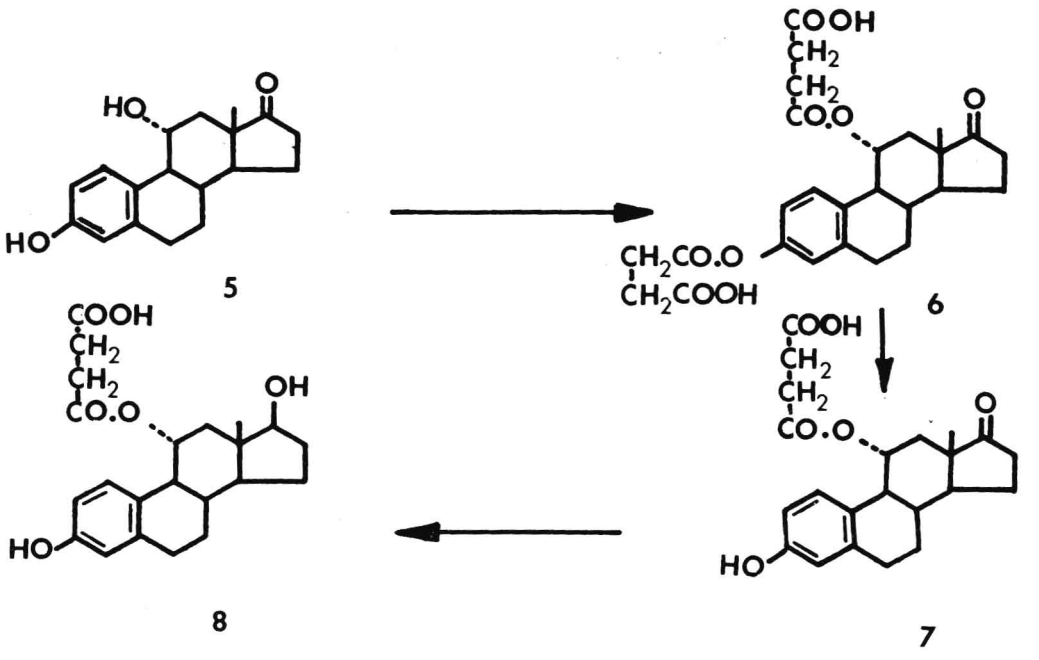

Fig. 2. Synthesis of oestradiol-11α-hemisuccinate.

The 11α-hemisuccinoxy derivatives of progesterone, androstenedione, testosterone, oestrone and oestradiol have all been conjugated with bovine serum albumin and used to raise antisera. The specificities of the antisera so formed (except that from androstenedione) have been described elsewhere and compared with antisera from other haptens of the hormones [27-31].

There is no doubt that we have come a long way since the original Mamoli observation. It is now possible to carry out a wide range of steroid transformations with micro-organisms and cell-free extracts and these processes provide access to many steroids substituted at unusual sites which would not otherwise have been so readily available.

Micro-organisms thus offer a very specialised facility for forming steroids which are not readily available by other means and which may have interesting applications for hapten formation. However, even with the wide knowledge of microbial enzyme transformations which has been built up during the last four decades we have not reached the stage where a new steroid can be made predictably from an untried combination of microbe and steroid substrate. The best way of applying our present knowledge in this field is to use well tried conversions for making otherwise inaccessible compounds and if necessary to modify them chemically. It could be very costly and unrewarding to set out to make a new compound without any previous experience in the field. Microbiological techniques are very specialised and require special equipment and expertise. The organisms themselves can be very capricious and like human beings do not always behave as expected. From the