SOME ASPECTS OF THE BIOCHEMISTRY OF OESTROGENS

A Thesis presented for the Degree

of

DOCTOR OF PHILOSOPHY

by

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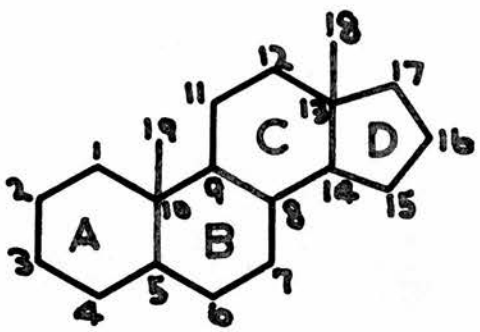
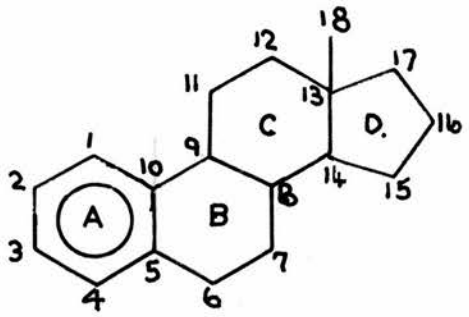
PREFACE

Steroids are compounds which contain the perhydrocyclopentenophenanthrene nucleus, the carbon atoms of which are numbered as shown:-



The steroids include a wide range of naturally occurring compounds among which are the sterols, bile-acids, sex-hormones, adrenocortical hormones etc.

The work presented in this thesis is concerned with one group of the sex hormones, the oestrogens, which are steroids containing eighteen carbon atoms, the angular methyl group attached to C-10 having been lost in the aromatization of the A-ring. The oestrogens therefore contain the following nucleus:-



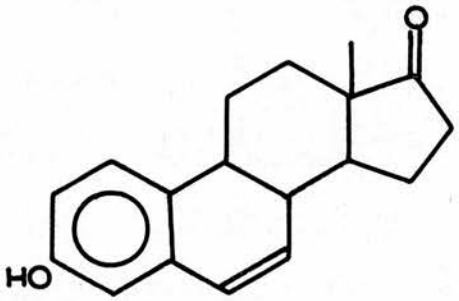
This nucleus bears a number of substituent groups giving rise to a range of derivatives. The nucleus is a reasonably flat structure and can be represented as planar. Thus as drawn above, one is looking at the "upper surface" of the nucleus and groups attached to it which project towards this "upper surface" (as does the angular methyl group attached to C-13) are said to have the β configuration and bonds joining them to the nucleus are drawn as a solid line. Conversely, groups which project towards the "lower surface" of the nucleus are said to have the α configuration and the bonds joining them to the nucleus are drawn as a dotted line. Groups whose configuration is not known are given the prefix ξ and their bonds are drawn as wavy lines. The position and configuration of each substituent is designated by the number of the carbon atom to which it is attached and the appropriate Greek letter, these designations being placed immediately before the description of the type of substituent,

e.g. 6α-hydroxyoestrone.

Positions 1,2, 3 and 4 of the oestrogen nucleus occur in the phenolic A ring and as this ring is a flat structure whose subsidiary bonds are completely coplanar with the ring, the question of configuration of any substituents in these positions does not arise.

The prefix △ is often used in trivial nomenclature to indicate unsaturation, the symbol being followed by the number of the lowest carbon atom involved e.g.

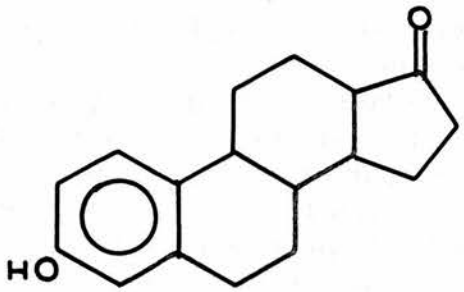
△-6 dehydro-oestrone would be



This practice is now considered to be incorrect. In formal nomenclature the number of the lowest carbon atom is followed by the suffix -ene, e.g. the above compound is 3-hydroxy oestra-1,3,5 (10), 6-tetraen -17-one. Where the two unsaturated carbon atoms are not numbered consecutively in the nucleus, both are quoted, the higher one being placed in

brackets as with -5, (10)- in the above example.

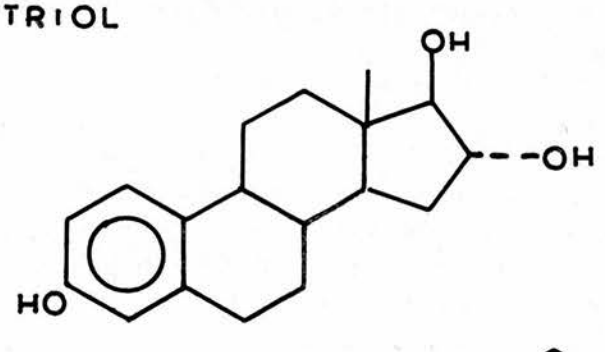
Elimination of the angular methyl group attached to C-13 in the oestrogens is indicated by the prefix 18-nor. Thus 18-noroestrone is

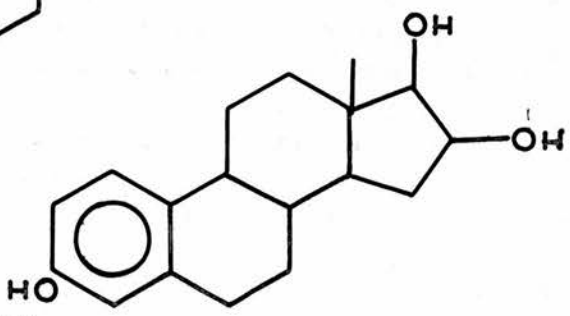


18-noroestrone

The prefix epi- indicates inversion of a substituent e.g. 16 epi-oestriol is the 16β-hydroxy epimer of oestriol in which the 16 hydroxy group has the α configuration.

OESTRIOL





16-EPI-OESTRIOL.

The trivial names and systematic names of the three most common oestrogens are:-

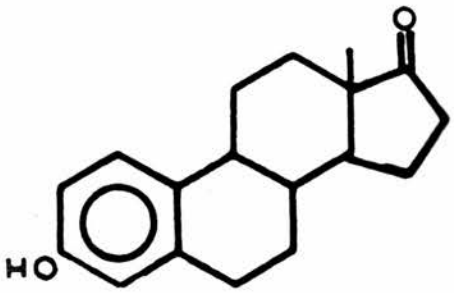
oestrone	3-hydroxyoestra- 1: 3 : 5 (10) trien-17-one.
oestradiol 17β	Oestra -1 : 3 : 5 (10) triene -3: 17β-diol.
oestriol	Oestra -1 : 3 : 5 (10) -triene 3 : 16α : 17β-triol.

SECTION 1

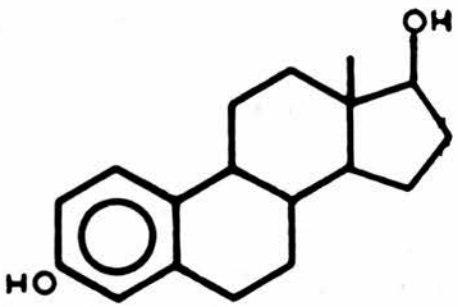
(1) INTRODUCTION

Until 1954 only four phenolic steroids of the oestrogen group had been isolated from natural sources. The first of these compounds to be obtained in a pure crystalline form and to be fully characterized was oestrone, which was isolated initially from human pregnancy urine by Doisy, Veler and Thayer (1929); by Butenandt (1929); and by Dingemanse, de Jongh, Kober and Laqueur (1930). Oestrone has subsequently been isolated from an extensive range of natural sources including mare pregnancy urine (Cartland, Meyer, Miller and Rutz, 1935) stallion urine, (Deulofeu and Ferarri, 1934) (Häussler, 1934), human male urine, (Dingemanse, Laqueur and Mühlbock, 1938), human placenta, (Westerfeld, MacCorquodale, Thayer and Doisy, 1938), stallion testes, (Beall, 1940), bovine adrenal glands, (Beall, 1939), bile of pregnant cows, (Pearlman, Rakoff, Cantarow and Paschkis, 1947) and from the urine of pregnant goats, (Klyne and Wright, 1957)

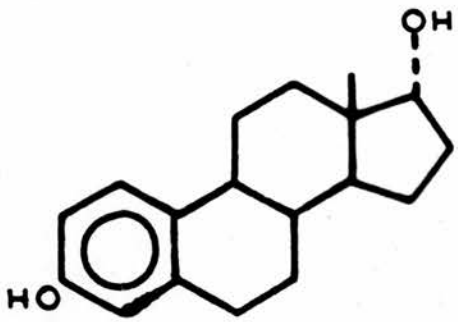
Shortly after the first isolation of oestrone a second oestrogen, oestriol, was isolated in the pure form from human pregnancy urine by Marrian (1930) and by Doisy, Thayer, Levin and Curtis (1930). Oestriol was also subsequently isolated from human placenta, (Butenandt and Browne, 1933), but has not yet been shown to be present in the ovaries of any species and neither is it present in the urine of the pregnant mare.



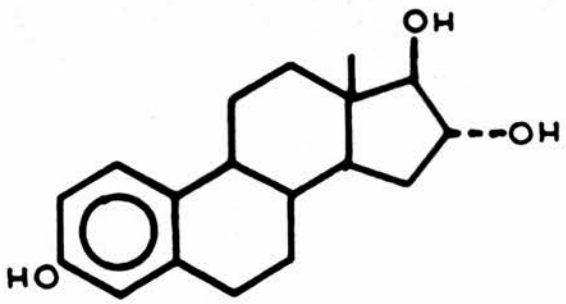
OESTRONE.



OESTRADIOL—17β.



OESTRADIOL—17α.



OESTRIOL.

FIG. 1.

The third "classical" oestrogen, oestradiol-17β was isolated in 1935 from the urine of pregnant mares (Wintersteiner, Schwenk and Whitman 1935). This compound has been further isolated from human pregnancy urine (Huffman, MacCorquodale, Thayer, Doisy, Van S. Smith and Smith 1940), human placenta (Huffman, Thayer and Doisy 1940), horse testes (Beall 1940), and stallion urine (Levin 1945; Levin 1949).

The isomeric oestradiol-17α was first isolated from the urine of pregnant mares (Hirschmann and Wintersteiner 1938) and has also been isolated from the urine of the pregnant goat (Klyne and Wright 1957) which contains none of the more common β-epimer. Reduction of oestrone to oestradiol-17α has been observed in the rabbit (Pearlman and Pearlman 1944), (Heard, Bauld and Hoffman 1941) and Stroud (1939) isolated a non-ketonic phenolic metabolite of oestrone, from the urine of rabbits receiving large injections of oestrone, which he suggested was oestradiol-17α. As yet this epimer has not been detected in human urine.

The structures of these four compounds are shown in Fig. 1.

Since 1954 no fewer than fourteen compounds of the oestrogen group have been isolated from, or detected in, human urine. This rapid development was due initially to the presentation of two very important pieces of work.

The first of these was the isolation by Marrian and Bauld (1954) of 16-epi-oestriol from human pregnancy urine. This isolation was made possible by using the technique of partition chromatography on celite columns, together with the highly specific Kober reaction as modified by Brown (1952) and Bauld (1954). The isolation of 16-epi-oestriol by the Edinburgh group led them to use these techniques in a search for other oestrogen derivatives in human pregnancy urine, especially those which might be logical intermediates between oestrone and oestriol, and oestrone and 16-epi-oestriol respectively.

The second piece of work was that of Beer and Gallagher (1955) who showed that following the administration of oestradiol-17β-[16-C^{14}] to humans, as much as 60 % of the administered radioactivity was recovered in urinary phenolic fractions. As only approximately 30% of this activity could be accounted for by the then-known oestrogens, it could only be concluded that urine contained other metabolites of oestradiol-17β which were of considerable quantitative importance. This, of course, lent fresh interest to the search for new urinary oestrogen derivatives.

Following the isolation of 16-epi-oestriol, Marrian and Bauld suggested that oestriol and 16-epi-oestriol might both be metabolic reduction products of 16-oxo-oestradiol-17β, a compound which had been prepared synthetically

(Huffman and Lott 1948). Accordingly, a search was initiated by Watson and Marrian (1955) for 16-oxo-oestradiol-17β in pregnancy urine. They detected a ketonic Kober-chromogen which could not be distinguished from 16-oxo-oestradiol-17β by partition chromatography and counter-current distribution in the solvent systems they used. Following the isolation of this material (Marrian, Loke, Watson and Panattoni 1957) the major component was shown to be identical with 16α-hydroxy-oestrone which had been prepared synthetically from oestrone (Leeds, Fukushima and Gallagher 1954). This material was found to contain approximately 20% of 16-oxo-oestradiol-17β, but as it was found that 16α-hydroxyoestrone undergoes rapid rearrangement into 16-oxo-oestradiol-17β under alkaline conditions, it was concluded that the 20% of the latter had been artifactually produced from the former during the course of its isolation. However, following the report of Levitz, Spitzer and Twombly (1956) on the detection, by reverse isotope dilution, of 16-oxo-oestradiol-17β-[16-C^{14}] and using fractionation procedures which did not entail the use of alkali, Layne and Marrian (1958) isolated this compound as well as 16α-hydroxyoestrone from human pregnancy urine. As the procedures used were specifically designed to preclude its artifactual formation from 16α-hydroxyoestrone, 16-oxo-oestradiol-17β was therefore established as a further natural oestrogen metabolite.

At the same time Layne and Marrian were conducting a search for 16β-hydroxyoestrone in human pregnancy urine as being the logical intermediate between oestrone and 16-epi-oestriol. This compound was detected by Brown, Fishman and Gallagher (1958) in human urine following the administration of oestradiol-17β-[16-C^{14}], and was also detected by Layne and Marrian (1958) as a spot corresponding to 16β-hydroxyoestrone by paper chromatography in the system chloroform/formamide provided that no alkali treatment had been used in the fractionation procedure. This chromatographic system had been shown to be capable of separating 16α-hydroxyoestrone, 16β-hydroxyoestrone and 16-oxo-oestradiol-17β and by its use, isolation of the compound in the pure crystalline form was achieved shortly afterwards.

More recently, Nocke, Breuer and Knuppen (1961) have isolated 17-epi-oestriol and 16,17-epi-oestriol from urine following the injection of 16α-hydroxyoestrone and 16β-hydroxyoestrone respectively to human males. This followed their previous finding that these compounds are produced in vitro with slices of human liver and ovary from 16β-hydroxyoestrone and 16-oxo-oestrone (Breuer, Knuppen and Pangels 1959; Breuer and Nocke 1959). 17-epi-oestriol has also been isolated from the urine of pregnant women as has 16,17-epi-oestriol (Breuer and Pangels 1961).

A metabolite of a rather unusual type was found by Kraychy and Gallagher (1957) in human urine following the administration of oestradiol-17β-[16-C^{14}]. Counter-current distribution of an ether extract of enzymically hydrolyzed human urine gave a peak of radioactivity with a partition coefficient different from that of either oestrone or 16-oxo-oestrone. Data from analyses and spectroscopy suggested that the compound was a methoxy derivative of oestrone and this was confirmed by identity of the compound with 2-methoxy-oestrone (Fishman 1958), prepared by the method of Loudon and Scott (1953) for the 0-hydroxylation of phenols. This work was confirmed by an independent group of workers (Engel, Baggett and Carter 1957).

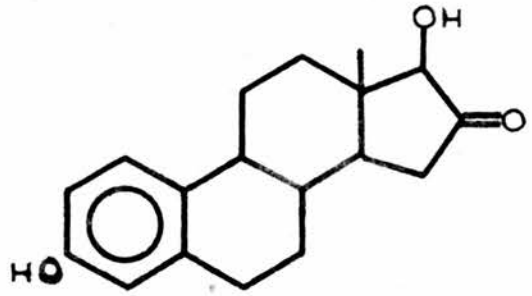
That 2-methoxylation of the A-ring seems to be a fairly general metabolic pathway was shown by the subsequent isolation from urine of 2-methoxyoestriol (Fishman and Gallagher 1958), 2-methoxyoestradiol-17α (Stimmel 1959) and the detection in urine of 2-methoxyoestradiol-17β (Frandsen 1959).

Fishman, Cox and Gallagher (1960) have also detected the somewhat similar 2-hydroxyoestrone in human urine, following the administration of oestradiol-17β-[16-C^{14}] to normal males. Although this compound has very similar partition characteristics to those of oestradiol-17β, the authors were able to separate the two by fractional crystallization after acetylation.

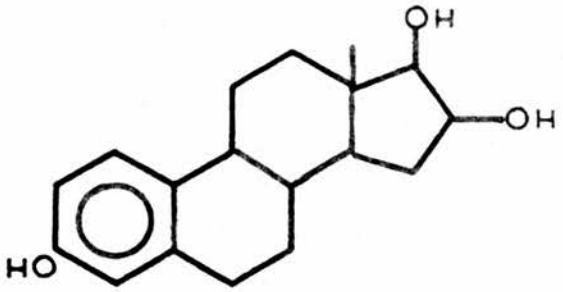
In trying to devise a method for the estimation of 16α-hydroxyoestrone and 16-oxo-oestradiol-17β in urine by column partition chromatographic analysis of ketonic phenolic extracts of enzymically-hydrolyzed late pregnancy urine, Loke, Watson and Marrian (1957) detected a Kober chromogen which was more polar than 16α-hydroxyoestrone and 16-oxo-oestradiol-17β. This they designated KC6, being the sixth Kober chromogen detected by the Edinburgh group in extracts of late pregnancy urine. Preliminary experiments on the purification of KC6 showed that it was in fact a mixture of two different chromogens, KC6A and KC6B, which could be separated by leaching the whole fraction with chloroform at -20°C. The more soluble KC6B was obtained in an amorphous form by this treatment, leaving a solid residue of KC6A which could be crystallized from methanol-benzene. That these fractions were two separate and distinct compounds was shown by their behaviour in the Kober reaction and on paper chromatography before and after sodium borohydride reduction.

KC6A, a positive Kober chromogen, gave a single spot having an RF value of 0.50 in the system methanol : water 66 : 33 /chloroform : benzene 36 : 64. Following reduction with sodium borohydride in methanol, the product showed an increase of about 200% in the intensity of the colour given in the Kober reaction, and gave a spot having the same RF value as the original material in the above system.

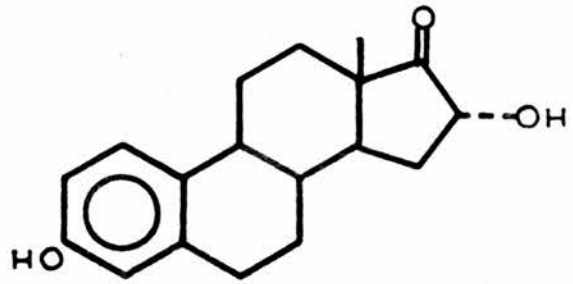
KC6B, also a positive Kober chromogen, gave a spot having an RF value of 0.50 in the above chromatography



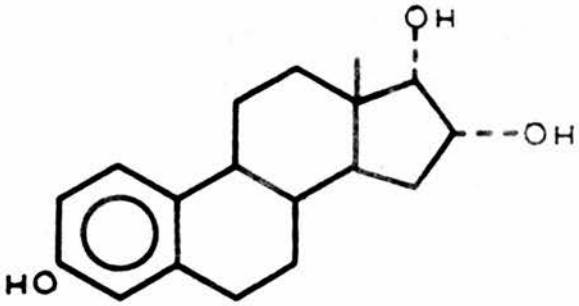
16-OXO-OESTRADIOL-17β.



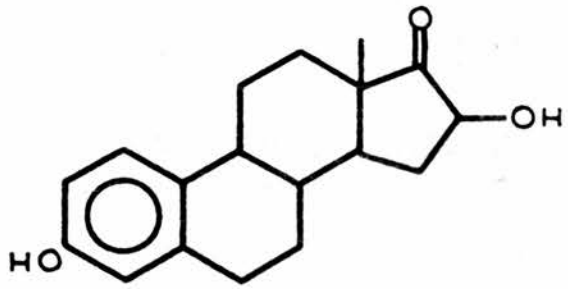
16-EPI-OESTRIOL.



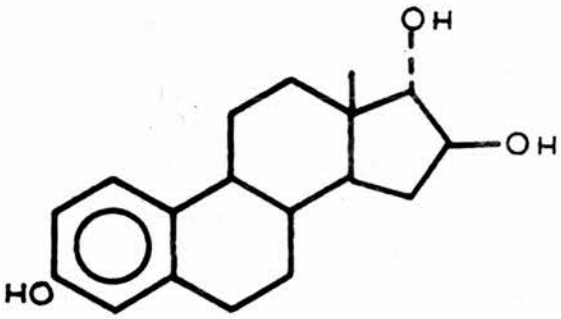
16α-HYDROXY-OESTRONE.



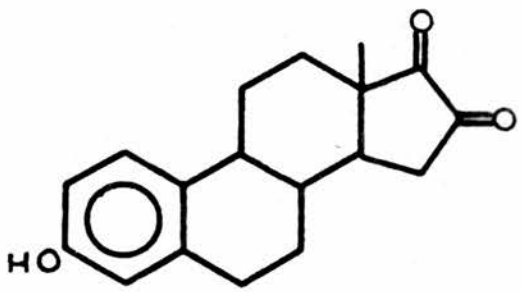
17-EPI-OESTRIOL.



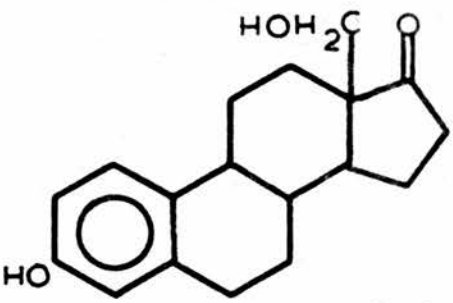
16β-HYDROXY-OESTRONE.



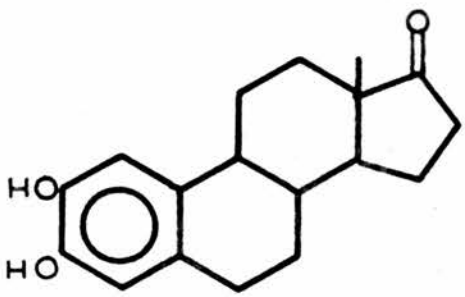
16,17-EPI-OESTRIOL.



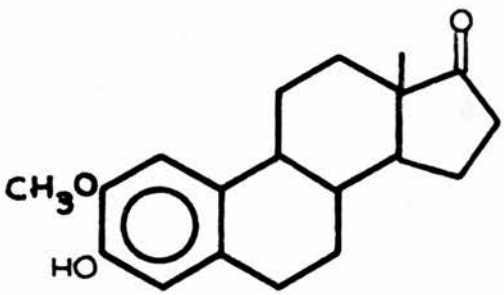
16-OXO-OESTRONE.



18-HYDROXY-OESTRONE.



2-HYDROXY-OESTRONE.



2-METHOXY-OESTRONE.

FIG. 2.

system. However, following reduction with sodium borohydride in methanol, the intensity of colour given by the product in the Kober reaction was approximately 40% less than that given by the original material, whilst on chromatography in the above system the RF value of the spot given was reduced to 0.15.

KC6A was subsequently isolated in the crystalline form and shown to be 18-hydroxyoestrone (Loke and Marrian 1958; Loke, Marrian, Johnson, Meyer and Cameron 1958).

KC6B was not obtained in the pure form but for reasons which will be given later it was suggested that it might be a 6-hydroxyoestrone.

The structures of these more recently isolated oestrogen derivatives are shown in Fig. 2.

In 1940 Longwell and Wintersteiner reported a method for the introduction of an oxygen function at C6 of oestradiol-17β by treatment of the diacetate with chromic acid at room temperature. However, it was not until some considerable time later that the report of Mueller and Rumney (1957) suggested that phenolic steroids substituted at C6 might have some biological significance. By using a carrier technique these authors showed that when oestradiol-17β-[16-C^{14}] was incubated with mouse liver microsomes it was converted into a 6-hydroxyoestradiol-17β and that this was further metabolised into 6-oxo-oestradiol-17β and a 6-hydroxyoestrone. As the 6-hydroxyoestradiol-17β so

produced was chromatographically identical with the compounds formed by sodium borohydride reduction of 6-oxo-oestradiol-17β, these 6-hydroxy derivatives were assigned the β-configuration.

Subsequent work by Wintersteiner and Moore (1959) suggested that these compounds should be designated 6-"α"-hydroxy-derivatives. This followed from their preparation of the epimeric 6-hydroxy-3,17β-oestradiols from 6-oxo-3, 17β-oestradiol by sodium borohydride reduction in methanol and by catalytic reduction in a neutral medium. By the former method of preparation 6"α"-hydroxyoestradiol-17β-triacetate could be obtained in good yield by direct crystallization of the acetylated reduction product. Chromatography of the mother-liquors failed to yield any of the 6"β"-hydroxyoestradiol-17β-triacetate. Catalytic reduction with platinum oxide in ethanol gave a mixture which after acetylation and chromatography yielded the 6"β"-hydroxyoestradiol-17β-triacetate as the sole crystallizable product, but in only approximately 20% of the theoretical yield. Meerwein-Ponndorf reduction with aluminium isopropylate yielded both epimers in approximately equal amounts but in low yield. They were separated by acetylation and chromatography on alumina.

Although the data obtained did not permit the definite assignment of absolute configurations to the two epimers, the authors suggested that the designations chosen

were probably correct on the following grounds; the epimer formed in good yield on sodium borohydride reduction should be the thermodynamically more stable one i.e. the 6α-epimer with the quasi-equatorial hydroxyl group. The apparently preferential formation of the 6β-epimer by catalytic reduction could be explained by approach of the catalyst from the relatively less-hindered α-face of the molecule. However, the sequence of elution of the two triacetates after absorption on alumina is the opposite of what one would expect if the former were the quasi-equatorial and the latter the quasi-axial epimer (Barton 1953). The authors suggested therefore that these designations be retained in inverted commas pending the acquisition of more definite configurational evidence.

This evidence was subsequently supplied by Breuer, Knuppen and Pangels (1961) in a most elegant manner which finally permitted the definite assignment of absolute configurations to the 6-hydroxyl-groups in the oestrogens. They utilised the aromatising enzyme preparation of Ryan (1959) to convert 6α, and 6β-hydroxylated C-19 steroids to the corresponding phenolic steroids, separating the epimers by paper chromatography in the system chloroform: ethyl acetate : 5 : 1/ formamide. As the configuration of the 6-hydroxyl groups in the neutral steroids is fully established (Balant and Ehrenstein 1952), they were therefore able to assign definite configurations to the two epimeric 6-hydroxy-

oestradiol-17β's. These configurations were in agreement with those proposed by Wintersteiner and Moore (1959).

In the course of his preliminary investigations on KC6B, Loke (1958) was unable to isolate pure crystalline material, but from the impure semi-crystalline material which he did obtain he gathered the following information:-

(1) It is a ketonic, phenolic Kober chromogen.

(2) The sodium borohydride reduction product of KC6B is of about the same polarity as oestriol in the system methanol : water 66 : 33/chloroform : benzene 36 : 64. Its methyl ether however is more polar than that of oestriol.

(3) KC6B shows a decrease of c.a. 40 % in the intensity of colour given in the Kober reaction following sodium borohydride reduction.

(4) It is stable to treatment with N-NaOH at room temperature.

(5) On treatment with acidified methanol it gives an "oestrone-like" product i.e. a spot of approximately the same polarity as oestrone in the system chloroform : benzene 1 : 1 /formamide. This does not revert entirely back to KC6B on treatment with aqueous acid.

(6) KC6B does not reduce blue tetrazolium.

(7) In the Kober reaction it shows only one peak at 512.5 mμ, unlike 18-hydroxyoestrone which shows another peak at 420 mμ.

The above evidence, together with the chromatographic mobility of the compound was consistent with the suggestion that it might be a 6-hydroxyoestrone. However, in addition to the lack of pure crystalline material on which to work, Loke was further hindered in his efforts to identify KC6B by the lack of authentic 6-hydroxyoestrone for comparative purposes. Although a satisfactory method exists for the introduction of a 6-keto-group into the oestrogen molecule (Longwell and Wintersteiner 1940) the subsequent reduction of this group whilst retaining a ketone group at C-17 is extremely difficult, due to the greater reactivity of the latter. Loke attempted to synthesize 6-hydroxyoestrone by subjecting 6-hydroxyoestradiol-17β-triacetate to Oppenauer oxidation followed by hot acid hydrolysis, to give a mixture of 6-hydroxyoestrone and 6-dehydro-oestrone. These were separated on a celite column and although he managed to obtain a crystalline product in this way, the authenticity of the material was never proved conclusively. However, the infra-red spectrum of this material was found to be essentially the same as that given by KC6B.

The synthesis of 6α-hydroxyoestrone was reported by Knuppen and Breuer (1961) and full details of the method used by these authors are given in Section 11 of this thesis.

From Loke's work on KC6B it seemed probable that human pregnancy urine might contain a 6-hydroxyoestrone.

This of course suggested the possibility that human pregnancy urine might also contain other 6-hydroxylated-oestrogen derivatives and therefore, as a preliminary, 6-oxo, and 6α-hydroxy-derivatives of oestradiol-17β and oestriol were prepared by the method of Longwell and Wintersteiner (1940) and their properties were examined in some detail. In particular the partition coefficients of these compounds were examined for the systems ether/water and ethyl-acetate/water, and the values obtained showed that these 6-hydroxylated-oestrogen derivatives were more water-soluble than had been expected. This was especially true of 6α-hydroxyoestriol and it was apparent that if this compound were present in human pregnancy urine it would not be extracted by the procedures commonly used for extraction of oestrogens from urine.

Accordingly, Marrian and King (unpublished) devised an extraction procedure based on the partition ratios found for 6α-hydroxyoestriol, which would have given c.a. 60% of any 6α-hydroxyoestriol present and applied this to a pool of enzymically hydrolyzed late pregnancy urine. Examination of the extract by paper chromatography showed that there was nothing present corresponding to 6α-hydroxyoestriol. It was concluded therefore that 6α-hydroxyoestriol was not present in human pregnancy urine in significant amounts and this line of enquiry was therefore abandoned.

The main line of enquiry in the work presented in

this thesis was to try to get more conclusive evidence for the presence of 6α, or 6β-hydroxyoestrone in the KC6B fraction of human pregnancy urine in order to confirm Loke's preliminary work. It was initially intended to do this by preparing a sufficient quantity of KC6B-concentrate to enable the isolation of a crystalline product, which could then be purified to such a degree as to give unambiguous results in the elucidation of its structure. Unfortunately this was not possible and the problem had to be attacked by a different method which would give a conclusive result without involving actual isolation. This was eventually found to be possible due to the unique nature of the 6-oxygenated-oestrogen derivatives and full details of the method used will be found in Section 111 of this thesis.

(2) EXPERIMENTAL METHODS

(a) Preparation of Ketonic, Phenolic Fractions from Late Pregnancy Urine.

Late pregnancy urine i.e. urine from the thirty-second week onwards, was collected and worked up to give ketonic, phenolic fractions as described by Loke et al. (1958). The urine was worked up in four-litre batches, the pH of each batch being adjusted to 4.6 with glacial acetic acid, followed by the addition of 100 mls.of M. acetate buffer, pH 4.6. Hydrolysis was then effected by incubation at 37°C for forty-eight hours with 4×10^6 Fishman Units of β-glucuronidase per litre (the enzyme was prepared as the acetone-dried powder from the common limpet, patella vulgata by the method of Dodgson and Spencer (1953). It was assayed for β-glucuronidase activity with phenolphthalein glucuronide as substrate by the method of Fishman (1948).

To each litre of urine was added 1 ml. of Bradosol (β-phenoxyethyldimethyldodecyl-ammonium-bromide; Ciba Laboratories Ltd.) and 150 gs. of sodium chloride to minimise emulsion formation. The urine was then extracted with ether, washed with 5% sodium bicarbonate solution and water, dried over anhydrous sodium sulphate and finally distilled to about one-eighth of the original volume.

This ether residue was then subjected to a phenolic separation by extracting three times with an equal volume of chilled N. sodium hydroxide. This was immediately run into

enough chilled 10 N. sulphuric acid to make the final solution acid to litmus and was then extracted with ether, washed with 5% sodium bicarbonate and water, dried over anhydrous sodium sulphate and distilled to dryness.

The phenolic residues thus obtained were pooled to give a final residue equivalent to 100 litres of urine and each such residue was then subjected to a Girard separation as described later. In order to ensure complete removal of non-ketonic material the ketonic fraction was subjected to a second Girard separation.

(b) Girard Separation.

The steroid was dissolved in ethanol and one-fifth of the volume of glacial acetic acid was added. An excess of Girard Reagent-T (trimethylammonium-hydrazide-chloride) was then added and the mixture allowed to stand overnight at room temperature. The solution was then chilled and to it was added chilled water containing enough N. sodium-hydroxide to neutralise nine-tenths of the acid present (determined by previous titration). This solution was then extracted three times with an equal volume of ether, which was washed once with one-third volume of water, the water washing being added to the aqueous phase. The ether was further washed twice with one-third volume of 5% aqueous sodium bicarbonate, once with one-third volume of water, dried over anhydrous sodium sulphate and taken to dryness to give the NON-KETONIC fraction.

The aqueous phase from this extraction was acidified to litmus with 10 N. sulphuric acid and allowed to stand at room temperature for one hour in order to hydrolyze the Girard complex. The solution was then extracted three times with an equal volume of ether and the ether washed twice with one-third volume of 5% sodium bicarbonate solution and once with one-third volume of water. It was then dried over anhydrous sodium sulphate and taken to dryness to give the KETONIC fraction.

(c) Column Partition Chromatography of Ketonic Phenolic Fractions.

Large-scale partition chromatograms on celite columns in the solvent system benzene : hexane 80 : 20/ methanol : water 70 : 30, were carried out at 25°C as described by Marrian et al. (1957). The ketonic phenolic residue from approximately 200 litres of urine was applied to each column and the eluate collected in 40 ml. fractions. An aliquot was taken from each fraction for estimation by the Kober reaction and the total eluate was seen to be divisible into three main regions corresponding to oestrone plus 2-methoxyoestrone; the α-ketols; and the KC6 region. The appropriate fractions from each region were then pooled together and taken to dryness.

(d) Paper Chromatography.

A very large number of chromatographic systems have been used in the course of this work and explicit details

of each one will be given where necessary. However, these systems fall into two distinct types:- impregnated paper systems such as those used by Zaffaroni, Burton and Keutmann (1950) and systems of the Bush (1952) type. In the former type of system the paper was impregnated with the stationary phase, e.g. formamide, by dipping it through a trough containing a 2 : 1 methanol : formamide solution, laying it on a horizontal sheet of glass, and blotted by covering with a piece of dry paper and laying another sheet of glass on top of this. The paper was then dried horizontally at 37°C for 45 minutes to remove excess methanol. The steroid, in methanolic solution, was then spotted on to the paper which was equilibrated in the tank for at least thirty minutes before adding mobile phase. Whatman No. 42 was the paper usually employed for this type of system.

For the Bush type of systems the steroid was spotted on to dry paper and this was then allowed to equilibrate in the tank, containing both mobile and stationary phases, for at least two hours and preferably overnight before adding mobile phase. A wide variety of chromatography papers were used depending upon the type of system.

Steroids were applied to the papers in methanolic solution by means of a 0.1 ml. blood pipette calibrated in divisions of 0.01 ml., the tip of which had been drawn out to a fine capillary. In order to minimise the area of the spot,

the methanol was dried off immediately with a jet of compressed air or nitrogen .

(e) Location of Oestrogens on Chromatograms.

The method most commonly employed in the present work for locating oestrogens on chromatograms was that of Davies and Mitchell (1954). The paper was sprayed with a solution of 4 : 1 water : Folin and Ciocalteu's reagent (B.D.H. Ltd.) and was then suspended in an atmosphere of ammonia in order to develop the blue colour characteristic of phenols.

The potassium cyanide/potassium ferricyanide reagent of Barton, Evans and Gardner (1952) was found to be more sensitive than Folin and Ciocalteu's reagent but suffered from the disadvantage of being less suitable for preserving a permanent record, as the whole paper eventually became a uniform blue colour. The paper was sprayed with a freshly-prepared solution of a mixture of equal volumes of 1% aqueous potassium cyanide and 1% aqueous potassium ferricyanide and was then allowed to stand in air for a few minutes, when the oestrogen spots developed an intense blue colour.

Oestrogen methyl ethers were detected by immersing the paper in a porcelain dish, the bottom of which had been first covered with a film of fuming sulphuric acid. Examination of the paper under ultra-violet light revealed the oestrogens as spots of intense yellow fluorescence.

(f) Kober Reaction

The Kober reaction as modified by Brown (1955) and Bauld (1956) was used. In all cases, unless otherwise stated, Bauld's "oestriol reagent" was used. Approximately 50 mgs. of hydroquinone was added to the tube containing a dry residue of the oestrogen and 2.6 mls. of the reagent added. The tube was heated for twenty minutes in a vigorously boiling water bath with frequent shaking. It was then cooled and a further 50 mgs. of hydroquinone was added, followed by the addition of 0.7 mls. of water. The tube was well shaken and again heated in the bath for fifteen minutes with frequent shaking. The final solution was cooled and the optical density read against a reagent blank on the Unicam S.P. 500, in 1 cm. light path glass cells, over the range 400 mμ - 550 mμ. The absorption maximum of the typical Kober chromogen is at 512.5 mμ and in the presence of urinary contamination the correct absorption at this wavelength was determined by applying the colour correction of Allen (1950). The average of the readings at 480 mμ and 545 mμ was subtracted from that at 512.5 mμ to give the corrected reading at this wavelength.

(g) Ittrich Extraction of Kober Colour.

In the presence of heavy contamination by urinary pigments the Kober colour was in some cases extracted by the method of Ittrich (1958). The normal Kober colour reaction was carried out as above and the final solution was

diluted with an equal volume of water. This was chilled by immersing in a bath of ice-water and half of the total volume of a 4% solution of p-nitrophenol in tetrachloroethane was added. The mixture was then vigorously shaken for one minute and placed in the dark at 0°C until it could be centrifuged in a refrigerated centrifuge at 2,000 r.p.m. for 10 minutes. The upper aqueous layer was then removed and the lower layer containing the pink Kober colour was read against a reagent blank on a Unicam S.P. 500, in 1 cm. light path glass cells, over the range 500 mμ - 580 mμ. The Kober colour shows a maximum absorption at 536 mμ in this solvent and it also exhibits a striking greeny-yellow fluorescence. The Allen colour correction was applied as before, taking the average of the readings at 508 mμ and 564 mμ.

(h) Methylation of Oestrogens.

Methylation was effected by the method of Brown (1955). To the dry residue of oestrogen in a test-tube was added 10 mls. of 0.4N sodium-hydroxide plus 0.18 gs. of boric acid and the solution was allowed to stand at 37°C for five minutes. Dimethyl sulphate (0.2 mls) was then added, the tube was thoroughly shaken and it was then allowed to stand at 37°C for twenty minutes. A further 0.2 mls of dimethyl sulphate was then added together with 0.4 mls. of 5N sodium hydroxide and the tube was kept at 37°C for another thirty minutes. Hydrogen peroxide (0.5 mls.) and 2 mls. of 5N sodium hydroxide were added and the solution

extracted once with 20 mls. of benzene, washed three times with 10 mls. of water, dried over anhydrous sodium sulphate and taken to dryness.

(i) Infra-red Spectroscopy.

Infra-red spectra were determined on a Perkin-Elmer Infracord Spectrophotometer. The dry oestrogen residue was dissolved in 100 µls. of specially purified and dried chloroform and this was read in a micro-cell having rock-salt faces against a similar cell containing the same pure chloroform. Prior to reading the oestrogen solution, a determination was made with both cells containing only chloroform, in order to detect any bands caused by discrepancies between the two cells. The spectra of unknown compounds were compared directly with those given by authentic standards determined under the same conditions.

(j) Sulphuric Acid Spectra.

To the dry residue of oestrogen in a test-tube was added 4 mls. of concentrated A.R. sulphuric acid and the tube was allowed to stand in the dark at 25°C for two hours. The solution was then read against a reagent blank, in 1 cm light path silica cells, on the Unicam S.P. 500 or the Unicam S.P. 700 automatic recording spectrophotometer, over the range 200 mµ - 520 mµ. In some instances the solutions were transferred back to the appropriate tubes and allowed to stand in the dark at 25°C and the spectra determined again after a further twenty-two hours.

(k) Melting Point Determinations.

Unless otherwise stated, all melting point determinations were made in sealed evacuated capillaries and the values quoted are uncorrected for emergent stem.

(l) Fast Black Salt K Derivatives.

Fast Black Salt K derivatives of the oestrogens were made by the method of Heftmann (1950). Approximately 10 μgs of oestrogen in 0.1 mls. of methanol plus 0.1 mls. of 10% sodium carbonate and 0.2 mls. of a freshly-prepared saturated solution of Fast Black Salt K (diazotised p-nitrophenyl azo-dimethoxy aniline) were heated in a boiling water bath for ten minutes. After cooling, the derivatives were extracted once with 0.2 mls. of benzene and this extract was spotted directly on to Whatman No.1 paper. This was then chromatographed in the system 2 : 1 toluene : petroleum ether (40^0 - 60^0)/ethanol : water 30 : 70.

(m) Pipe-Stem Crystallizations.

The material to be crystallized was transferred to a small weighed tube and weighed. Crystallization was then carried out with a suitable solvent or mixture of solvents. Meanwhile, a length of the stem from a clay-pipe was taken and the ends were rounded off with a file. The stem was then thoroughly cleaned by washing exhaustively with water and then boiling for three hours with six separate portions of methanol. The clean pipe-stem was then placed in a stoppered tube and cooled in the refrigerator. Following

crystallization of the material, the pipe stem was then placed in the small tube such that its end rested on the bottom of the tube, dipping into the mother liquors. The whole was then placed in a large stoppered tube and kept in the refrigerator until the mother liquors had been absorbed by the pipe stem. This was then carefully withdrawn from the small tube, tapping carefully to remove any adhering crystals, and the mother liquors recovered by eluting with hot methanol.

SECTION 11

(1) 6-OXYGENATED DERIVATIVES OF OESTRONE

(a) 6-oxo-oestrone-acetate.

Oestrone (2.007 gs) was acetylated with acetic anhydride in pyridine by refluxing for thirty minutes under an air condenser. After cooling overnight, the acetate was precipitated by addition of crushed ice and water, filtered off, washed copiously with water until free of pyridine and dried in vacuo. The crude acetate (2.324 gs) melted at 125° - 127°C. This was dissolved in 8.0 mls.of glacial acetic acid and a mixture of 1.98 gs.of chromium trioxide dissolved in 1.6 mls.of water plus 12 mls.of glacial acetic acid was added and allowed to stand at room temperature for twenty-four hours. Excess chromic acid was reduced with 3.0 mls.of ethanol and the mixture was diluted with 200 mls.of water before being extracted with ether. The extract was washed with 50 ml.portions of saturated aqueous sodium bicarbonate until the washings showed a pink colour, then with 5% (w/v) aqueous sodium carbonate saturated with sodium bicarbonate, and finally with water. Evaporation of the dried ether residue gave 1.425 gs.of crude non-acidic residue, which on crystallization from 6.0 mls.of methanol yielded 1.065 gs.of crystals melting at 178° - 190°C. This was recrystallized from 15.0 mls.of methanol to give 0.304 gs.of material melting at 192° - 195°C.

This material was combined with the products of three similar preparations and the whole was recrystallized overnight at 0°C from methanol to yield crystals melting at 194° - 196°C; (α) $^{18.0}_{D}$ + 34.65° in ethanol (C, 0.247); Found:- C, 73.8; H, 6.8. Calculated for $C_{20}H_{22}O_4$:- C, 73.6 ; H, 6.8%.

The ultra-violet absorption spectrum in ethanol showed maxima 246 - 249 mμ (E 10,785) and at 297 - 300 mμ (E 2,300). This curve very closely resembles that quoted by Longwell and Wintersteiner (1940) for 6-oxo-oestradiol-17β-diacetate.

(b) 6-oxo-oestrone.

The mother liquors from the final crystallization of 6-oxo-oestrone-acetate were taken to dryness and recrystallized from 10 mls.of methanol to give 123.7 mgs.of material melting at 183° - 192°C. This was hydrolyzed with 3.0 mls.of 20% (w/v) potassium hydroxide in methanol for 24 hours at room temperature under an atmosphere of nitrogen. After dilution with 20 mls.of water, the solution was acidified to phenolphthalein with carbon-dioxide and extracted three times with 10 ml.portions of ethyl acetate. After two washings with 5 ml.portions of water, the extract was dried over sodium sulphate and evaporated to dryness to give 100.9 mgs.of residue. Crystallization of this residue twice from methanol yielded 32.4 mgs.of crystals melting at 250°-257°C. Analysis of this material showed it to be impure and

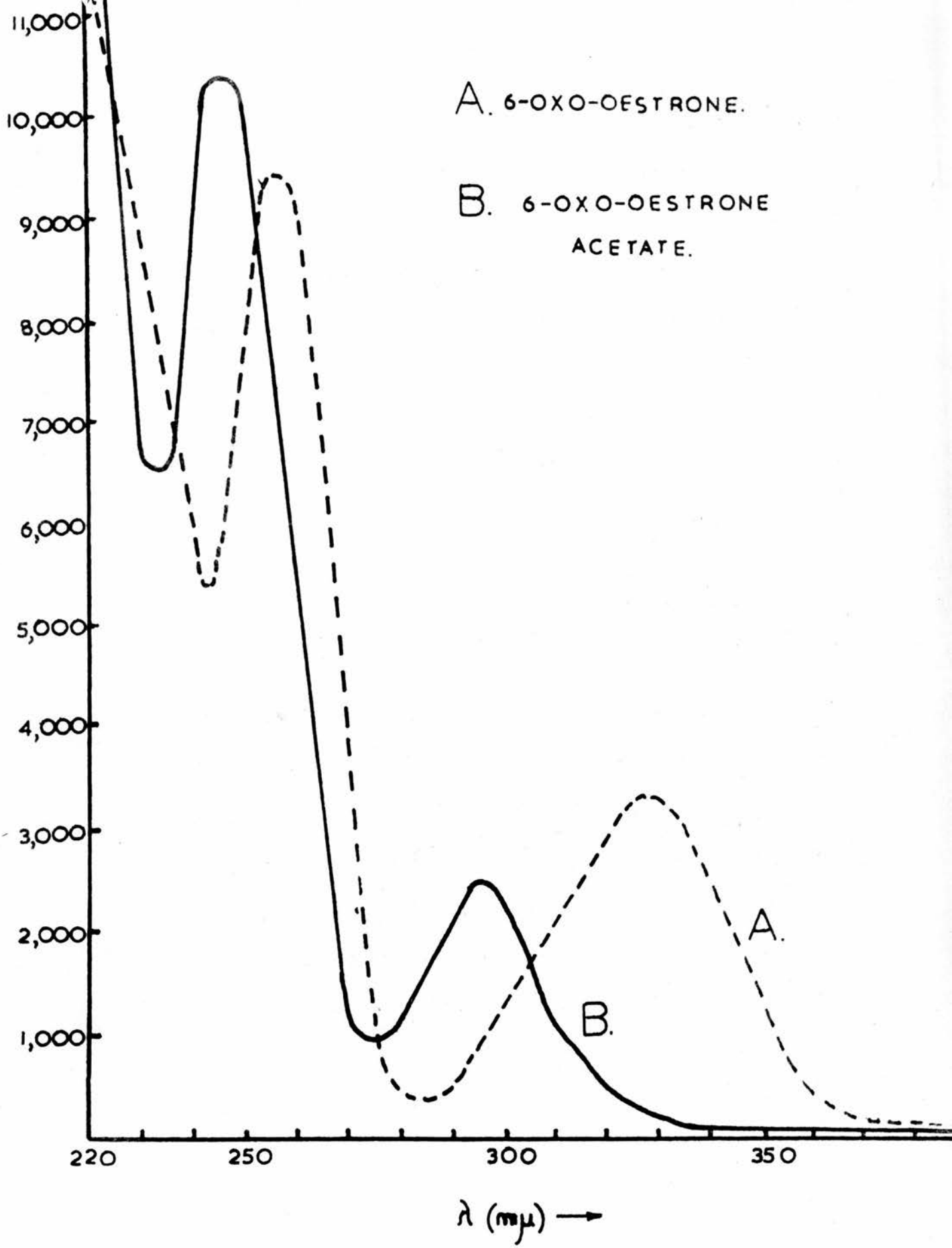

A. 6-OXO-OESTRONE.
B. 6-OXO-OESTRONE ACETATE.
11,000
10,000
9,000
8,000
7,000
6,000
5,000
4,000
3,000
2,000
1,000
220
250
300
350
λ (mμ) →
A.
B.

FIG. 3.

as it looked very brown and dirty it was recombined with the mother liquors and decolourized by heating in methanolic solution with activated charcoal. This gave 56.9 mgs.of residue which after two crystallizations from methanol yielded 36.5 mgs.of crystals melting at 245° - 246°C (sealed evacuated capillary); (α) $^{22.0}_{D}$ + 97.45 in ethanol (C, 0.5136); Found: C, 75.4; H, 6.3. Calculated for $C_{18}H_{18}O_3$:- C, 76.6, H, 6.4%. The ultra violet absorption spectrum in ethanol showed maxima at 255 mμ (E 9441) and 327 mμ (E 3282), as shown in Fig. 3.

(c) 6α-hydroxyoestrone.

Soloway, Deutsch and Gallagher (1953) described the selective reduction of steroid 3, 20-diketones by sodium borohydride. Reduction of 6-oxo-oestrone with sodium borohydride in methanol at room temperature showed that the oxygen function at the 17-position was reduced in preference to that at the 6-position as the product of the reaction was just less polar than 16-epioestriol in the system chloroform/ formamide, whilst its ultra-violet absorption spectrum in ethanol showed maxima at 256 mμ and 328 mμ, characteristic of 6-oxo-oestrogens (Longwell and Wintersteiner 1940).

It was imperative therefore to find some method of protecting the oxygen function at the 17-position before subjecting that at the 6-position to reduction and it was decided that this might be achieved by formation of a Δ 16-17-enol acetate. Moffett and Weisblat (1952) found that

isopropenyl acetate, in the presence of an acidic catalyst, converted many steroid ketones to enol-acetates of the same type as those reported by the use of other reagents, e.g. acetic anhydride with acetyl chloride (Inhoffen 1936) (Westphal 1937), or acetic anhydride with sodium or potassium acetate (Ruzicka and Fischer 1936), or acetic anhydride with p-toluene-sulphonic-acid (Marshall, Kritchevsky, Lieberman and Gallagher 1948) (Kritchevsky and Gallagher 1949).

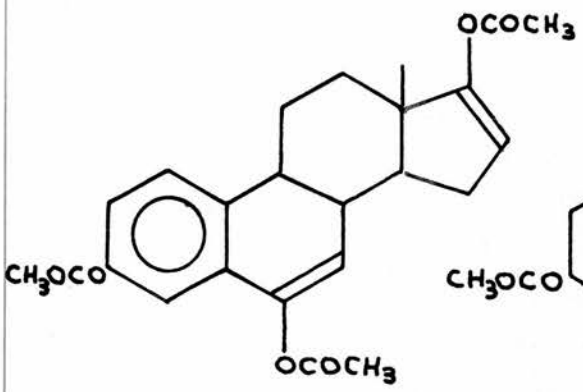
Accordingly 6-oxo-oestrone-acetate (29.99 mgs) and p-toluene-sulphonic-acid (107.0 mgs) in 20 mls of isopropenyl acetate were refluxed under an air condenser. After two hours, 2 mls.of isopropenyl acetate was very slowly distilled off from the mixture and refluxing was then continued. After six hours when 6 mls.of isopropenyl acetate had been distilled off, a further 10 mls.of isopropenyl acetate was added and the process repeated to give a total refluxing time of twelve hours and a total distillate volume of 12 mls. The reaction mixture was extracted with ether which was then washed with saturated bicarbonate and water, dried over sodium sulphate and evaporated to dryness.

Purification of the product was effected by chromatography on a column of 5 gs.of acid-washed alumina equilibrated in benzene. The material was applied in 3.0 mls. of benzene, eluting with the same solvent and taking 5 ml. fractions. Cuts 3, 4 and 5 were pooled and taken to dryness to give 23.5 mgs.of residue which on crystallization from ethanol yielded 7.3 mgs.of crystals melting at 212^0 - 217^0C.

Analysis of this material showed C, 71.6; H, 6.4.

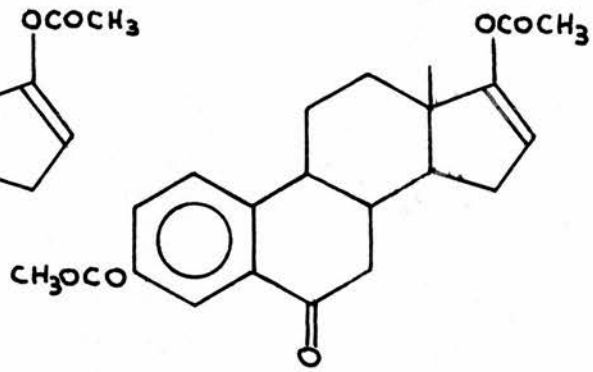
Enol acetylation of 6-oxo-oestrone acetate might conceivably have yielded either of two products, or a mixture of both, as follows:-

1. -the dienol triacetate of 6-oxo-oestrone.



C - 70.2%
H - 6.4%

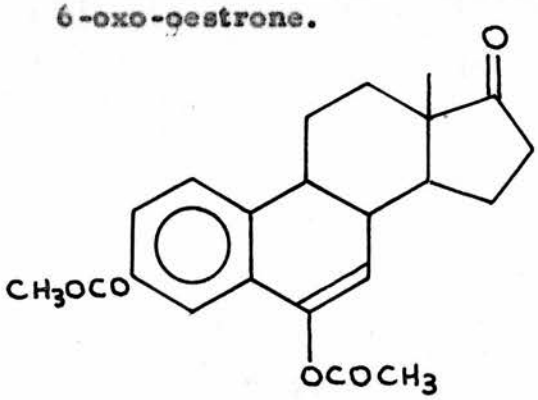
11. -the mono-enol diacetate of 6-oxo-oestrone.



C - 71.7%
H - 6.6%

The analysis figures for the reaction product strongly suggest that it has structure 11. There remains however an alternative structure to that shown in 11 which is given below:-

111. - the alternative mono-enol diacetate of 6-oxo-oestrone.



This structure will of course have the same carbon and hydrogen values as that shown in 11.

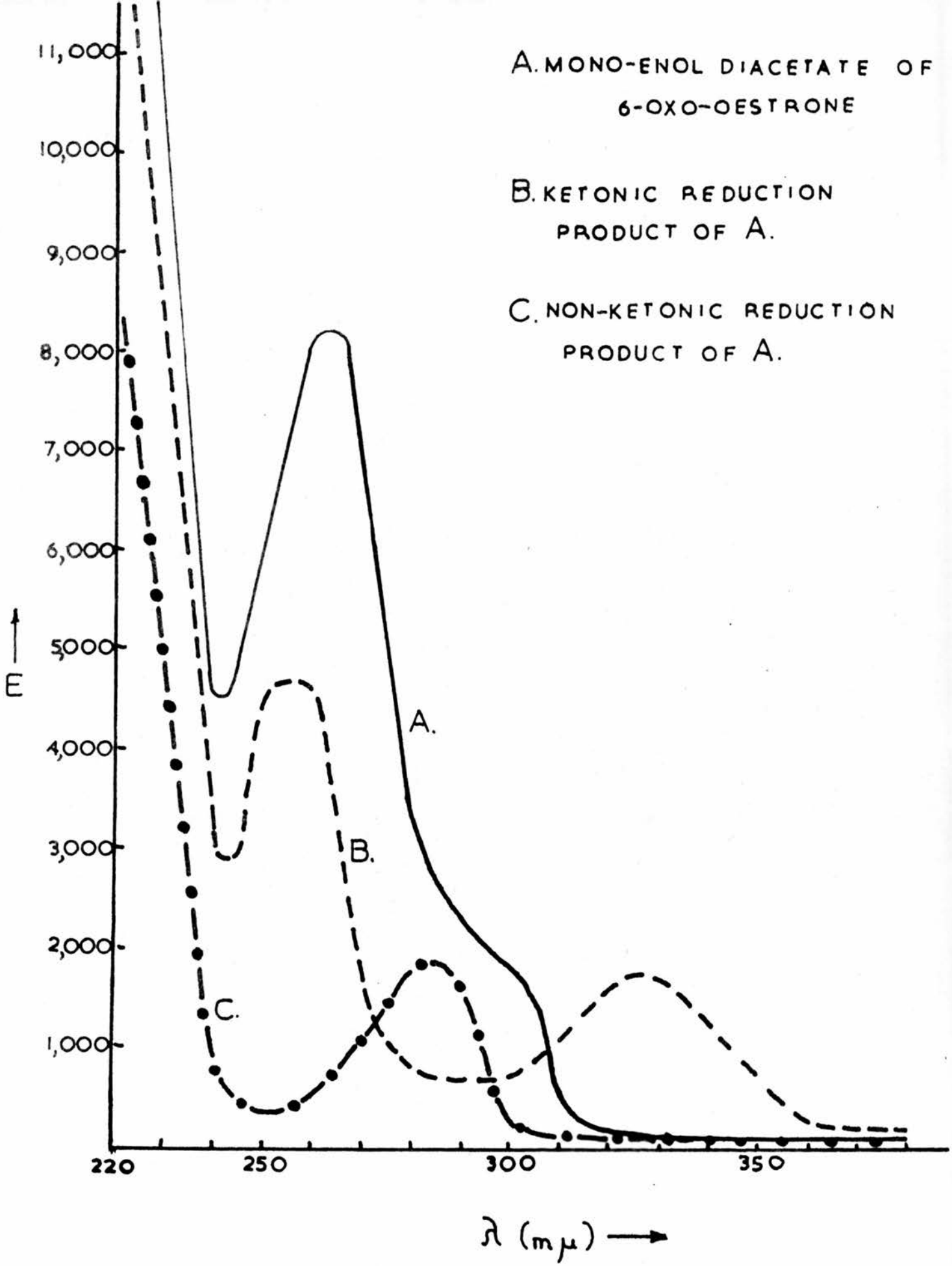

A. MONO-ENOL DIACETATE OF 6-OXO-OESTRONE
B. KETONIC REDUCTION PRODUCT OF A.
C. NON-KETONIC REDUCTION PRODUCT OF A.
11,000
10,000
9,000
8,000
7,000
6,000
5,000
4,000
3,000
2,000
1,000
E
A.
B.
C.
220
250
300
350
λ (mμ) ⟶

FIG. 4.

The ultra-violet absorption spectrum of the reaction-product in ethanol showed a single maximum at 261 - 262 mμ; with shoulders at 285 - 290 mμ and 295 - 300 mμ. Longwell and Wintersteiner (1940) showed that acetylation of 6-oxo-oestrone to give the diacetate, results in a shift in the ultra-violet absorption maxima towards a lower wavelength. Thus the diacetate gives the same type of curve with maxima at 250 mμ and 300 mμ. Thus the ultra-violet absorption spectrum given by the reaction product does not conform at all well to that which one would expect from structure 11 which has a 6-oxygen function, (see Fig. 4).

Sodium borohydride reduction of this material in methanol under various conditions gives rise to a number of products. By chromatography in the system chloroform/formamide and by their ultra-violet absorption spectra in ethanol these were shown to be mainly a 6-hydroxyoestradiol-17β; 6-oxo-oestradiol-17β and a little 6-oxo-oestrone. A similar result was given by lithium aluminium hydride reduction in tetrahydrofuran. It was obvious that the oxygen function at the 17-position was not in fact being protected against reduction by the enol-acetate group which had been introduced into the molecule, and this line of work was therefore abandoned.

It was decided to utilise the remainder of the mono-enol acetate in an attempt to prepare 6α-hydroxyoestrone by an

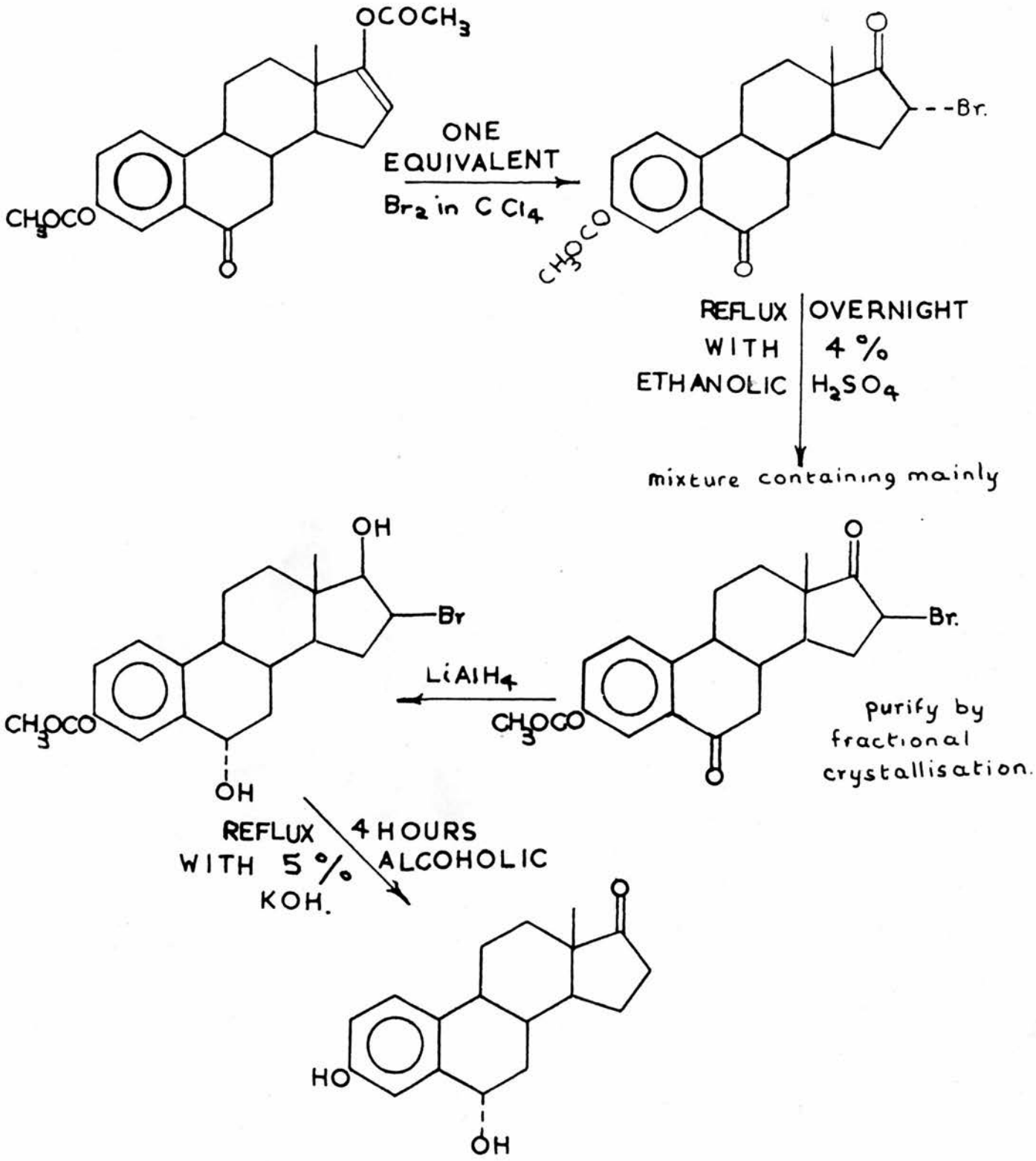
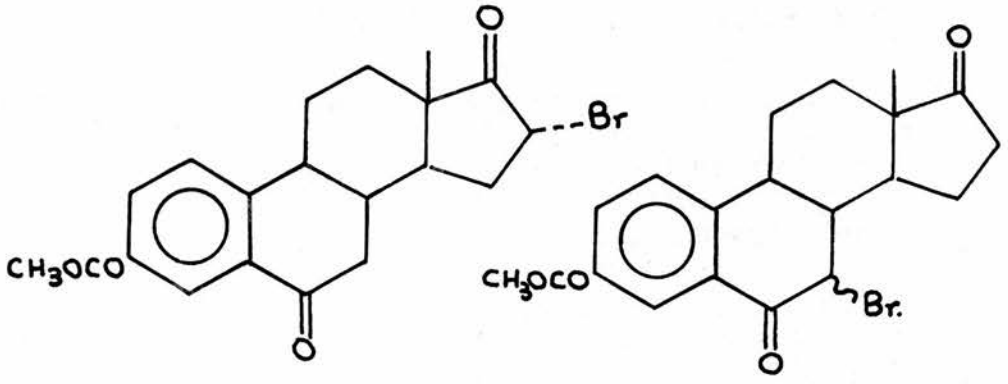

OCOCH3
CH3OCO
ONE
EQUIVALENT
Br2 in C Cl4
--Br.
CH3OCO
REFLUX
WITH
ETHANOLIC
OVERNIGHT
4 %
H2SO4
mixture containing mainly
OH
Br
CH3OCO
OH
LiAlH4
Br.
CH3OCO
purify by
fractional
crystallisation.
REFLUX
WITH 5 %
KOH.
4 HOURS
ALCOHOLIC
HO
OH

FIG. 5.

alternative method based on the work of Fishman and Biggerstaff (1958). These authors showed that bromination of the 17-enol-acetate of oestrone-acetate gave the 16α-bromo-derivative which on refluxing in acid solution was isomerized to give a mixture containing mainly the 16β-bromo-derivative. This could be separated from the 16α-epimer by fractional crystallization using the optical rotation in chloroform as a check on purity. Lithium aluminium hydride reduction followed by refluxing in alkaline solution of both epimers gave different products. The 16α-bromo-epimer gave a mixture containing the 16β-, 17β-oxide plus a little of the 17-ketone, whilst the 16β-bromo-derivative gave only the 17-ketone. The suggested method for the synthesis of 6α-hydroxyoestrone by this method is shown in Fig. 5.

Accordingly, 47 mgs. of the previously prepared mono-enol-diacetate of 6-oxo-oestrone was dissolved in 5 mls. of carbon tetrachloride and the solution chilled in ice. To this was added a solution of 20 mgs. of bromine in 1.0 ml. of carbon tetrachloride. The bromine was not completely decolourized and a precipitate rapidly formed in the mixture, which was allowed to stand at 0°C for fifteen minutes. The solvent was evaporated off in vacuo with slight warming, leaving a very pale yellow solid. This was washed with chilled methanol and desiccated to give a solid melting at 208° - 218°C with a very brown melt. Crystallization from 40% chloroform in methanol at 0°C overnight yielded 27.9 mgs.

of crystalline material melting at 210° - 211°C. Found 59.9; H, 5.2; Br, 20.0; Calculated for $C_{20}H_{21}O_4Br$: C, 59.3; H, 5.2; Br, 19.7%. This could represent either:-



6.2 mgs.of this bromination product was refluxed for twelve hours on a boiling water bath with 4% (w/v) ethanolic sulphuric acid. The ethanol was evaporated under air and the solution was diluted with water and extracted with chloroform. The extract was washed with 5% (w/v) aqueous bicarbonate and then with water, dried over anhydrous sodium sulphate and taken to dryness to give 4.7 mgs.of dark green residue. This was dissolved in 1.0 mls.of tetrahydrofuran and added dropwise to 1.0 mls.of a solution of 5.58 mgs.of lithium aluminium hydride in 5.9 mls.of tetrahydrofuran. After standing at room temperature for fifteen minutes 10 mls.of ethyl acetate plus 5 mls.of chilled 2N sulphuric acid were added, transferring to a separating funnel. The aqueous layer was run off, the ethyl acetate was washed with 5% (w/v) aqueous sodium bicarbonate, then with water, and was dried over anhydrous sodium sulphate.

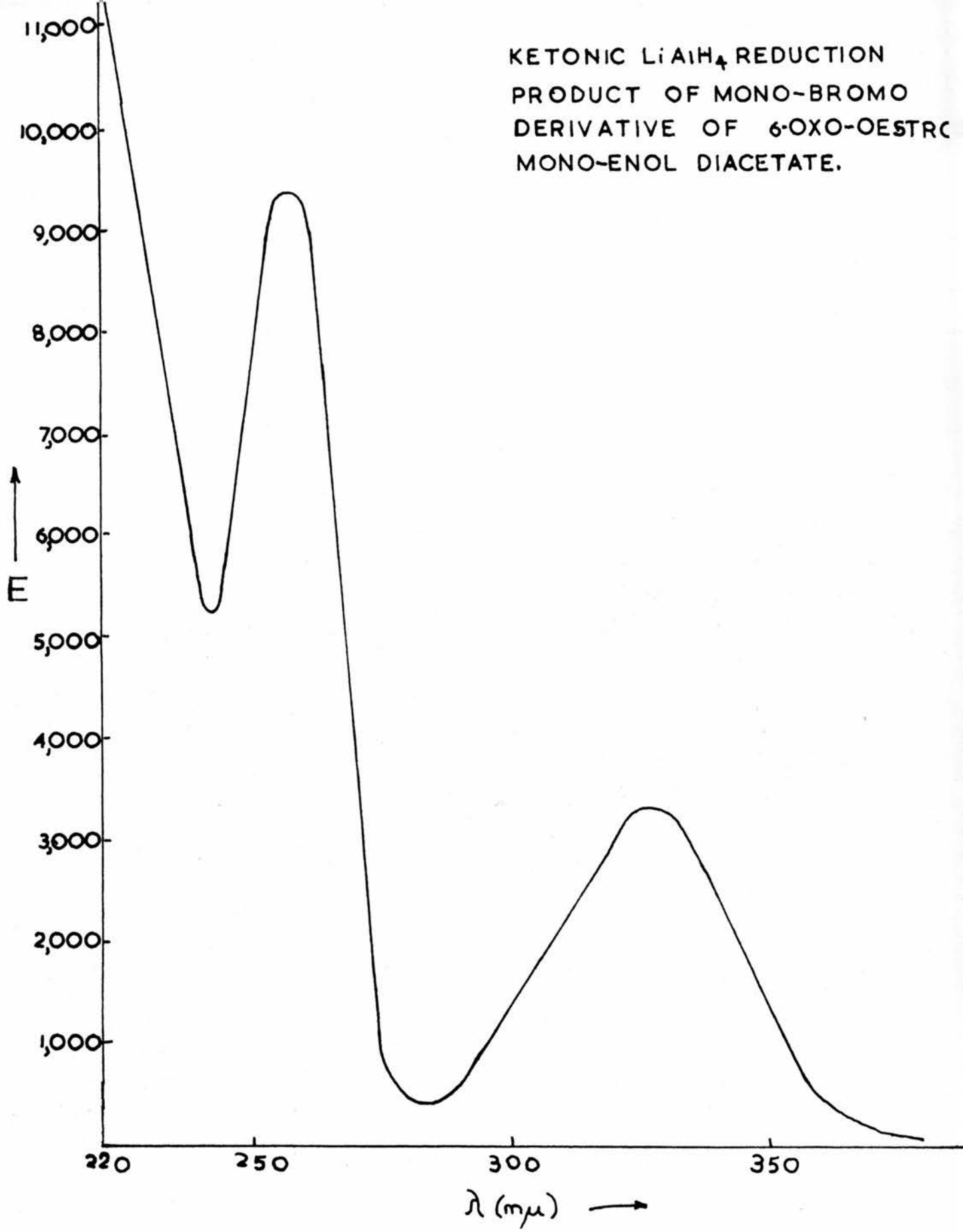

KETONIC LiAlH4 REDUCTION
PRODUCT OF MONO-BROMO
DERIVATIVE OF 6-OXO-OESTRC
MONO-ENOL DIACETATE.
11,000
10,000
9,000
8,000
7,000
6,000
5,000
4,000
3,000
2,000
1,000
E
220
250
300
350
λ (mμ)

FIG. 6.

before being taken to dryness.

The residue was then refluxed with 2.0 mls.of 5% (w/v) ethanolic potassium hydroxide for four hours. The solution was diluted with 10 mls.of water, acidified to phenolphthalein with carbon dioxide and extracted with ethyl acetate. This was washed with water, dried over sodium sulphate and taken to dryness to give 3.7 mgs.of residue.

Chromatography of this material in the system chloroform/formamide showed three spots; one running as 6-oxo-oestradiol-17β and two others more polar than 16-epi-oestriol. In the Zimmermann reaction the material showed a completely negative curve over the range 370 mμ - 550 mμ with no indication of a peak in the 515 mμ region. Some of this material was subjected to the Girard separation and the ultra-violet absorption spectrum of the ketonic fraction in ethanol showed two maxima at 256 mμ and 326 mμ. This is identical with the curve quoted by Longwell and Wintersteiner (1940) for 6-oxo-oestradiol-17β. (See Fig. 6).

The evidence clearly indicates therefore that the oxygen function at the 17-position has again been reduced in preference to that at the 6-position. This lends support to the suggestion derived from the previous results, that enol-acetylation has taken place at the 6-7-position rather than at the 16-17-position as was hoped.

The difficult synthesis of 6α-hydroxyoestrone was achieved by Knuppen and Breuer (1961). As it was found that

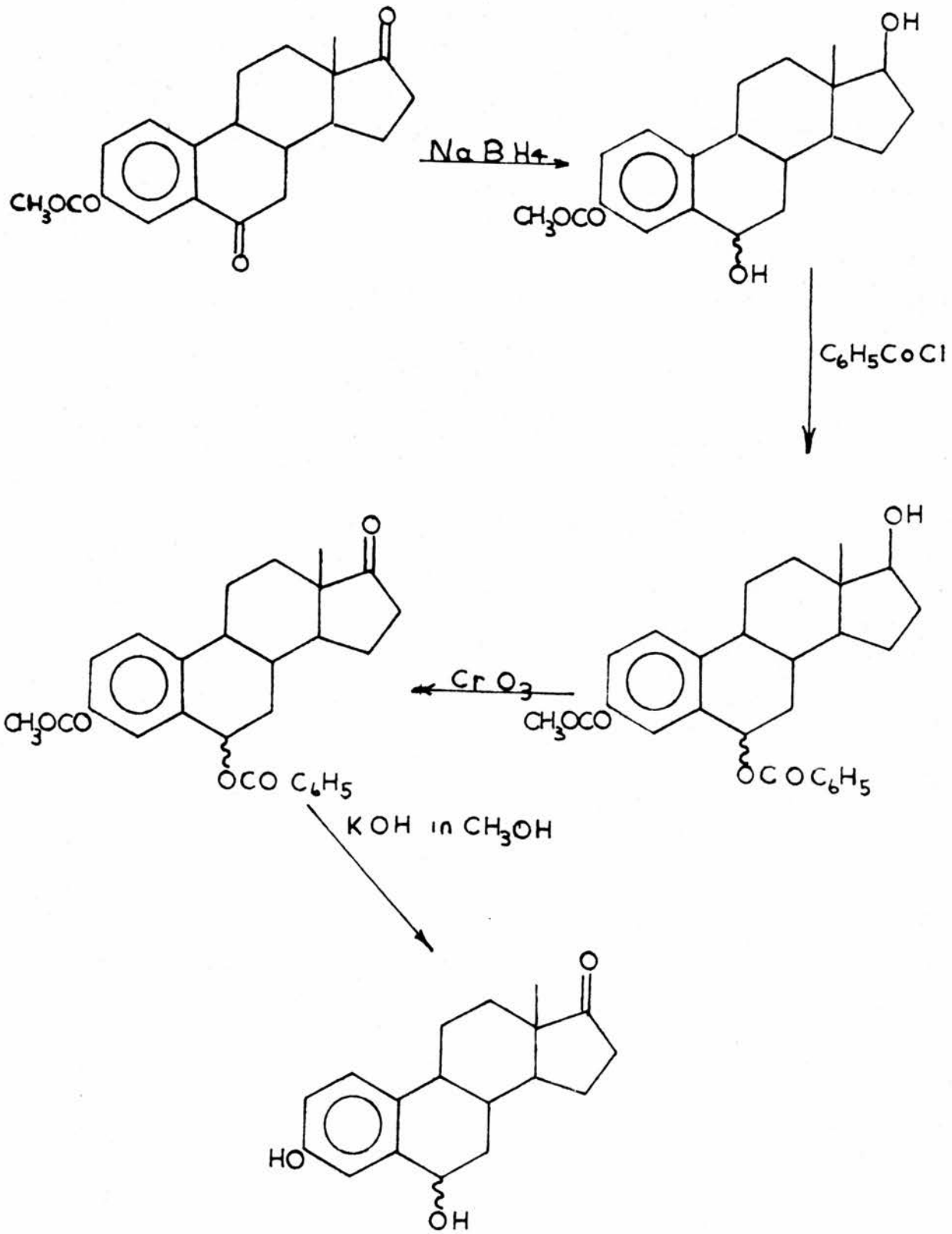


FIG. 7.

selective reduction of the 6-keto-group of 6-oxo-oestrone with sodium borohydride or lithium aluminium hydride in methanol led to the formation of 6-oxo-oestradiol-17β, whilst selective oxidation of the 17-hydroxyl- group of 6-hydroxyoestradiol-17β yielded 6-oxo-oestradiol-17β, the authors suggested that the 6-hydroxyl-group behaved as a secondary alcohol group in which the hydrogen atom is more reactive than that of the secondary alcohol at C-17. The activity of the 6-hydroxyl group is enhanced by the proximity of the aromatic ring and this would be reinforced by the presence of an acetoxy-group at C-3. On these grounds they suggested that the 6-hydroxyl-group should undergo the Schotten-Baumann reaction. Direct benzoylation of 6-hydroxy-oestradiol-17β is of course not possible as the phenolic hydroxyl group at C-3 is much more reactive than the hydroxyl group at C-6 and, on account of its insolubility in an aqueous medium, the resulting 3-mono-benzoate precipitates immediately and the reaction proceeds no further. The final procedure used by Knuppen and Breuer for the synthesis of 6α-hydroxyoestrone is summarized in Fig. 7.

A sample of this material was kindly prepared by Dr. M.M. Coombs of the Imperial Cancer Research Fund, by the method of Knuppen and Breuer (1961).

(2) 6-OXYGENATED DERIVATIVES OF OESTRADIOL-17β

(a) 6-oxo-oestradiol-17β-diacetate.

Oestradiol-17β (2.008 gs) was acetylated by refluxing with 5 mls. of acetic anhydride in 5 mls. of dry pyridine under air for thirty minutes. The acetate was precipitated with crushed ice and water, filtered off, washed copiously with water and dried in vacuo overnight to give 2.6015 gs. of crude acetate melting at 105° - 108°C.

This was dissolved in 9.0 mls. of glacial acetic acid and a solution of 2.205 gs. of chromium trioxide in 1.8 mls. of water plus 13.2 mls. of glacial acetic acid was added and the mixture allowed to stand at room temperature for twenty-four hours. The reaction mixture was diluted with 200 mls. of water plus 3 mls. of ethanol to reduce excess chromic acid and was then extracted with ether. The extract was washed with saturated aqueous bicarbonate until the washings showed a pink colour, and then with 5% (w/v) sodium carbonate saturated with sodium bicarbonate and finally with water. After drying over sodium sulphate, the extract was taken to dryness to give 1.756 gs. of residue. This was subjected to the Girard separation to yield 0.943 gs. of non-ketonic residue and 0.561 gs. of ketonic residue. The latter was reacetylated by refluxing for thirty minutes under air with acetic anhydride and pyridine to yield 0.575 gs. of residue. After being decolourized by warming with activated charcoal in methanol,

the residue was crystallized to give 0.215 gs. of material melting at 160° - 166°C.

The crystalline material obtained from three similar preparations was combined and re-crystallized from methanol to give 405.6 mgs of material melting at 174° - 175°C (sealed evacuated capillary). The ultra-violet absorption spectrum of this material in ethanol showed two maxima at 300 mμ (E 1642) and 247 mμ (E 8178).

(b) 6α-hydroxyoestradiol-17β.

16.4 mgs. of 6-oxo-oestradiol-17β diacetate was dissolved in 12.9 mls. of methanol and this solution was added dropwise with stirring to a solution of 105.0 mgs. of sodium borohydride in 10.0 mls. of methanol. The mixture was allowed to stand for two hours at room temperature and was then acidified with 10% aqueous acetic acid. Two-thirds of the methanol was removed under a stream of air at room temperature and the residue was diluted with 20 mls. of water. The solution was extracted with ether, the ether was washed with 5% (w/v) aqueous sodium bicarbonate, and then with water. It was finally dried over sodium sulphate and evaporated to dryness.

The residue was hydrolyzed by standing at room temperature for twenty-four hours with 25 mls. of a 5% (w/v) aqueous solution of potassium hydroxide under an atmosphere of nitrogen. After diluting with 5 mls. of water, the solution

was acidified with carbon dioxide until acid to phenolphthalein and was then extracted with ethyl acetate. The extract was washed with water, dried over anhydrous sodium sulphate and taken to dryness to give 12.6 mgs. of residue, which melted at 243° - 246°C with evolution of gas. This residue was crystallized from acetone/benzene to yield crystals melting at 248° - 251°C. Found: C, 75.2; H, 8.5; Calculated for $C_{18}H_{24}O_3$:- C, 75.0; H, 8.4%.

(c) 6α-hydroxyoestradiol-17β-diacetate.

42.3 mgs. of 6α-hydroxyoestradiol-17β was acetylated by refluxing with 0.4 mls. of acetic anhydride in 0.4 mls. of pyridine for nineteen hours. The acetate was precipitated with crushed ice and water, filtered off and washed copiously with water. This crude material melted almost completely at 55° - 70°C and then started to re-solidify at 70° - 90°C giving a complete melt at 130° - 140°C. The 60.3 mgs. of residue was crystallized at 0°C from 3 mls. of 80% aqueous methanol to give material melting at 135° - 141°C.

4.9 mgs. of this crystalline material was subjected to a "pipe-stem" crystallization from methanol to give 4.2 mgs. of crystals melting at 120° - 125°C. Following desiccation for two days, the melting point was found to be 141° - 146°C. After a further two days in the desiccator the crystals melted at 120° - 133°C, but on cooling, the melt was seen to re-crystallize into long, blunt-ended crystals which were quite different in appearance to the initial small granular crystals.

On reheating the melt, these crystals melted at 140° - 145°C. This strongly suggests that 6α-hydroxyoestradiol-17β diacetate can exist in two polymorphic forms which have different melting points.

(3) 6-OXYGENATED DERIVATIVES OF OESTRIOL

(a) 6-oxo-oestriol-triacetate.

1.96 gs. of crude oestriol, prepared from late pregnancy urine, was acetylated with acetic anhydride and pyridine at room temperature for twenty-four hours and the product recrystallized once from hexane. The triacetate (1.87 gs) was dissolved in 6.5 mls. of glacial acetic acid and to this solution was added 1.7 gs. of chromium trioxide dissolved in 9.7 mls. of glacial acetic acid plus 1.4 mls. of water. After standing at room temperature for twenty-four hours, the excess chromic acid was reduced by the addition of 2 mls. of ethanol and after diluting with 200 mls. of water the mixture was extracted with ether. The extract was washed with 50 ml. portions of saturated aqueous sodium bicarbonate until the washings showed a pink colour, then with 50 mls. of aqueous 5% (w/v) sodium carbonate saturated with sodium bicarbonate, and finally with 50 mls. of water. After being dried over anhydrous sodium sulphate, the extract was taken to dryness to give 1.0 gs. of residue. This was subjected to the Girard separation and the ketonic fraction was re-acetylated, decolourized by warming with activated charcoal in methanol and re-crystallized from methanol to give 0.24 gs. of crystalline material melting at 135° - 138°C.

This was combined with 0.49 gs. of similarly prepared material and after re-crystallization from methanol

0.57 gs. of material was obtained, melting at 137° - 139°C. (a) $[\alpha]_D^{16.5}$ - 41° in ethanol (C, 0.493). Found: C, 67.3; H, 6.5; Calculated for $C_{24} H_{28} O_7$:- C, 67.3; H, 6.6%.

The ultra-violet absorption spectrum of this material in ethanol showed maxima at 249 mμ (E 9150) and 298 mμ (E2260).

(b) 6-oxo-oestriol.

6-oxo-oestriol triacetate (110 mgs) was hydrolyzed with 2.0 mls. of 20% (w/v) potassium hydroxide in methanol for twenty-four hours at room temperature in an atmosphere of nitrogen. After dilution with 30 mls. of water, the solution was acidified to phenolphthalein with carbon-dioxide and extracted three times with 10 ml. portions of ethyl acetate. After two washings with 5 ml. portions of water, the extract was dried over sodium sulphate and evaporated to dryness. Crystallization of the product twice from methanol/benzene yielded 39 mgs. of crystals, melting point 240° - 242°C, with slight yellowing. (a) $[\alpha]_D^{16.0}$ - 7° in ethanol (C, 0.504). Found: C, 71.5; H, 7.1; Calculated for $C_{18} H_{22} O_4$:- C, 71.5; H, 7.3%.

The ultra-violet absorption spectrum in ethanol showed maxima at 257 mμ (E 7895) and 325 mμ (E 3025).

(c) 6α-hydroxyoestriol.

A solution of 84.5 mgs. of 6-oxo-oestriol in 8.0 mls. of methanol was added dropwise to a solution of 70.7 mgs. of

sodium borohydride in 6.5 mls. of methanol. After standing for two hours at room temperature the solution was acidified to litmus with 10% (v/v) aqueous acetic acid and evaporated almost to dryness under a stream of air at room temperature. The residue was diluted with 3 mls. of water; the suspended crystals were filtered off with suction, washed with water and dried in vacuo.

Some difficulty was experienced in crystallizing the product but eventually it was found that by evaporating a solution of the material in 50% (v/v) aqueous methanol to just under half-volume, well-formed needles separated out on cooling to room temperature. These were filtered off with suction, washed with water and dried in vacuo to give crystals melting at 242° - 245°C with evolution of gas (change of form at 140° - 150° C). (a) $[\alpha]_D^{14.0}$ + 84° in ethanol (C, 0.498). Found: C, 71.4; H, 7.6; Calculated for $C_{18}H_{24}O_4$:- C, 71.0; H, 7.9%.

The ultra-violet absorption spectrum in ethanol showed a single maximum at 282 mμ (E 2415).

(d) 6α-hydroxyoestriol tetra-acetate.

40 mgs. of 6α-hydroxyoestriol (melting point 244° - 248°C) was acetylated at room temperature for twenty-four hours with acetic anhydride and pyridine. The crude acetate was purified by filtering in benzene solution through a short column of acid-washed alumina and crystallizing twice from methanol. The product melted at 159° - 161°C, (a) $[\alpha]_D^{16.0}$ - 12°

in ethanol (C, 0.506). Found: C, 66.2; H, 6.8; Calculated for $C_{26}H_{28}O_8$:- C, 66.1; H, 6.8%.

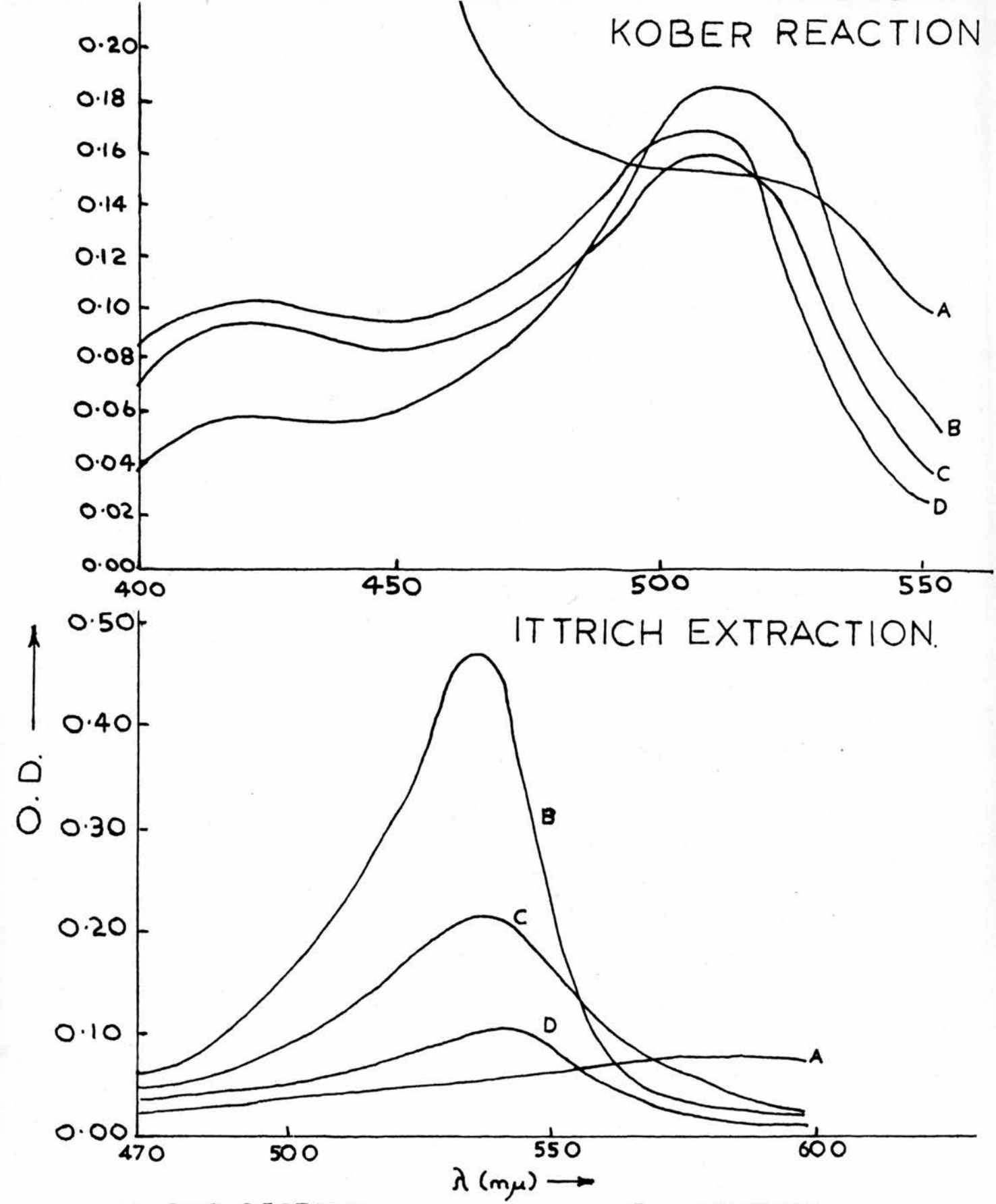
(4) BEHAVIOUR OF 6-OXYGENATED OESTROGEN DERIVATIVES IN THE KOBER - REACTION.

Each of the 6-oxygenated oestrogen derivatives was subjected to the Kober reaction, carried out by the method of Brown (1952) and Bauld (1954) using the "oestriol reagent" of the latter.

In one such experiment with 6α-hydroxyoestrone, 6α-hydroxyoestradiol-17β, and 6α-hydroxyoestriol all three compounds showed the normal pink colour at the end of the reaction. The absorption spectrum given by the final chromogen was normal in each case showing a single absorption maximum at 517 mμ - 518 mμ.

In a second experiment in which 20 μgs. of 6α-hydroxyoestrone and 10 μgs. of oestriol were subjected to the Kober reaction at the same time and under identical conditions, the former showed a single maximum at 510 mμ - 512 mμ whilst the absorption maximum given by oestriol was 511 mμ - 513 mμ. The optical density shown by 6α-hydroxyoestrone was 80.5 % of that shown by the same weight of oestriol.

When 20 μgs. each of 6-oxo-oestrone, 6-oxo-oestradiol-17β and 6-oxo-oestriol were subjected to the Kober reaction it was found that neither of the compounds showed a normal positive Kober colour. It was interesting that in the



A.— 6-OXO OESTRIOL.

B.— OESTRIOL.

C.— 6α-HYDROXYOESTRIOL

D.— Δ6-DEHYDRO-OESTRON

Fig. 8.

first stage of the reaction after approximately two minutes heating the solution containing 6-oxo-oestradiol-17β was slightly red in colour. The solution of 6-oxo-oestriol was less red, whilst the 6-oxo-oestrone solution showed only a slight trace of redness. These colours had faded considerably by the end of the first stage and by the end of the second stage all three compounds gave clear yellow solutions which were indistinguishable from the blank. When the optical densities of these solutions were determined on an S.P.500 spectrophotometer it was found that they showed no absorption maximum in the region 510 mμ - 520 mμ.

It is of interest to note that in the Kober reaction Δ 6-dehydro-oestrone showed the normal Kober colour giving an absorption spectrum with maximum at 510 - 511 mμ. These absorption curves are shown in Fig. 8, together with the correspon ding curves of the extracted Kober colours (Ittrich, 1958).

(5) PARTITION OF OESTROGEN DERIVATIVES BETWEEN ORGANIC SOLVENTS AND WATER

Following the observation that when the water washings of an ether extract of 6-oxo-oestriol were allowed to evaporate by standing for some time at room temperature, crystals corresponding to 6-oxo-oestriol in chromatographic mobility were obtained, it was decided to investigate the partition coefficients of the various 6-oxygenated-oestrogen derivatives in the systems ether/water and ethyl acetate/water.

The steroid (accurately weighed within the range 100 μgs. - 300 μgs) was spread in a thin film on the inside of a flask by evaporation from methanolic solution and was then shaken at 25° C with 10 mls. of each phase (which had been pre-equilibrated) until all the steroid had dissolved. The mixture was then carefully transferred to a separating funnel and after separation of the two phases, 6.0 mls. of each was removed and evaporated to dryness in vacuo in a small flask. Each residue was then dissolved in 3.0 mls. of pure ethanol and the concentration of steroid determined from the extinction at 281 mμ or at 325 mμ (6 oxo-oestrogens) using samples of the corresponding phase without oestrogen as a blank in each case. The concentration of steroid was derived from the expression,

TABLE 1

$$K = \frac{\text{Concentration of Steroid in Organic Phase}}{\text{Concentration of Steroid in Aqueous Phase}}$$

Compound	Temperature	K Ether/Water	% age error	Temperature	K Ethyl Acetate/Water	% age error
Oestriol	26.0°	7.85	19.0	26.0°	24.9	27.0
6α-hydroxyoestriol	25.0°	0.122	13.0	25.0°	0.899	15.0
6-oxo-oestriol	26.7°	0.668	8.4	26.7°	5.54	0.6
16-epi-oestriol	23.9°	35.8	8.8	23.9°		0.8
Oestradiol-17β	23.3°	78.5	8.0	23.3°	52.2	7.0
6-oxo-oestradiol-17β	23.3°	22.8	7.0	23.3°		13.0
6-hydroxyoestradiol-17β	23.3°	5.8	10.4	23.3°	15.7	1.2
16-oxo-oestradiol-17β	23.9°	65.8	1.2	23.9°	207.5	0.12
11β-hydroxyoestradiol-17β	23.9°	5.6	7.6	23.9°	15.4	2.0
oestrone	24.4°	91.1	0.52	24.4°		8.08
6-oxo-oestrone	25.0°	27.7	10.8	25.0°	100.4	1.5
11β-hydroxyoestrone	25.6°	7.4	5.6	25.6°		5.6
16α-hydroxyoestrone	26.1°	16.0	13.6	26.1°		3.6
16β-hydroxyoestrone	26.1°	13.9	12.0	26.1°		10.0

$$E = \frac{0.D}{c \times d}$$

Where,

E = Molecular Extinction Coefficient

0.D = Optical Density

c = Molar Concentration of Solution

d = length of light path in cms.

As we used 1 cm cells,

$$\therefore \quad c = \frac{0.D}{E}$$

The results of these determinations are shown in Table 1.

(6) DISCUSSION

The method of Longwell and Wintersteiner (1940) for the introduction of an oxygen function at C-6 of oestradiol-17β-diacetate has been found to be equally successful when applied to the acetates of oestrone and oestriol. Thus, approximately half of the starting material was converted into acids in the oxidation step, whilst from the neutral fraction, 20% - 28% of the starting material was recovered as ketonic material. The final weights of 6-oxo-oestrogen-acetate obtained were not quite so high as the 17.13% quoted by these authors, being 15.1% for 6-oxo-oestrone-acetate; 10.7% for 6-oxo-oestradiol-17β diacetate; and 12.4% for 6-oxo-oestriol-triacetate respectively.

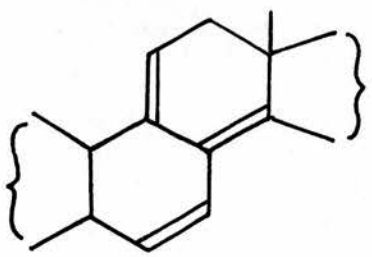
One of the most striking features of the 6-oxo-oestrogens is the very characteristic ultra-violet absorption spectrum shown by these compounds in ethanolic solution, with maxima at 326 mμ and 257 mμ. The maximum at 326mμ has been attributed to the extension of the conjugated system of the phenolic A-ring by the double bond at C-6 (Longwell and Wintersteiner 1940).

Fieser and Fieser (1959) have given a summary of the calculations of the wavelength of maximum absorption in various chromophore systems, based upon the addition of values associated with various features of the system. These

values have been derived from observation of a large number of chromophore systems. The oestrogen molecule consists of a homo-annular diene system in Ring A, which is extended by a third double bond. This system has two alkyl residues attached to it extending from C-11 to C-10; and from C-6 to C-5. From the values quoted by Fieser and Fieser (1959) we find:-

Parent homo-annular diene		253
Double bond extending the conjugation		30
Ring residues	C-11 to C-10	5
	C-6 to C-5	5
	Total	293

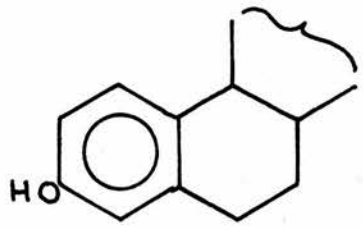
This calculated value is too high as the absorption maximum of the oestrogens in ethanol is 281 mμ. However, as these authors have shown, where cross-conjugation occurs the increment of 30 mμ for the extension of a diene system by a third double bond does not apply, the cross-conjugation exerting a hypsochromic effect of 10 mμ, e.g.



Calculated value = 293 mμ; Observed value = 285 mμ.

The extension of the diene system of the oestrogen molecule is not cross-conjugated in the same way as that in the above example, but nevertheless it is not a straightforward extended conjugated system, as it is enclosed in the form of a ring. The difference between the observed value of 281 mμ and the calculated value of 293 mμ probably reflects the degree to which the extension of the conjugation of the diene system is hindered by virtue of being in the form of a homoannular triene.

Comparison of the chromophore system of a 6-oxygenated oestrogen with that of an unsubstituted oestrogen suggests that the introduction of an oxygen function at C-6 should partly remove this hindering effect, due to the fact that the chromophore system is extended by the exo cyclic double bond which is conjugated with the homoannular triene system.



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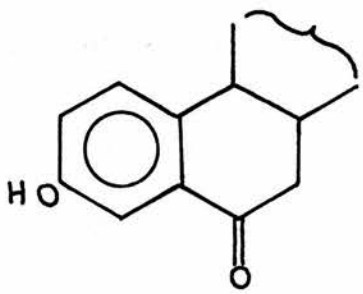


From the values quoted by Fieser and Fieser (1959) we find:-

Parent homo-annular diene		253
Double bond in Ring A extending the conjugation		30
Double bond at C-6 extending the conjugation		30
Ring residues	C-11 to C-10	5
	C-7 to C-6	5
Exo-cyclic location of double bond at C-6		5
	Total	328

This value is in good agreement with the maximum at 326 mμ shown by the 6-oxygenated-oestrogens. These compounds also contain the separate chromophore system of an α,β-unsaturated ketone, due to the oxygen function at C-6. Addition of the values relevant to such a system gives:-

Parent α,β unsaturated ketone	215
Double bond extending the conjugation	30
Exo-location of double bond at C-6	5
Total	250

This value is in reasonable agreement with that of the second absorption maximum shown by the 6-oxygenated-oestrogens at 256 mμ. Thus the observed ultra-violet absorption spectrum shown by these compounds in ethanol might be explained on the basis of:-

(1) Extension of the chromophore system of the A-Ring by an exo-cyclic double bond at C-6.

(2) Formation of a second chromophore system, that of an α,β-unsaturated ketone, by the introduction of an oxygen function at C-6.

The ultra-violet absorption spectra of the 6-oxygenated-oestrogens are unique, in that there is no other position in the oestrogen molecule whereby introduction of an oxygen function will give the same chromophore systems. Therefore the ultra-violet absorption spectra of these compounds provides a very sound basis for their identification. Reduction of the oxygen function at C-6 to give a 6-hydroxyl group leads to a reversion to the chromophore system found in the unsubstituted oestrogens with an expected absorption maximum at 281 mμ. This is in fact what is found for the 6-hydroxy-oestrogens.

In agreement with the observations of Longwell and Wintersteiner (1940) it was found that introduction of an oxygen function at C-6 brought about a marked decrease in dextro-rotation. Again this effect was nullified by reduction of the ketone group to a secondary alcohol group e.g. 6-oxo-oestriol (a) $\overset{25^0}{D}$ - 70 in ethanol; 6α-hydroxy-oestriol (a) $\overset{25^0}{D}$ + 84 in ethanol. That this would appear to be a general effect in the steroid molecule is shown by the

analogous case of testosterone, (a) $^{25^0}_{D}$ + 109 in ethanol (Ruzicka and Wettstein 1935) and 6-oxo-testosterone (a) $^{25^0}_{D}$ - 58 in ethanol (Butenandt and Riegel 1936). This decrease in dextro-rotation due to introduction of an oxygen function at C-6 is lessened and in some cases nullified by reduction to a 6-hydroxy group (Fieser and Fieser 1959). It is interesting to note that this reversion towards dextro-rotation is much less pronounced for 6β-hydroxy groups than for 6α-hydroxy groups.

From the results of the attempted synthesis of 6α-hydroxyoestrone it can only be concluded that the attempted protection of the oxygen function at C-17 by enol-acetate formation was unsuccessful. Although it was shown that this material was a mono-enol-diacetate of 6-oxo-oestrone, the possibility that enol-acetylation might have taken place at the 6,7-position could not be precluded. This seemed likely, as numerous reductions of the material with both sodium borohydride and lithium aluminium hydride unfailingly yielded 6-oxo-oestradiol-17β. Evidence in support of this suggestion was furnished by the ultra-violet absorption spectrum of this material in ethanol, in which the characteristics of an oxygen function at C-6 were absent. Still further evidence in support of the suggestion that enol-acetylation had taken place at the 6,7-position was furnished by failure of the attempted synthesis of 6α-hydroxyoestrone by the method based on the work of Fishman and Biggerstaff

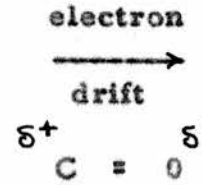
(1958), which again yielded 6-oxo-oestradiol-17β. Analysis of the initial bromination product showed it to be a mono-bromo derivative of 6-oxo-oestrone and yet the subsequent steps in the sequence yielded 6-oxo-oestradiol-17β. This could only be explained by introduction of bromine at C-7 rather than at C-16, due to the location of the enol-acetate group at C-6 to C-7.

A most interesting feature of the 6-oxygenated oestrogen derivatives is their relatively high solubility in aqueous media. Examination of the partition coefficients found for the systems ether /water and ethyl acetate/water shows that introduction of a 6-hydroxyl group into oestriol makes the molecule 98.4% less soluble in ether than oestriol itself, whilst a 6-oxo-group makes the molecule 91.5% less soluble in ether than oestriol. Similar calculations for oestradiol-17β show that the solubility of the molecule in ether is lessened by 92.6% following introduction of a 6-hydroxyl group and 71.0% following the introduction of a 6-oxo-group. For oestrone the solubility in ether is lessened by 69.6% for introduction of a 6-oxo group.

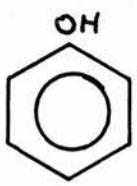
It is of considerable interest to compare the effects of the introduction of oxygen functions and hydroxyl functions at C-6 with the introduction of these same functions at other points in the steroid nucleus. Thus introduction of a β-hydroxyl group at C-11 of oestradiol-17β lessened the solubility of the molecule in ether by 92.9%. This is almost

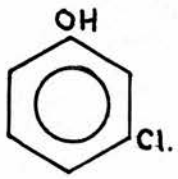
identical with the figure of 92.6% found for the introduction of a hydroxyl group at C-6. Furthermore comparison of the values found for oestriol with those for oestradiol-17β show that the former is 90% less soluble in ether than the latter, again the figure for the introduction of a third hydroxyl group being of the same order as before. These data suggest that the position of the extra hydroxyl group may not be significant in determining the increased solubility of the resultant molecule in aqueous media, and that the observed effect is due almost entirely to the hydroxyl group itself.

In comparison with these results it was found that introduction of an oxygen function at C-16 lessened the solubility of oestradiol-17β in ether by 16.2%. This is very much less than the value of 71.0% found for introduction of an oxygen function at C-6, which suggests that the position at which an oxygen function is introduced might be of far more importance in determining the relative water solubility of the resulting compound than the group itself. Due to lack of appropriate compounds, this suggestion could not be verified further but evidence in support of such a concept can be provided on theoretical grounds from the nature of the ketone group itself, in which there is an electron-drift from carbon to oxygen, giving rise to a slight dipole within the group.



This dipole will give rise to an inductive effect transmitted to the bonds adjacent to the carbon atom of the ketone group and the extent to which this effect will be transmitted will depend upon the nature of these bonds. Thus C-16 is attached to saturated carbon atoms at C-15 and C-17 and therefore following the introduction of an oxygen function at C-16, the effects of the resulting ketone group will be nullified to a large extent. On the other hand C-6 is attached to a saturated carbon atom at C-7 but it is also attached to C-5 which forms part of the highly unsaturated system of the benzenoid A Ring. Following the introduction of an oxygen function at C-6 the inductive effect due to the resulting ketone group will therefore be transmitted throughout this conjugated system and will result in an increased dissociation of the phenolic hydroxyl at C-3, making the oestrogen more water-soluble. This effect is comparable to the introduction of a chlorine atom in the meta-position of phenol, the resulting compound being much more water soluble than phenol itself.





In this respect also therefore the fact that the 6-position is the only position in the oestrogen molecule in which an oxygen function

can extend the conjugation of the phenolic A Ring makes the 6-oxo-oestrogens unique in their especially high water solubility. As stated previously, this suggestion will require verification with other oxygenated oestrogen derivatives before it can be accepted per se.

In view of the results of this investigation into the partition characteristics of oestrogen derivatives it would seem advisable to consider the possible dangers of using extraction procedures involving arbitrarily-fixed proportions of organic solvents, in the investigation of the metabolism of oestrogens. This is particularly important when conclusions about the nature of metabolites formed (whether "free", conjugated or degraded to acidic substances) are based on their extractibility from aqueous media (c.f. Beer and Gallagher 1955; Valcourt, Thayer, Doisy, Elliott and Doisy 1955; Jellinck 1959). Any such conclusions must obviously be supported by further investigation by methods which will furnish more concrete evidence.

SECTION 111

(1) PREPARATION OF KC-6 FRACTION

The initial working up of late pregnancy urine including hydrolysis, extraction and column partition chromatography was exactly as that described by Loke, Marrian and Watson (1959). When processed in this way, 850 litres of late pregnancy urine yielded 259.3 mgs.of crude KC-6 fraction.

Attempted separation of the two components in this residue by leaching with chloroform at -20°C as described by Loke et al. (1959), proved unsuccessful due to the very gummy nature of the residue. Examination of the material in various paper-chromatographic systems showed that it might be possible to separate the two components in the system chloroform/formamide. When an aliquot of the KC-6 fraction was chromatographed for eleven hours in this system, it showed a number of minor spots and one compound spot having two centres of intensity at 12.6 cms.and 14.7 cms. from the origin respectively. It was decided therefore to subject the 259 mgs. of crude residue to a large-scale paper chromatographic separation in this system. The paper used was Whatman No. 42 which had been extracted for three days in a Soxhlet apparatus with a mixture of methanol/chloroform in equal parts, and then dried in air (Layne and Marrian 1958). The KC-6 residue was dissolved in methanol and the equivalent of 5 mgs. of the original residue was applied to a paper six inches

wide, as a narrow streak along the origin line. After running for eleven hours, a narrow strip was cut from each side of the paper and sprayed with Folin and Ciocalteu's reagent. Development with ammonia vapour showed that although the KC-6 spot had not completely separated into two components, there were two centres of intensity with a definite lessening of intensity in the centre of the spot. The papers were cut on either side of the main spot and this was divided by cutting in the centre along the line of least intensity. The more polar and less polar components of the KC-6 fraction were then eluted by combining the appropriate areas of paper from six chromatograms at a time and cutting these into pieces of approximately 1 cm^2. The paper was then shaken for one hour with 50 mls. of methanol, which was filtered through a sintered glass filter and taken to dryness. This procedure was repeated twice more for each lot of paper. The combined methanol eluates were finally concentrated to a volume of c.a. 25 mls., diluted with 20 mls. of water and extracted four times with 50 ml-portions of ether. This was washed four times with 20 ml-portions of water, dried over anhydrous sodium sulphate and taken to dryness.

The residue from the more polar material weighed 29.2 mgs. and had the appearance of a yellow gum which

appeared to contain a little white, semi-crystalline material; 43.3 mgs. of very gummy brown residue was obtained from the less polar material. Chromatography of these residues in the system chloroform /formamide showed that the former was very slightly contaminated by the less polar material whilst the latter showed only a very slight trace of contamination by the more polar material.

KOBER REACTION.

A.– 20μgs OESTRIOL

B – 60μgs "KC6B"

C – 60μgs "KC6B" AFTER $NaBH_4$ REDUCTION.

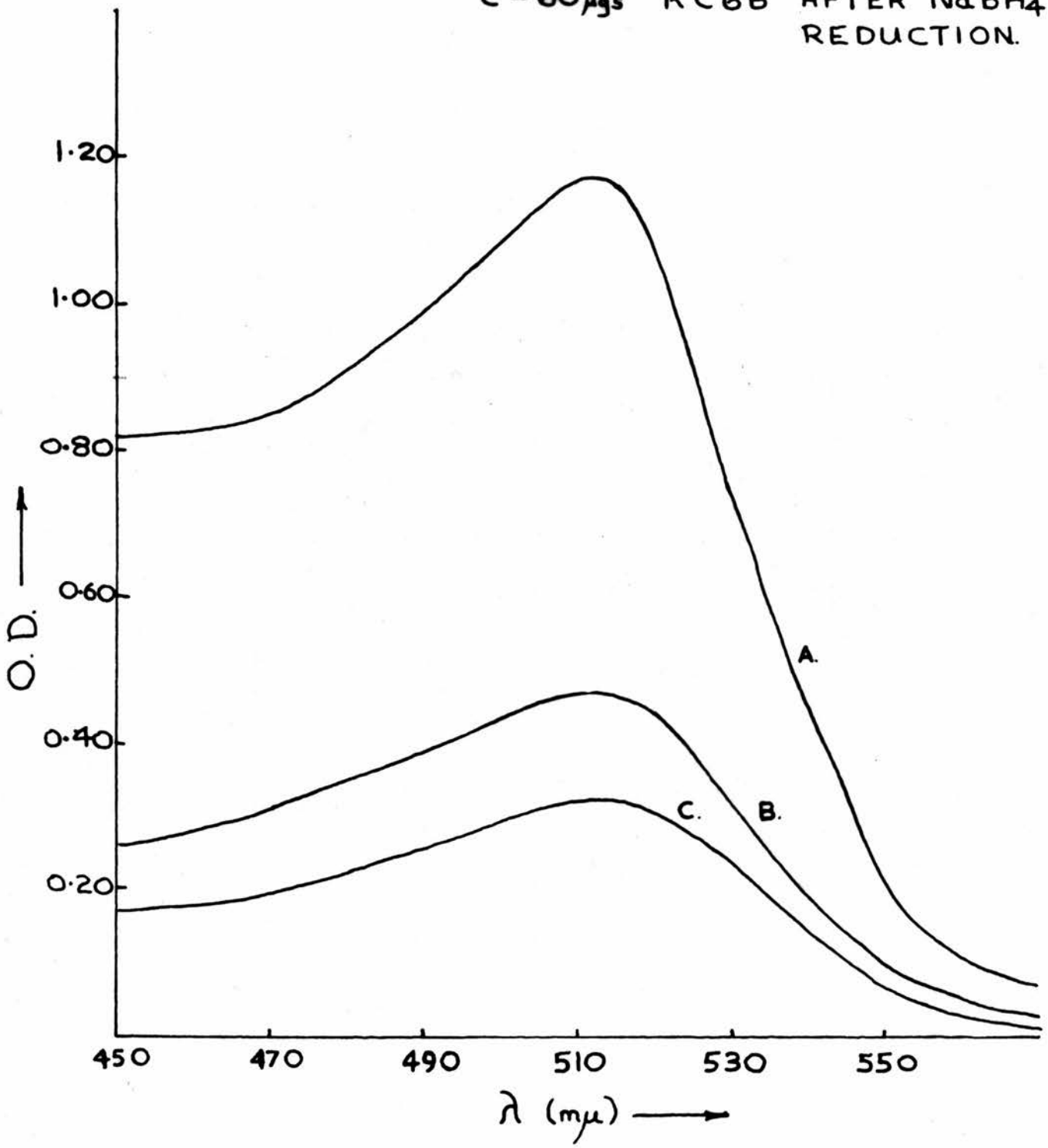


FIG. 9.

(2) INVESTIGATION OF THE LESS POLAR FRACTION

For comparative purposes, 300 μgs. of the less polar residue was reduced with sodium borohydride in methanol. Chromatography in the system chloroform/formamide showed that the parent material gave a single spot slightly less polar than 16-epioestriol; the reduced material also gave a single spot which was slightly less polar than oestriol. A second spot of the original residue, which had been run on the same paper, was sprayed with blue tetrazolium as specified by Mader and Buck (1952), but it showed no reducing power with this reagent.

In the Kober reaction, both the reduced and unreduced fractions gave normal Kober colours, having absorption maxima at 512 mμ. The original material showed 13.7% of the intensity given by an equivalent weight of oestriol and this was found to be diminished by 32% on reduction. (See Fig. 9)

The remainder of the original material was subjected to treatment with 10 mls. of N-NaOH in order to remove contaminating material from the more polar fraction. It was found that much of the solid would not dissolve at room temperature and on heating on a boiling water bath for thirty minutes a rather cloudy solution was obtained. This was made acid to phenolphthalein with carbon dioxide and extracted with ether to yield 17.7 mgs. of residue, which gave a normal Kober colour of 35% of the intensity shown by an equivalent weight of

oestriol. Attempted crystallizations of this material from methanol/benzene, acetone and ether were all unsuccessful and it was decided to attempt to purify it further by alumina chromatography.

The residue was dissolved in 4 mls. of 40 : 1 Benzene : Methanol and applied to a column of 3.0 gms. of alumina slurried in benzene (the alumina had been activated by heating at 100° C in vacuo and then deactivated by addition of 3% by weight of water). The column was eluted with 40 : 1 Benzene : Methanol taking 2 ml. cuts. These were taken to dryness in small weighed tubes to give two fractions of 12.2 mgs. and 0.5 mgs. In the Kober reaction the major fraction gave a very deep wine-coloured solution after c.a. 2 minutes heating and this colour persisted to the end of the second stage. On diluting the final solution with an equal volume of 78.8% aqueous sulphuric acid it showed a normal Kober spectrum, having a single absorption maximum at 512 mμ.

Chromatography of this material in the system chloroform/formamide showed a single spot less polar than 16-epioestriol, plus a very faint spot running just behind the solvent front. Separation of the main fraction from this non-polar contaminant was achieved by paper chromatography in the system chloroform/formamide. Elution of the appropriate areas of the papers, as before, yielded 5.1 mgs. of the main

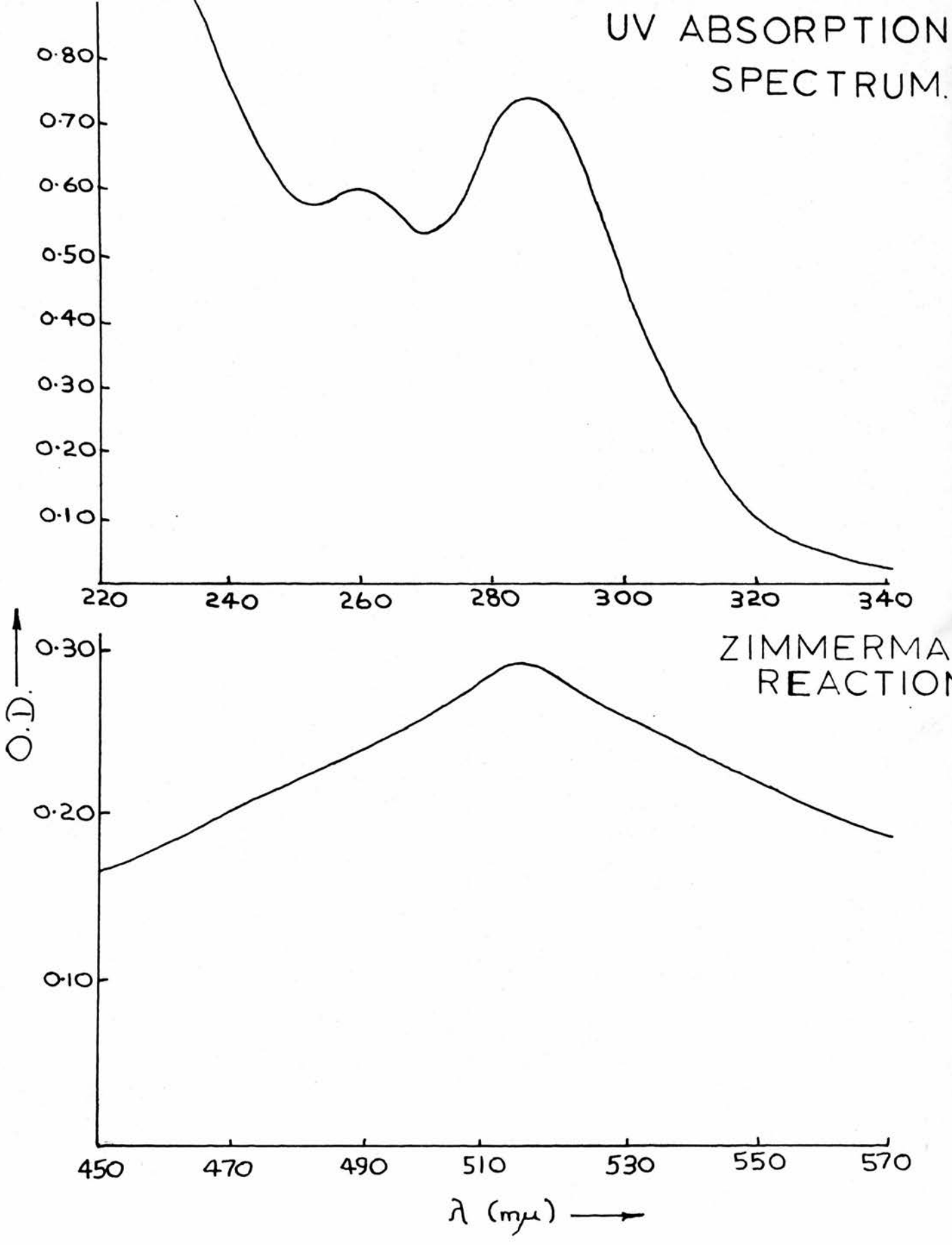

UV ABSORPTION SPECTRUM.
0·80
0·70
0·60
0·50
0·40
0·30
0·20
0·10
220
240
260
280
300
320
340
ZIMMERMAN REACTION
O.D.
0·30
0·20
0·10
450
470
490
510
530
550
570
λ (mμ) ⟶

FIG.10.

fraction and 4.2 mgs. of less polar material. In the Kober reaction, the former gave a normal Kober colour having an absorption maximum at 512 mμ, the intensity of the colour being 44% of that given by an equivalent weight of oestriol, the less polar material showed no absorption maximum at 512 mμ.

The main fraction residue was decolourized with charcoal in methanol to give 4.0 mgs. of a clear gummy residue. Attempted crystallizations of this residue from a variety of solvents proved unsuccessful. The ultra-violet absorption spectrum of this material in ethanol showed a main peak at 288 mμ with a secondary peak at 260 mμ. In the chloroform/formamide system it gave a single spot slightly more polar than 6-oxo-oestradiol-17β. In comparison, 11β-hydroxyoestrone gave a single spot slightly less polar than 6-oxo-oestradiol-17β.

In the Zimmerman reaction it showed an absorption maximum at 515 mμ which is the same maximum given by oestrone in this reaction. (See Fig. 10)

The remainder of this material was subjected to sodium borohydride reduction in methanol for direct comparison with 6α-hydroxyoestradiol-17β. The ultra-violet

absorption spectra of both compounds in ethanol were identical, having a single absorption maximum at 282 mμ. Chromatography in the system chloroform/formamide showed that the reduced material gave a single spot having the same mobility as 6α-hydroxyoestradiol-17β.

The evidence obtained from this batch of less polar material very strongly suggested that it might contain a 6-hydroxyoestrone, but conclusive evidence on this point was still lacking. It was felt that this could only be obtained by processing a much larger quantity of late pregnancy urine in order to obtain sufficient pure crystalline material to permit of a positive identification.

This work was therefore put in hand, but unfortunately, after 940 litres of urine had been processed it was found to be impracticable to process any further quantity of urine. The problem therefore had to be re-assessed in the light of the work already done and with the knowledge that the amount of less polar fraction available was insufficient to allow a direct isolation by classical methods.

(3) PRELIMINARY INVESTIGATION OF SECOND KC-6 RESIDUE

From a total of 940 litres of late pregnancy urine, 9.35 gms. of ketonic phenolic residue was obtained, which yielded 288.9 mgs. of KC-6 residue.

Chromatography of this material in the system chloroform /formamide showed that the KC-6 fraction was heavily contaminated with highly polar material which remained at the origin. It was found that under the new laboratory conditions, the chloroform/formamide system gave unreliable results when attempting to separate standard solutions of 18-hydroxyoestrone and 6α-hydroxyoestrone and it was therefore decided not to risk attempting a large-scale separation on paper as before. A large number of other systems of both the Bush-type and the Zaffaroni-type were investigated with a view to separating these compounds, but without success.

In view of the probability that separation of the two components of the KC-6 fraction would be adversely affected by the latter high concentration of very polar material present, it was decided to remove this by chromatography in the system Benzene : Hexane : Methanol : Water : 75 : 25 : 70 : 30,

using washed Whatman No.42 paper. The residue was applied to a total of thirty-six papers in this system, the papers being run for twenty-four hours. Strips cut from the edges of the papers and stained with Folin and Ciocalteu's reagent showed that a narrow intense band of material remained at the origin, with a second band approximately one third of the way down the paper. The area of each paper corresponding to this latter band was cut out and eluted with methanol, the eluates being combined and taken to dryness under nitrogen.

At this stage it was decided that owing to the difficulty encountered in attempting to separate authentic 6α-hydroxyoestrone and 18-hydroxyoestrone, it would be advantageous to remove any 18-hydroxyoestrone by alkali treatment of the residue.

In a preliminary experiment one two-hundred and fiftieth of the KC-6 residue was taken, together with 10 μgs. each of authentic 6α-hydroxyoestrone and 18-hydroxyoestrone in two separate tubes. Normal sodium hydroxide (2 mls) was added to each tube and they were then allowed to stand at room temperature for two hours. The solutions were diluted with water and normal hydrochloric acid was added to bring the pH to 7 by universal indicator paper. The solutions were then extracted with ethyl acetate and the extracts chromatographed on Whatman No.2 paper in the system Benzene : Methanol : Water, 100 : 50 : 50, against untreated KC-6

residue and standards of oestrone, 6α-hydroxyoestrone and 18-hydroxyoestrone. This showed that whilst the 6α-hydroxy-oestrone was unaffected by this treatment, 18-hydroxyoestrone gave a single spot having the same chromatographic mobility as oestrone. The untreated KC-6 residue showed a very elongated spot extending from the front of the 6α-hydroxy-oestrone standard to some distance behind this spot, whereas the NaOH-treated KC-6 residue showed a much smaller spot running as 6α-hydroxyoestrone plus a second spot running as oestrone.

An investigation was then made of the possibility of separating the KC-6B material from the material running as oestrone, which is produced on alkali treatment. This could have been achieved by paper chromatography but it was felt that an alumina column might be more convenient and give less contamination than separation using paper chromatography. Accordingly 1 mg. of oestrone and 1 mg. of 6α-hydroxyoestrone were dissolved in 5 mls. of 1% (v/v) Methanol : Benzene ; Alumina (10 gms) was activated by heating for thirty minutes in vacuo at 100° C and was then deactivated by shaking for one hour with 3% (v/wt) of water. This was slurried in benzene and poured into a column of 1 cm. internal diameter. The 5 mls. of steroid solution was added to the column and allowed to fall to the level of the alumina, eluate being collected immediately.

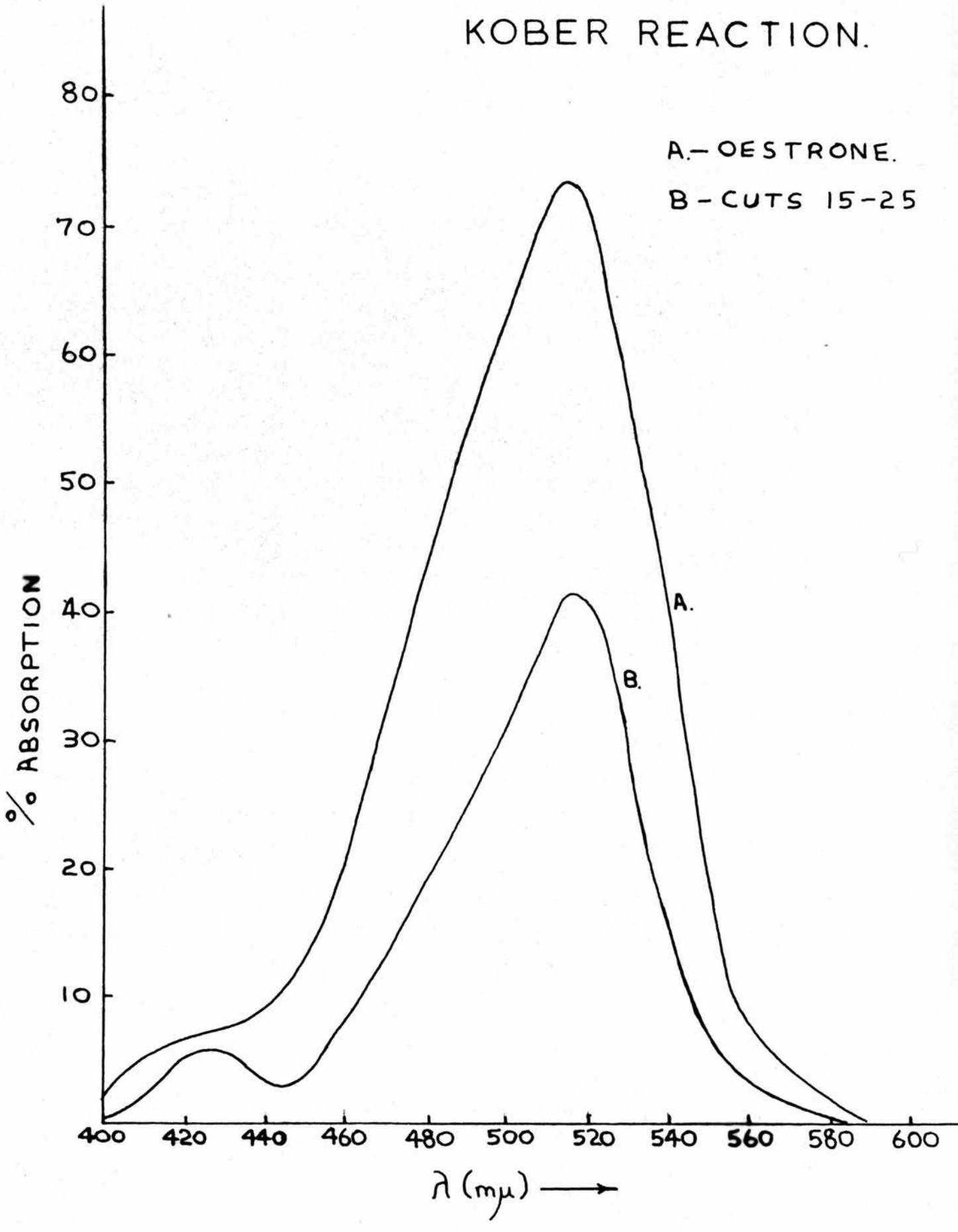

KOBER REACTION.
A.– OESTRONE.
B – CUTS 15–25
% ABSORPTION
80
70
60
50
40
30
20
10
A.
B.
400
420
440
460
480
500
520
540
560
580
600
λ (mμ) ⟶

FIG. 11.

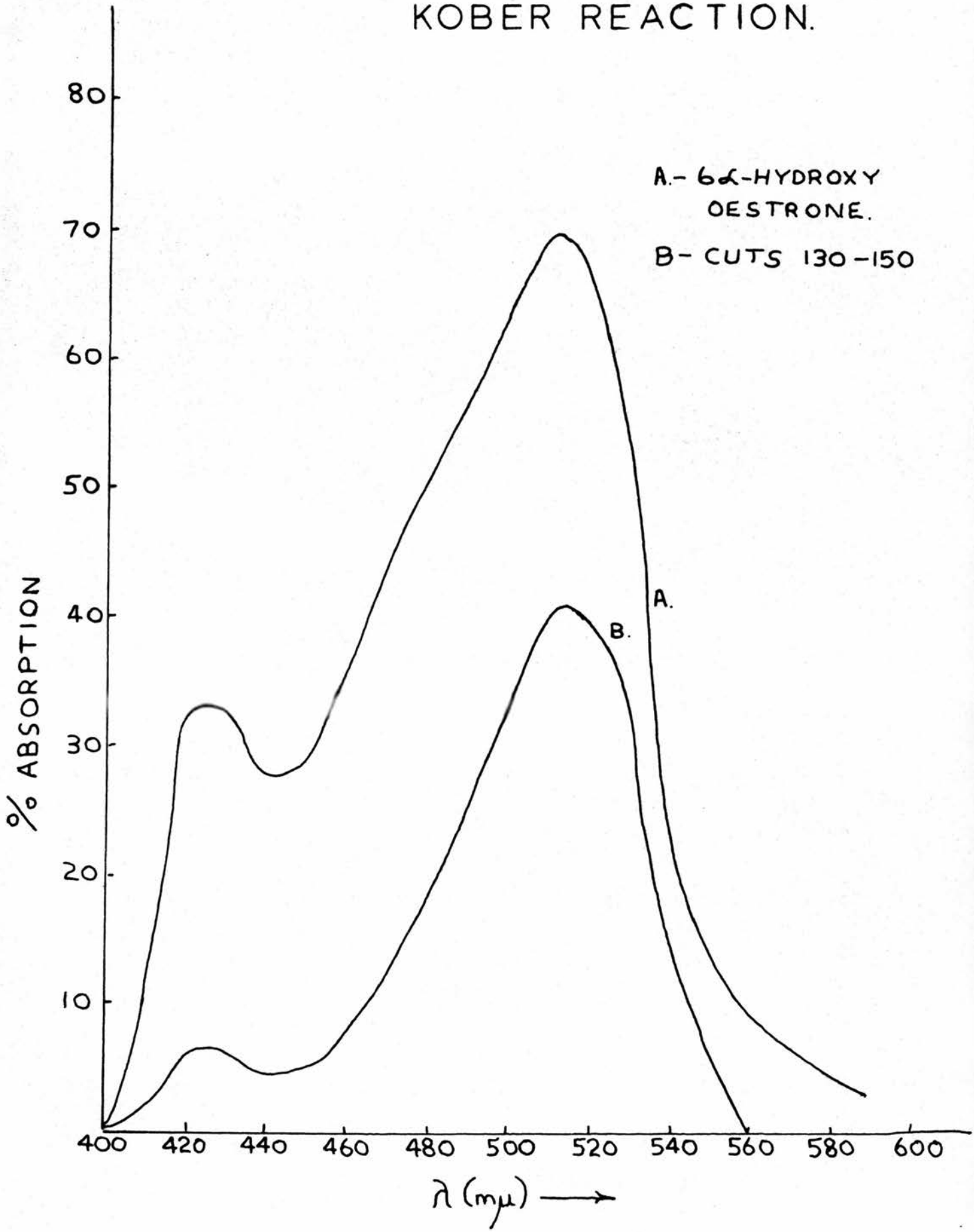

KOBER REACTION.
A.- 6α-HYDROXY OESTRONE.
B- CUTS 130-150
% ABSORPTION
80
70
60
50
40
30
20
10
A.
B.
400
420
440
460
480
500
520
540
560
580
600
λ (mμ) ⟶

FIG. 12.

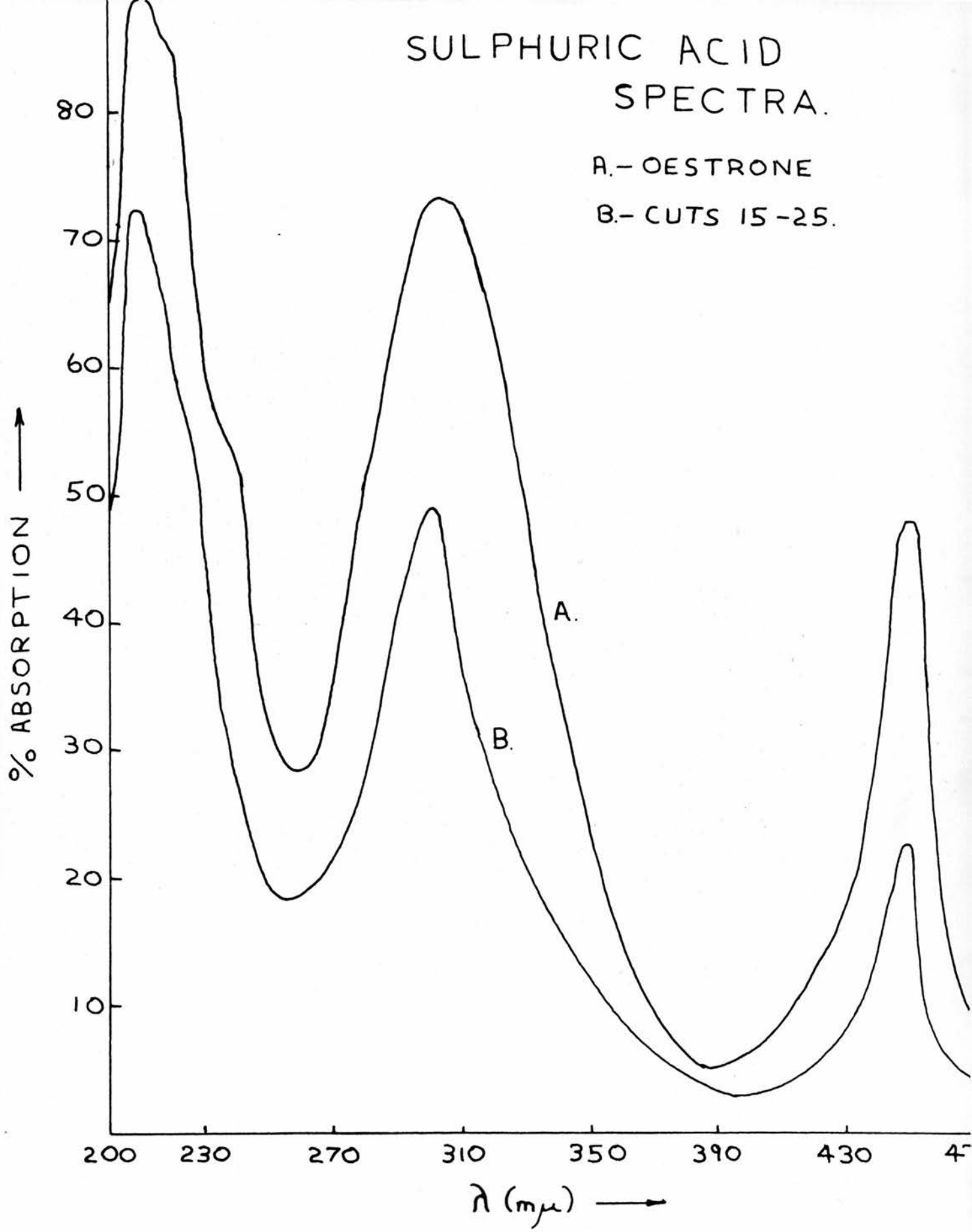

SULPHURIC ACID
SPECTRA.
A.- OESTRONE
B.- CUTS 15-25.
% ABSORPTION
80
70
60
50
40
30
20
10
A.
B.
200
230
270
310
350
390
430
λ (mμ)

FIG. 13.

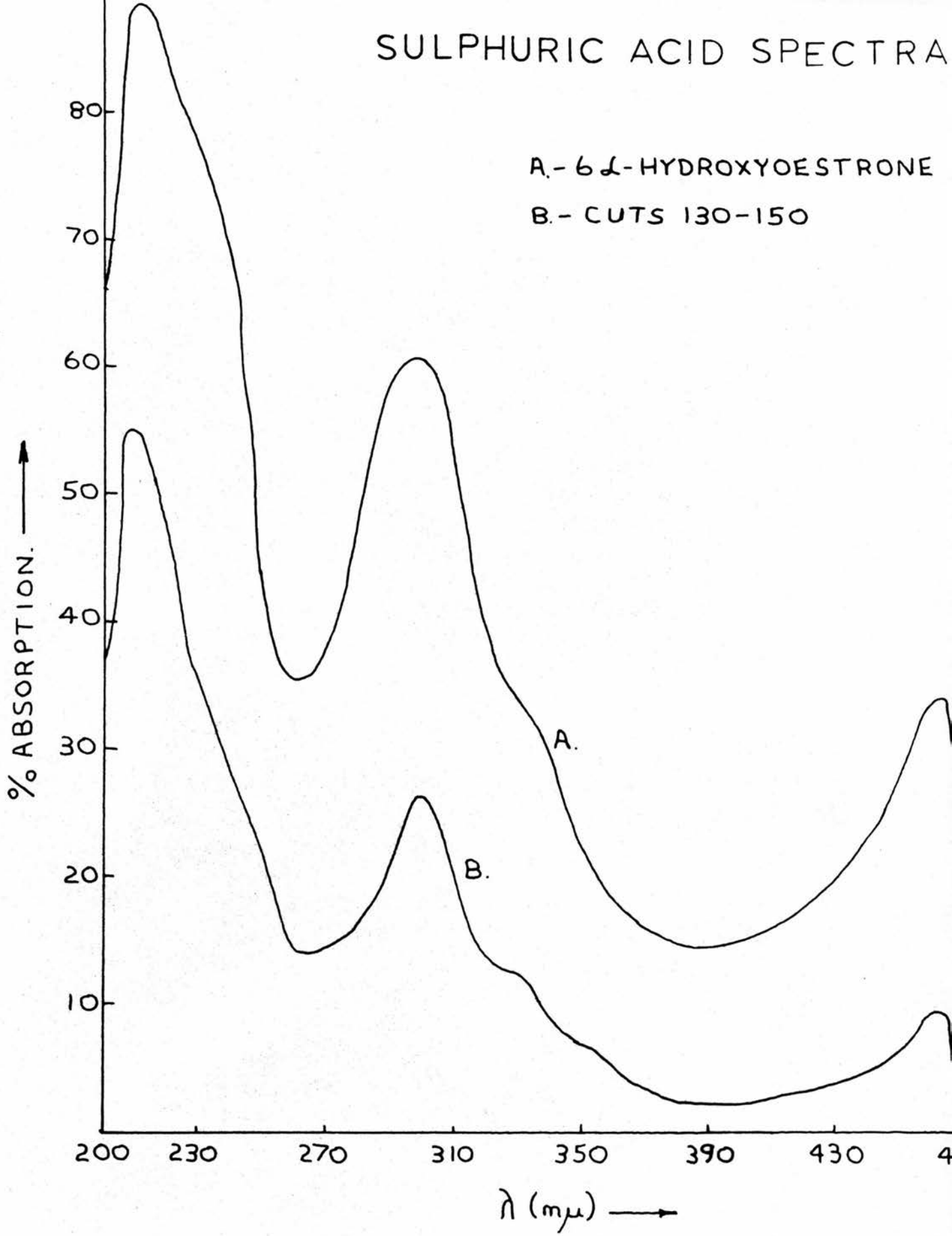

SULPHURIC ACID SPECTRA
A.- 6 α-HYDROXYOESTRONE
B.- CUTS 130-150
% ABSORPTION.
80
70
60
50
40
30
20
10
A.
B.
200
230
270
310
350
390
430
λ (mμ)

FIG. 14.

The column was eluted with 1% (v/v) Methanol : Benzene : taking 2 ml. cuts. After one hundred and thirty cuts had been collected, the elution was continued with 10% Methanol : Benzene. The cuts were taken to dryness under nitrogen, dissolved in 3.5 mls. of pure ethanol and their ultra-violet absorption spectra determined on a Unicam S.P. 700 recording spectrophotometer, over the range 250 mμ to 320 mμ. The curves so obtained showed that oestrone was eluted with its peak at cut 20 i.e. after 40 mls. and 6α-hydroxyoestrone was eluted with its peak at cut 140 i.e. after 280 mls.

Cuts 15 - 25 inclusive and cuts 130 - 140 inclusive were pooled and taken to dryness under nitrogen. Ultra-violet absorption spectroscopy and paper chromatography in the systems chloroform/formamide and Benzene : Methanol : Water, 100 : 50 : 50 showed these residues to be identical with authentic oestrone and 6α-hydroxyoestrone respectively. This was further confirmed by the Kober reaction and by their sulphuric acid spectra. (See Figs. 11, 12, 13 & 14)

It seemed feasible therefore to separate KC-6B from KC-6A by alkali treatment followed by alumina column chromatography and the next step was to prove the identity of KC-6B. From the previous investigation on KC-6B, together with the previous work of Loke (1958), it seemed likely that it might be a 6-hydroxyoestrone and it was decided to base our investigation

on this assumption. As a preliminary to investigation of the KC-6B material therefore it was decided to use standard 6α-hydroxyoestrone in an attempt to prove its structure conclusively by some means other than actual isolation of crystalline material.

(4) PRELIMINARY EXPERIMENTS ON AUTHENTIC 6α-HYDROXYOESTRONE

One method of attacking this problem arose from the previous work on the preparation of 6-oxygenated derivatives of oestrone, oestradiol-17β and oestriol. It was found that the 6-oxo-derivatives of each of these compounds have a very characteristic absorption spectrum in ethanol, showing maxima at 256 mμ and 329 mμ, these values being in excellent agreement with those quoted by Longwell and Wintersteiner (1940) for 6-oxo-oestradiol-17β. It was suggested that these characteristics could be explained by the double bond at C-6 extending the conjugation of the phenolic A-ring. Examination of the structure of the oestrogen molecule shows that the only possible position in which a double bond carrying an oxygen function can extend this conjugation directly, is at C-6 and this is in consequence a unique position in the molecule. It was felt therefore that if our basic assumption of the nature of the KC-6B was correct, then the unique nature of the oxygen function at C-6 would provide a sound basis for identification. However, if KC-6B were in fact a 6-hydroxyoestrone, it would be necessary to find a method for the oxidation of the hydroxyl group at C-6 to a ketone group.

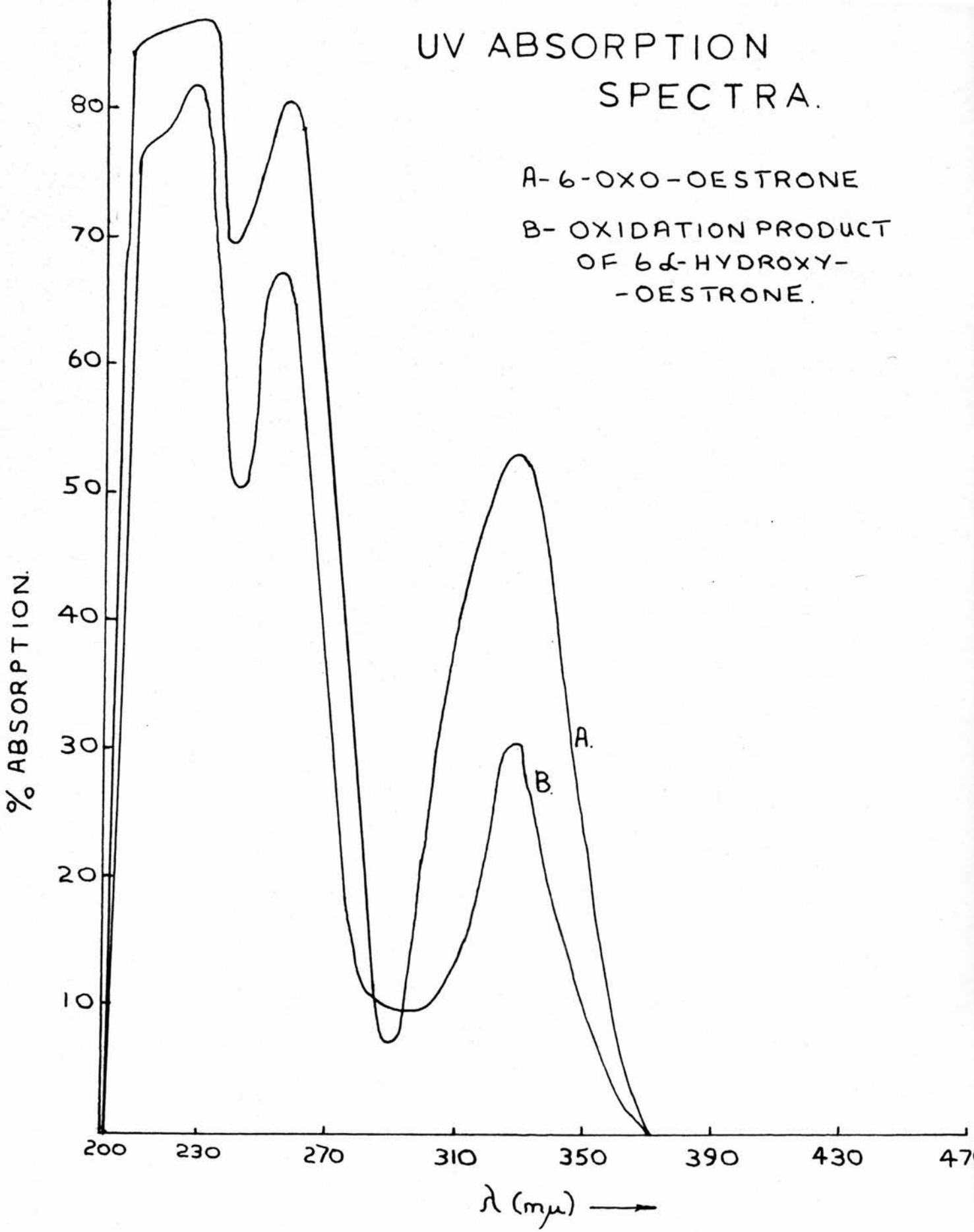

UV ABSORPTION SPECTRA.
A- 6-OXO-OESTRONE
B- OXIDATION PRODUCT OF 6α-HYDROXY--OESTRONE.
% ABSORPTION.
80
70
60
50
40
30
20
10
A.
B.
200
230
270
310
350
390
430
λ (mμ) ⟶

FIG. 15.

Iriate, Ringold and Djerassi (1958) described a method for the oxidation of the 7α-hydroxyl group of 7α-hydroxyoestrone to a 7-ketone group, using ice-cold 8 N chromium trioxide in sulphuric acid and it was decided to try this method with 6α-hydroxyoestrone.

Acetone was chilled to 0°C and 1 mg. of 6α-hydroxyoestrone was dissolved in 2 mls. of this solvent. Chromium trioxide (26.6 gs.) was dissolved in 100 mls. of 10 N sulphuric acid and this solution was also chilled to 0° C. The flask containing the steroid solution was then immersed in an ice-salt bath in order to bring the temperature below 0° C, and the chromic acid solution was added to it dropwise with stirring. After two drops had been added, the solution remained yellow and no more was added. The solution was allowed to stand, with stirring, for two minutes at 2° C and the reaction was then terminated by adding ethanol to the solution until it turned green. After diluting with water, the solution was extracted with ethyl acetate which was washed with 5% aqueous $NaHCO_3$ and water, dried over anhydrous sodium sulphate and then taken to dryness.

Comparison of this material with authentic 6-oxo-oestrone by ultra-violet spectroscopy showed them to be identical, both giving maxima at 256.5 mμ and 329 mμ. (See Fig. 15). From the intensities of the peaks in the standard solution and that of

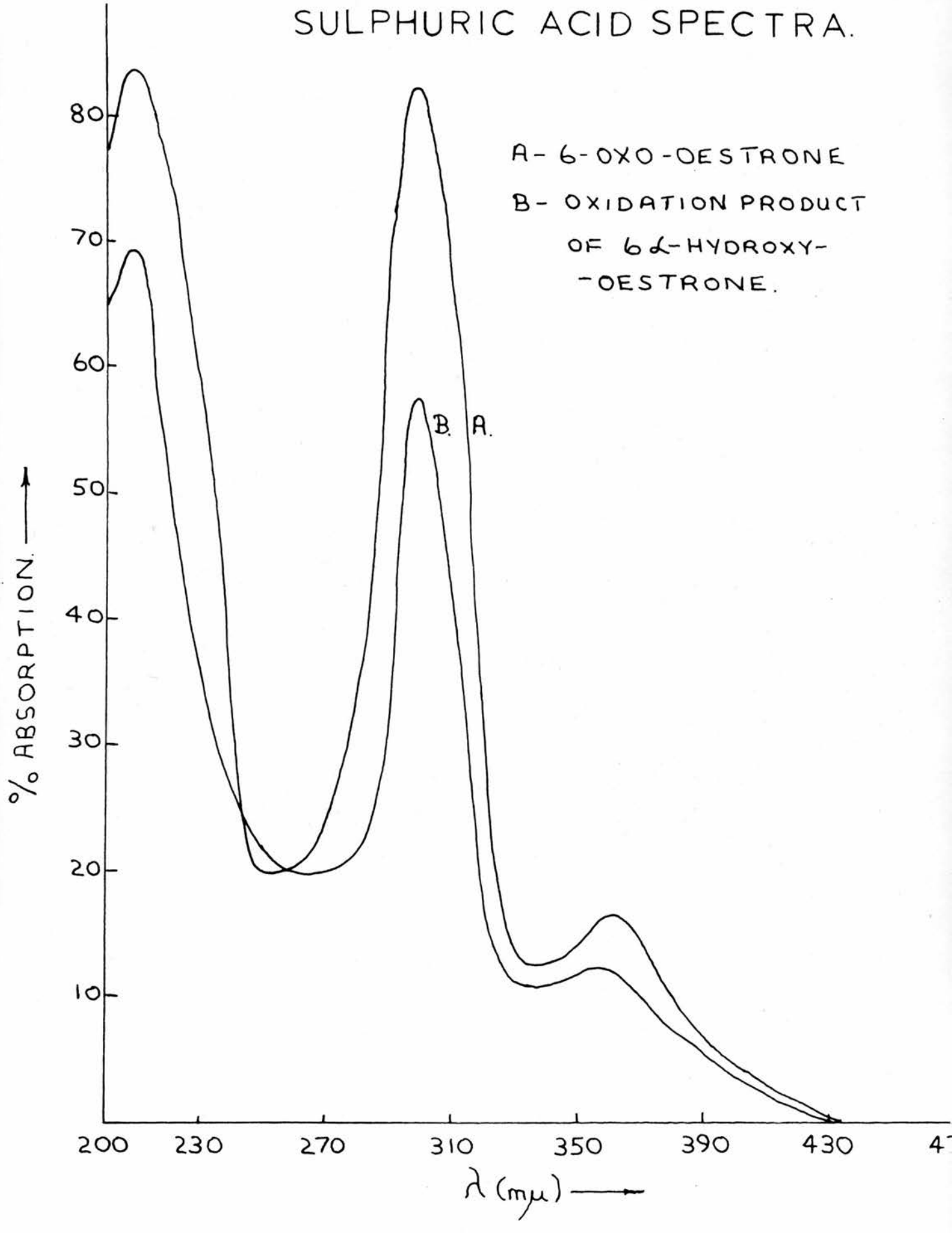

SULPHURIC ACID SPECTRA.
A- 6-OXO-OESTRONE
B- OXIDATION PRODUCT OF 6α-HYDROXY--OESTRONE.
B.
A.
% ABSORPTION
80
70
60
50
40
30
20
10
200
230
270
310
350
390
430
λ (mμ)

FIG.16.

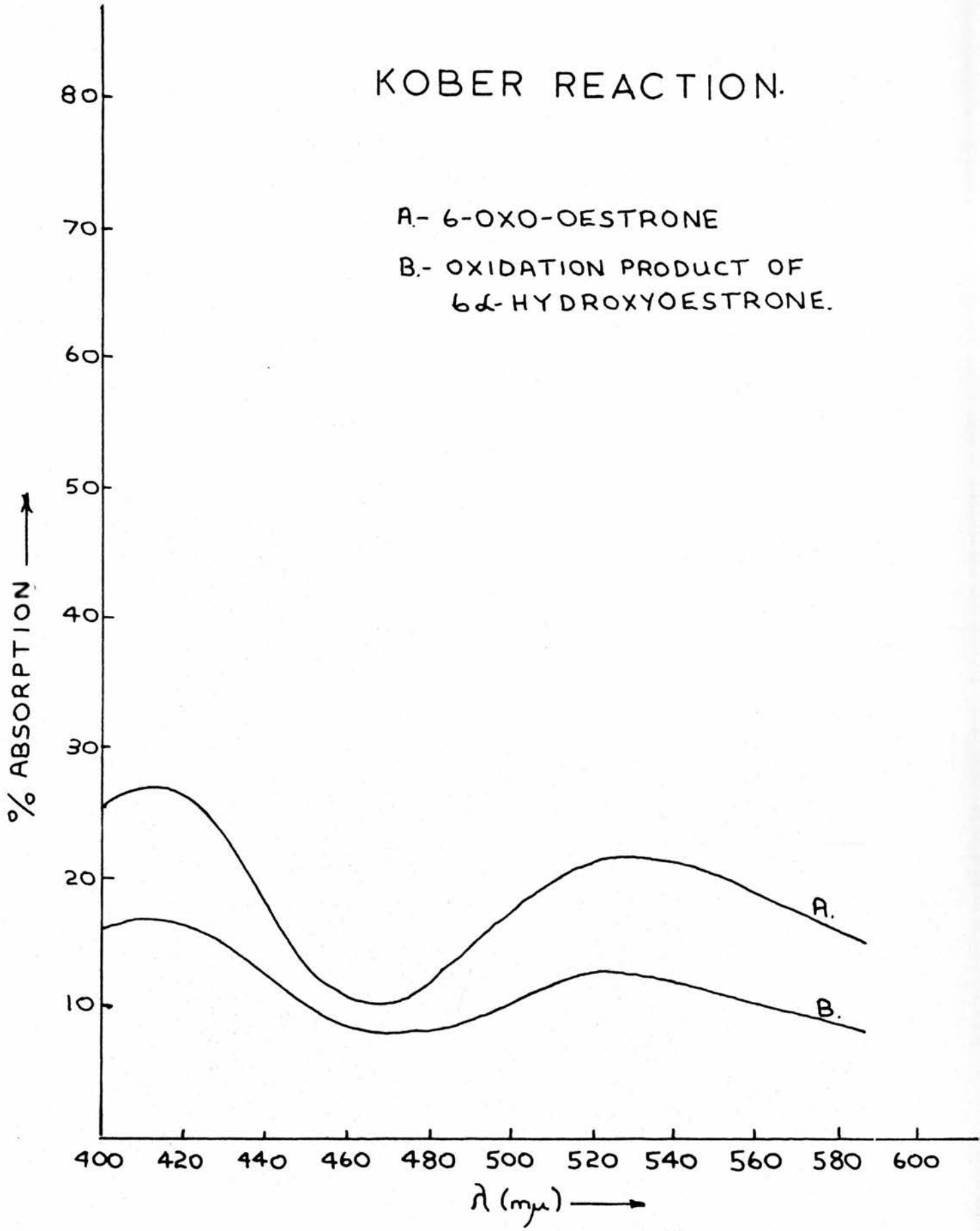

KOBER REACTION.
A.- 6-OXO-OESTRONE
B.- OXIDATION PRODUCT OF 6α-HYDROXYOESTRONE.
% ABSORPTION
80
70
60
50
40
30
20
10
400
420
440
460
480
500
520
540
560
580
600
λ (mμ)
A.
B.

FIG. 17.

the oxidation product, it appeared that the latter had been given in ca. 60% yield from the starting material.

Paper chromatography of the oxidation product in the system chloroform/formamide against authentic 6α-hydroxyoestrone and 6-oxo-oestrone, showed that it gave a single spot, corresponding in mobility exactly with the latter.

The oxidation product of 6α-hydroxyoestrone was also identical with authentic 6-oxo-oestrone in the Kober reaction and in its sulphuric acid spectrum. Furthermore, the intensities of the peaks given in these tests confirmed the estimated yield of ca. 60% on oxidation. (See Figs. 16 & 17)

(5) DETERMINATION OF THE STRUCTURE OF KC-6B

Sodium hydroxide (50 mls of N) was added to the 167.35 mgs of KC-6 residue and this was allowed to stand at room temperature for two hours. An equal volume of N hydrochloric acid was then added and the solution was extracted with ethyl acetate, the extract being washed with 5% aqueous sodium bicarbonate and water and taken to dryness.

A column was then prepared of 10 gms of alumina (which had been activated at 100^0 C in vacuo and then de-activated by shaking for one hour with 3% (v/wt) of water slurried in benzene. The residue of the sodium hydroxide-treated KC-6 material was dissolved in 1% Methanol : Benzene (v/v) and applied to the column, which was then eluted with the same solvent taking 2 ml cuts. After cut 116 the eluate was changed to 10% Methanol : Benzene and further fractions were collected up to cut 160. Each fraction was taken to dryness under nitrogen and desiccated over concentrated sulphuric acid for two days. They were then dissolved in 4 mls of ethanol and read against an ethanol blank on the Unicam SP 700 recording spectrophotometer, over the range 250 mμ - 320 mμ. From the curves obtained it was seen

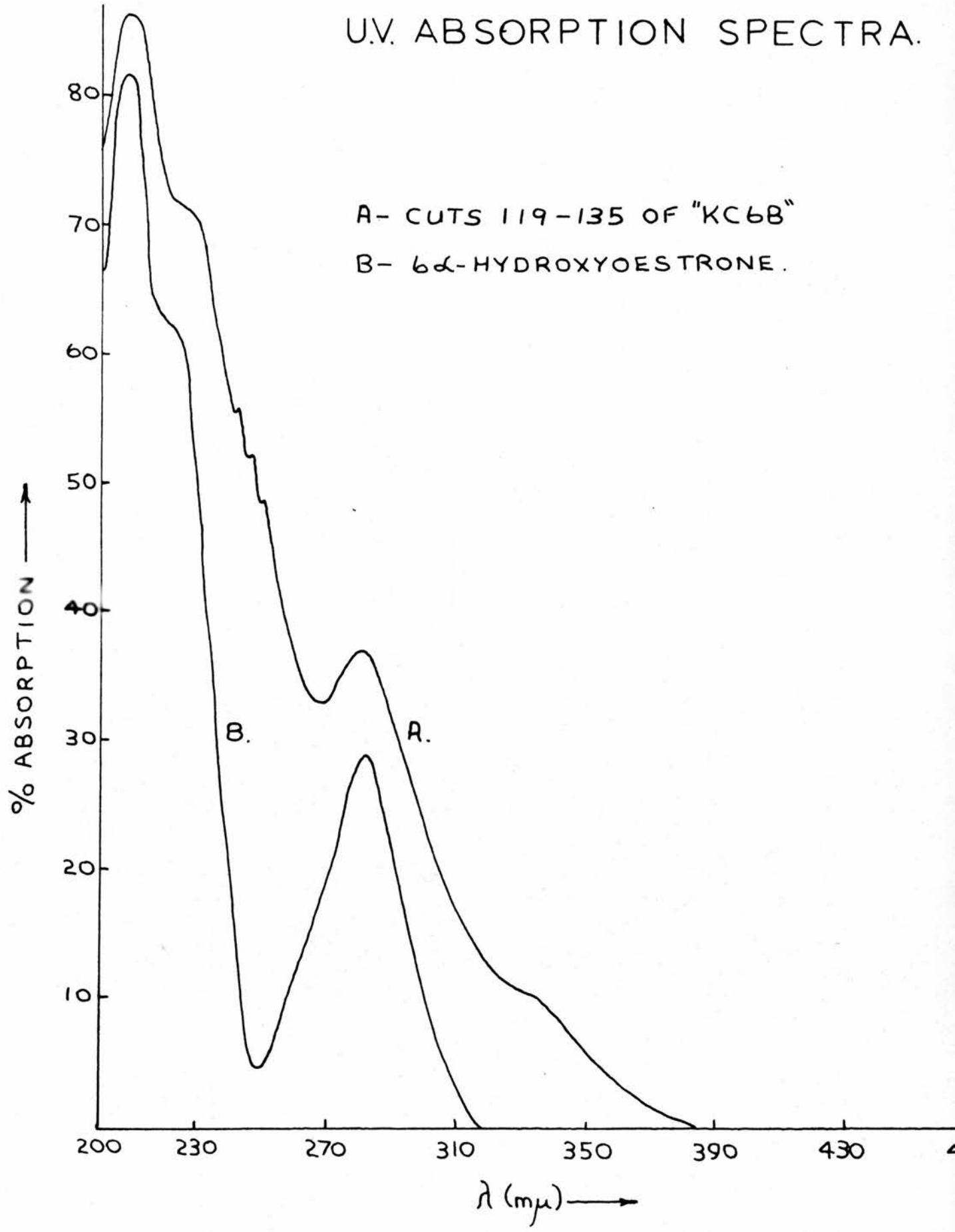

U.V. ABSORPTION SPECTRA.
A- CUTS 119-135 OF "KC6B"
B- 6α-HYDROXYOESTRONE.
% ABSORPTION
80
70
60
50
40
30
20
10
B.
A.
200
230
270
310
350
390
430
λ (mμ)

FIG. 18.

that two main fractions appeared at cuts 14 to 36 inclusive, and 119 - 135 inclusive. These cuts were therefore combined to give two fractions and taken to dryness under nitrogen.

The fraction from cuts 119 - 135 gave 9.85 mgs. of residue which on chromatography in the system 100 : Benzene/ 50 : 50 Methanol : Water, showed a single spot running as 6α-hydroxyoestrone. Examination of this material by ultra-violet spectroscopy in ethanolic solution in comparison with a standard solution of authentic 6α-hydroxyoestrone showed that they had almost identical spectra. Thus 6α-hydroxyoestrone showed a maximum at 207.5 mμ; a shoulder at 222 mμ; and a second maximum at 282.5 mμ. The KC-6B material showed a maximum at 207.9 mμ; a shoulder at 222 mμ and a second maximum at 282.5 mμ. However, the KC-6B fraction did not show such a low minimum between the 207.5 mμ and the 282.5 mμ peaks as the standard, as can only be expected in the presence of contaminating paper residue from previous paper chromatography. (See Fig. 18)

In the Kober reaction, using "oestriol reagent" (Brown, 1955) both the KC-6B material and standard 6α-hydroxy-oestrone gave a normal pink colour, having a maximum absorption at 515 mμ in both cases. Comparison of the intensities of the colours produced by equal weights of material showed that the KC-6B residue contained approximately 26% of

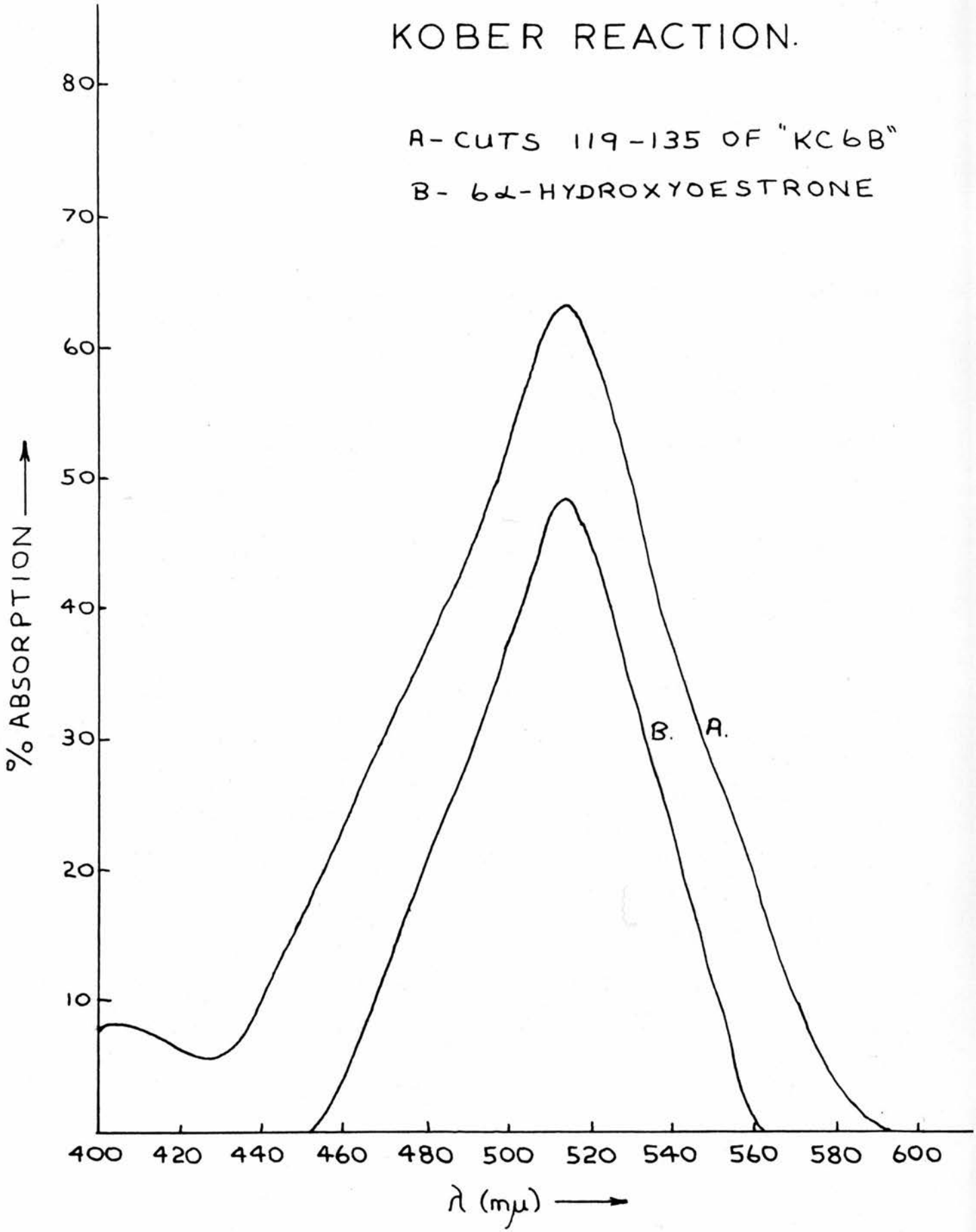

KOBER REACTION.
A- CUTS 119-135 OF "KC6B"
B- 6α-HYDROXYOESTRONE
% ABSORPTION
80
70
60
50
40
30
20
10
B.
A.
400
420
440
460
480
500
520
540
560
580
600
λ (mμ)

FIG.19.

Kober chromogen esti mated as 6α-hydroxyoestrone. (See Fig.19)

Infra-red spectroscopy of the KC-6B residue material in chloroform solution gave a very indefinite spectrum. This was not surprising considering the degree of impurity of the residue as shown by the Kober reaction. It showed two absorption bands at 1730 cms.$^{-1}$ and 1700 cms.$^{-1}$ plus a band at 1600 cms.$^{-1}$ which was much stronger than either of the other two.

At this stage it was decided to purify the KC-6B material further by paper chromatography. It was felt that the system used should have sufficient resolving power to separate 6-oxo-oestradiol-17β and 6α-hydroxyoestrone in order to obtain the degree of purification required. Two solvent systems were found to be capable of effecting this resolution, both using Whatman No. 2 paper:-

(1) "Bush B5" system - 100 : Benzene / 50 : 50 Methanol : Water.

(2) "Kushinsky" system - 70 : 30 Benzene : Heptane/60 : 40 Methanol : Water.

The former required a running time of three hours, whilst the latter required twenty hours, but as it gave a better resolution it was chosen in preference to the former. The Whatman No.2 paper used was previously washed by extraction with ethanol for three days in a Soxhlet extractor.

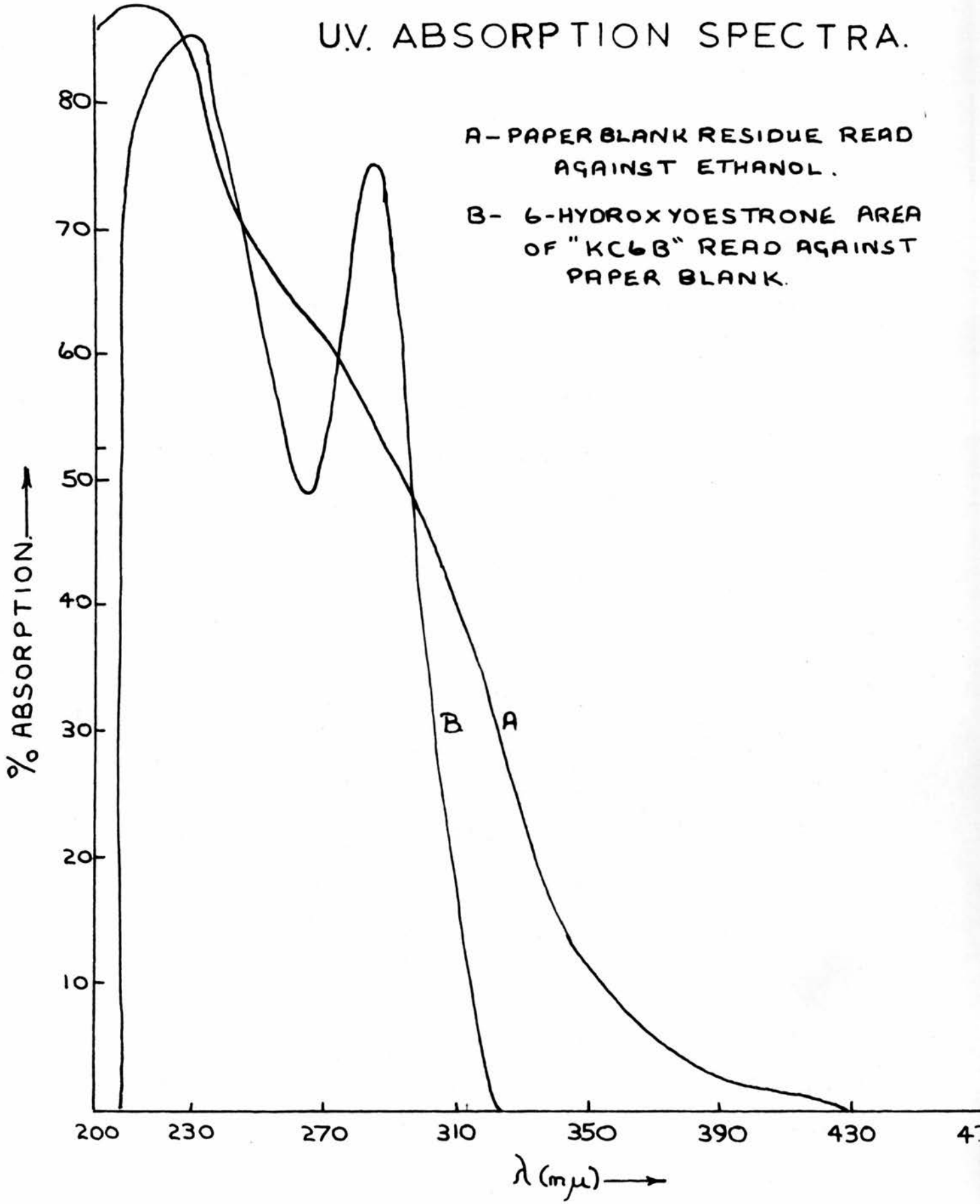

U.V. ABSORPTION SPECTRA.
A- PAPER BLANK RESIDUE READ AGAINST ETHANOL.
B- 6-HYDROXYOESTRONE AREA OF "KC6B" READ AGAINST PAPER BLANK.
% ABSORPTION
80
70
60
50
40
30
20
10
B
A
200
230
270
310
350
390
430
λ (mμ)

FIG. 20.

Compound spots of 10 μgs.each of 6α-hydroxyoestrone and 6-oxo-oestradiol-17β were applied to channels on each side of the paper. One quarter of the KC-6B residue was applied as a streak over two inches of the origin line in the centre of the paper which was then equilibrated for three hours and run overnight for eighteen hours. The side channels were removed and sprayed with Folin and Ciocalteu's reagent and the areas of the central strip corresponding to the positions of the standard spots were cut out and eluted with ethanol. An identical piece of blank washed paper which had been run in the same system was eluted with ethanol to serve as a solvent blank. The residues from these eluates were dissolved in pure ethanol and read on the Unicam S.P. 700 recording spectrophotometer against the paper blank residue as the reference solution. The 6α-hydroxyoestrone area residue gave a clearly defined spectrum having a single maximum of absorption at 285 mμ, whilst the 6-oxo-oestradiol-17β area residue gave a completely blank spectrum with no absorption over the range 250 mμ - 320 mμ. On reading this solution against pure ethanol as the reference solution it showed a non-specific absorption spectrum very similar to that given by a paper blank residue. (See Fig. 20)

Infra-red spectroscopy of the 6α-hydroxyoestrone

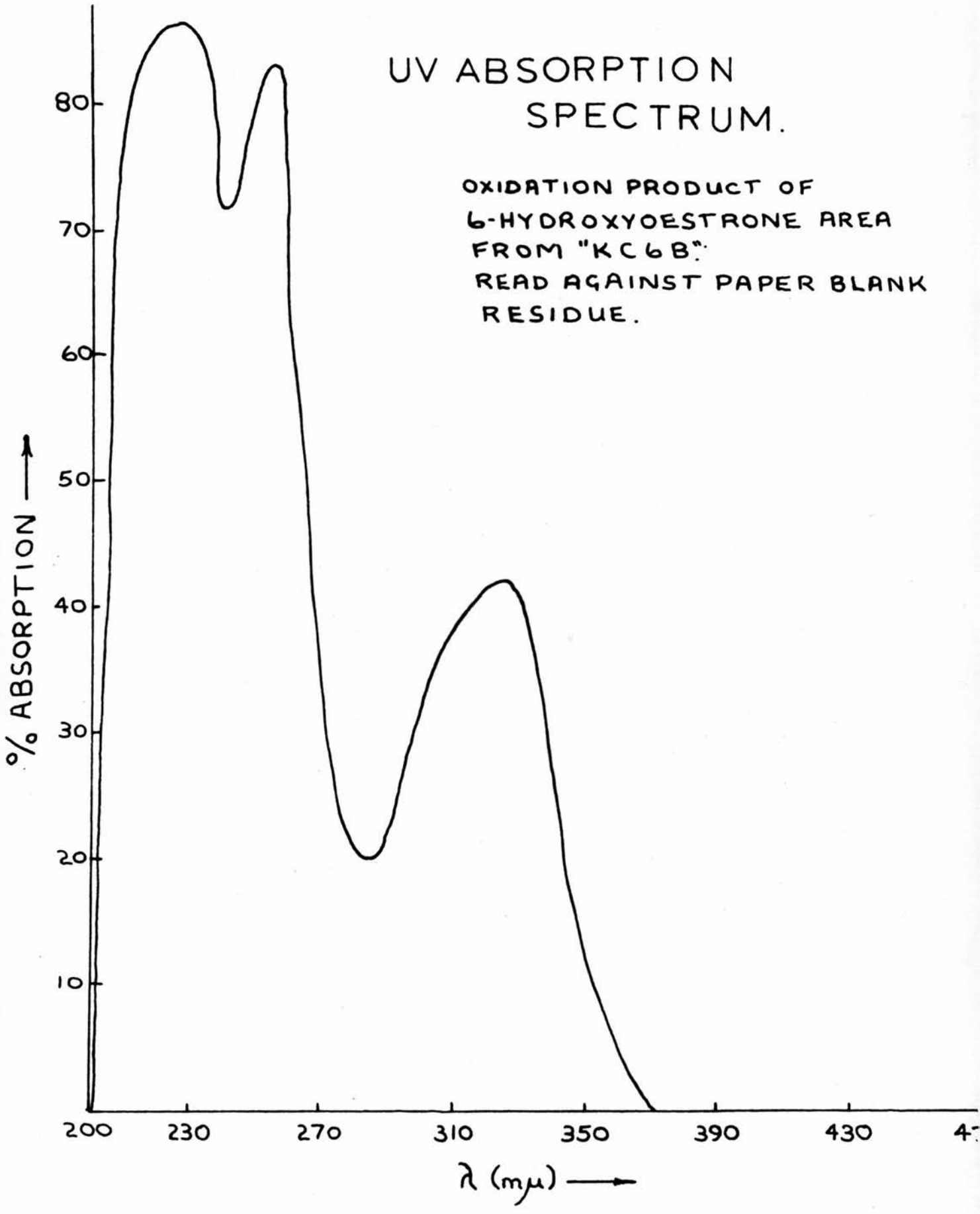

UV ABSORPTION SPECTRUM.
OXIDATION PRODUCT OF 6-HYDROXYOESTRONE AREA FROM "KC6B": READ AGAINST PAPER BLANK RESIDUE.
% ABSORPTION
80
70
60
50
40
30
20
10
200
230
270
310
350
390
430
λ (mμ)

FIG. 21.

area in chloroform solution gave a spectrum which was fully consistent with that given by standard 6α-hydroxyoestrone. Thus it showed an absorption band at 1725 cms.$^{-1}$ corresponding to a five-membered ring ketone, which was more intense than the aromatic ring stretching band at 1600 cms.$^{-1}$.

The residue from the 6α-hydroxyoestrone area was then subjected to oxidation by the method of Iriate et al.(1958) as described previously for authentic 6α-hydroxyoestrone. The acetone solution of residue required two drops of the chromic acid solution before turning yellow. It was then extracted as before and the oxidation product investigated by ultra-violet and infra-red spectroscopy.

The ultra-violet absorption spectrum in ethanolic solution when read against a solution of paper blank residue as reference showed two very clearly defined maxima at 256.7 mμ and 327 mμ. The positions of these maxima are in excellent agreement with the values quoted by Longwell and Wintersteiner (1940) for 6-oxo-oestradiol-17β and clearly indicate the presence of a 6-oxo-oestrogen derivative.(See Fig.21)

This was further confirmed by the infra-red spectrum in chloroform solution which showed absorption bands at 1660 cms.$^{-1}$ and 1600 cms.$^{-1}$ indicating a six-membered

ring ketone and aromatic ring stretching respectively. The band at 1660 cms.$^{-1}$ was less intense than that at 1600 cms.$^{-1}$, a feature which was found to be true for other 6-oxo-oestrogens. It also showed a strong absorption at 1720 cms.$^{-1}$ indicating the presence of a five-membered ring ketone, this band being more intense than that at 1600 cms.$^{-1}$. Thus the infra-red spectrum clearly indicates a compound having an aromatic ring and bearing a five-membered ring ketone group plus a six-membered ring ketone group. Taken in conjunction with the evidence from ultra-violet absorption spectroscopy this very strongly suggests that the oxidation product of the KC-6B residue is in fact 6-oxo-oestrone.

The Kober reaction, using "oestriol" reagent, was carried out on 20 μg.quantities of authentic samples of the following oestrogens; 6α-hydroxyoestrone, 6α-hydroxy-oestradiol-17β, 6-oxo-oestrone and oestriol. At the end of the second stage of the reaction, the solutions of 6α-hydroxy-oestrone, 6α-hydroxyoestradiol-17β and oestriol showed the normal pink Kober colour, whilst the 6-oxo-oestrone solution gave a pale clear brownish solution. The former three compounds also showed the normal type of oestrogen absorption curve having a single maximum at 516 mμ, whilst

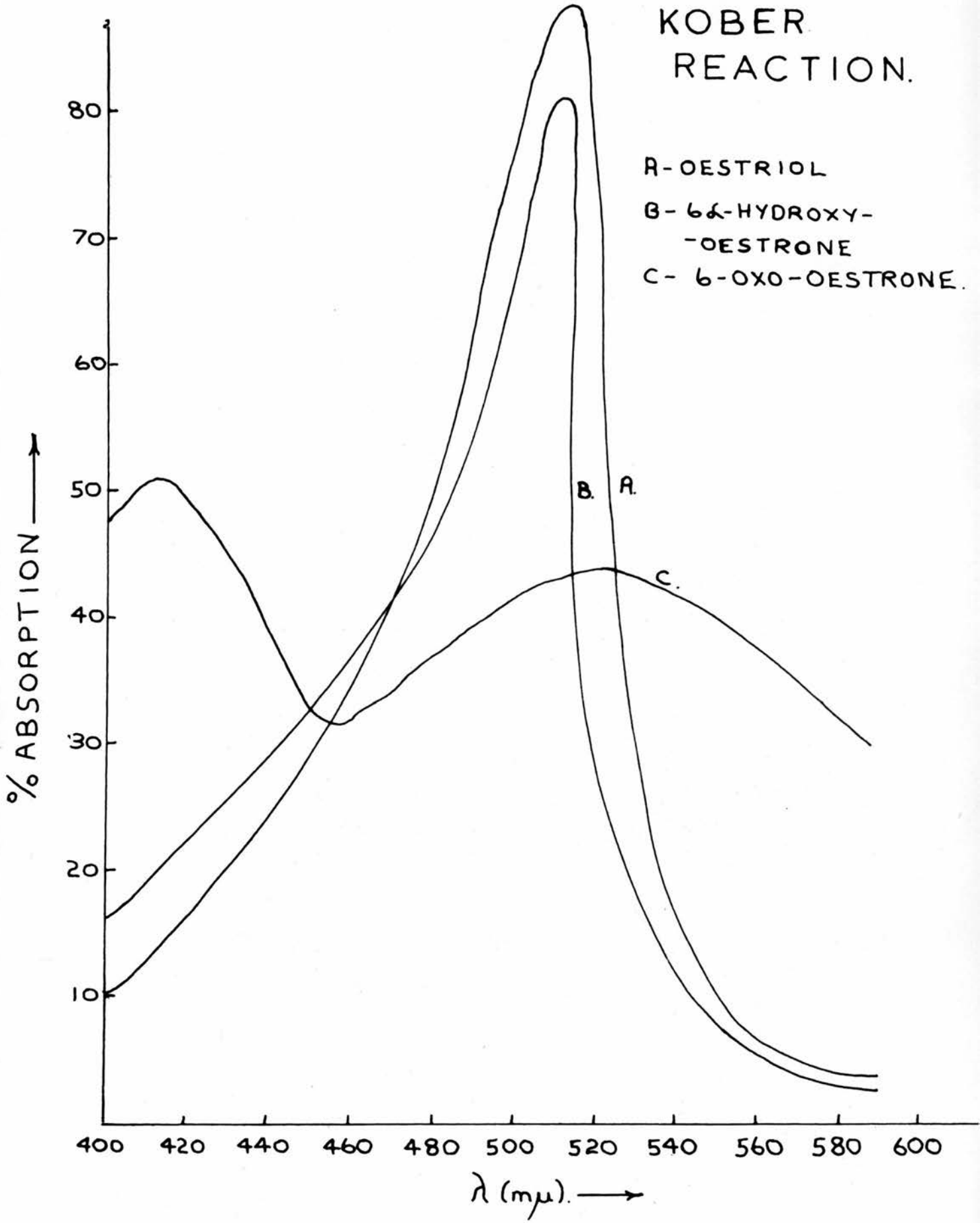

KOBER
REACTION.
A-OESTRIOL
B-6α-HYDROXY-
-OESTRONE
C-6-OXO-OESTRONE.
% ABSORPTION
80
70
60
50
40
30
20
10
B.
A.
C.
400
420
440
460
480
500
520
540
560
580
600
λ (mμ).

FIG. 22.

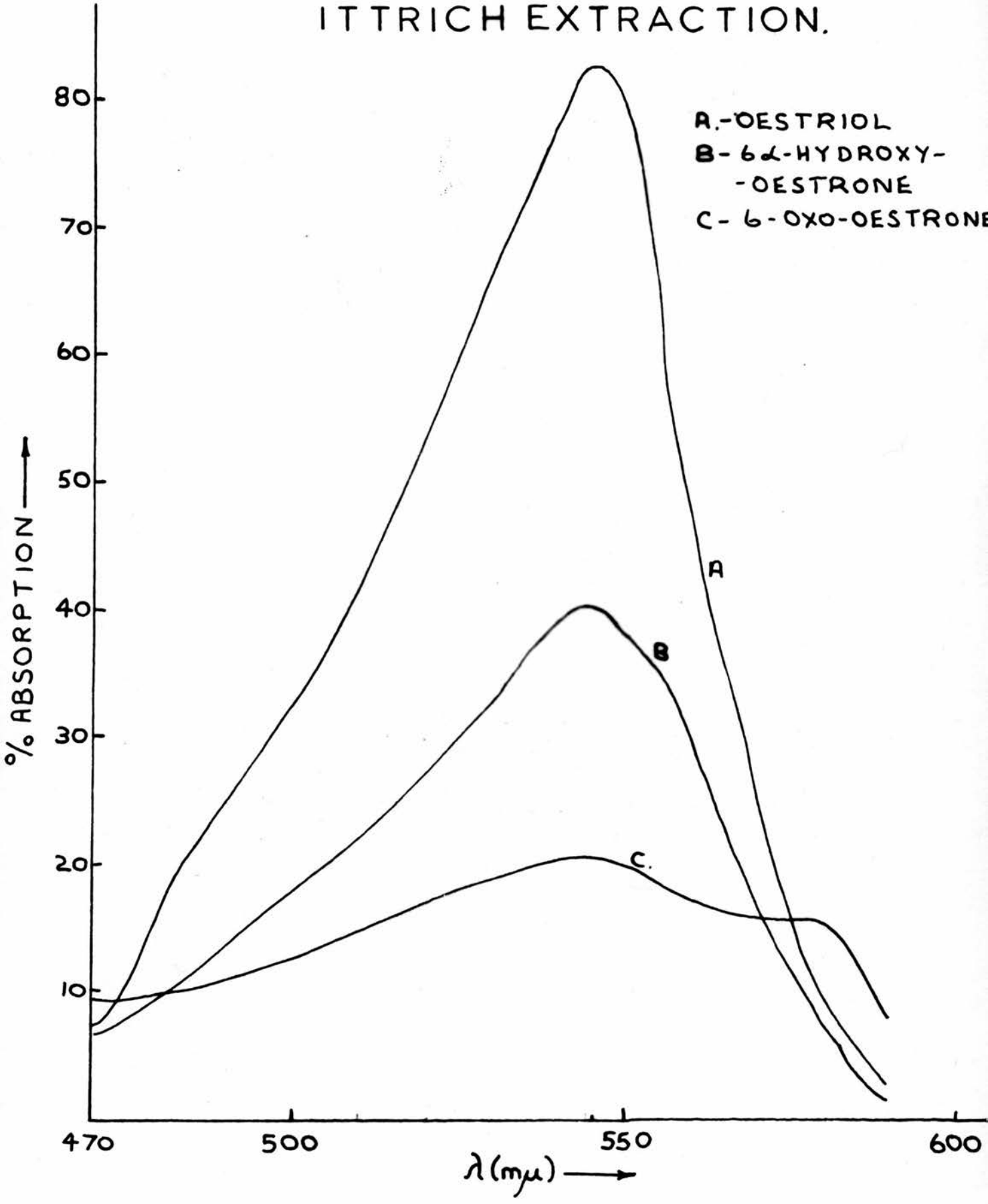

ITTRICH EXTRACTION.
A.-OESTRIOL
B- 6α-HYDROXY-
-OESTRONE
C- 6-OXO-OESTRONE
% ABSORPTION
80
70
60
50
40
30
20
10
A
B
C
470
500
550
600
λ(mμ)

FIG. 23.

6-oxo-oestrone gave a very abnormal curve having two broad maxima of low intensity at 413 mμ and 525.5mμ. When these colours were extracted by the method of Ittrich (1958), oestriol showed a very pink coloured solution which exhibited a marked greenish-yellow fluorescence. The 6α-hydroxyoestrone and 6α-hydroxyoestradiol-17β solutions were of a clear pink colour showing no fluorescence. The 6-oxo-oestrone solution was clear and colourless being indistinguishable from the reagent blank by inspection. Again, the former three solutions showed a normal type of absorption curve having a single maximum at 544 mμ, whilst the 6-oxo-oestrone solution gave an unusual spectrum with broad, low-intensity maxima at 454 mμ, 544 mμ and a shoulder at 581 mμ. (See Figs. 22 & 23)

The Kober reaction followed by extraction by the method of Ittrich (1958) was then carried out on the 6α-hydroxy-oestrone area from KC-6B and its oxidation product together with oestriol as a standard reference. The 6α-hydroxyoestrone area and oestriol solutions gave the normal pink Kober colours at the end of the reaction, whilst the extracted colours were the same as those found for the standard solutions i.e. both pink but the oestriol extract showing greenish-yellow fluorescence whilst the 6α-hydroxyoestrone area extract was clear pink showing no fluorescence. The oestriol solution

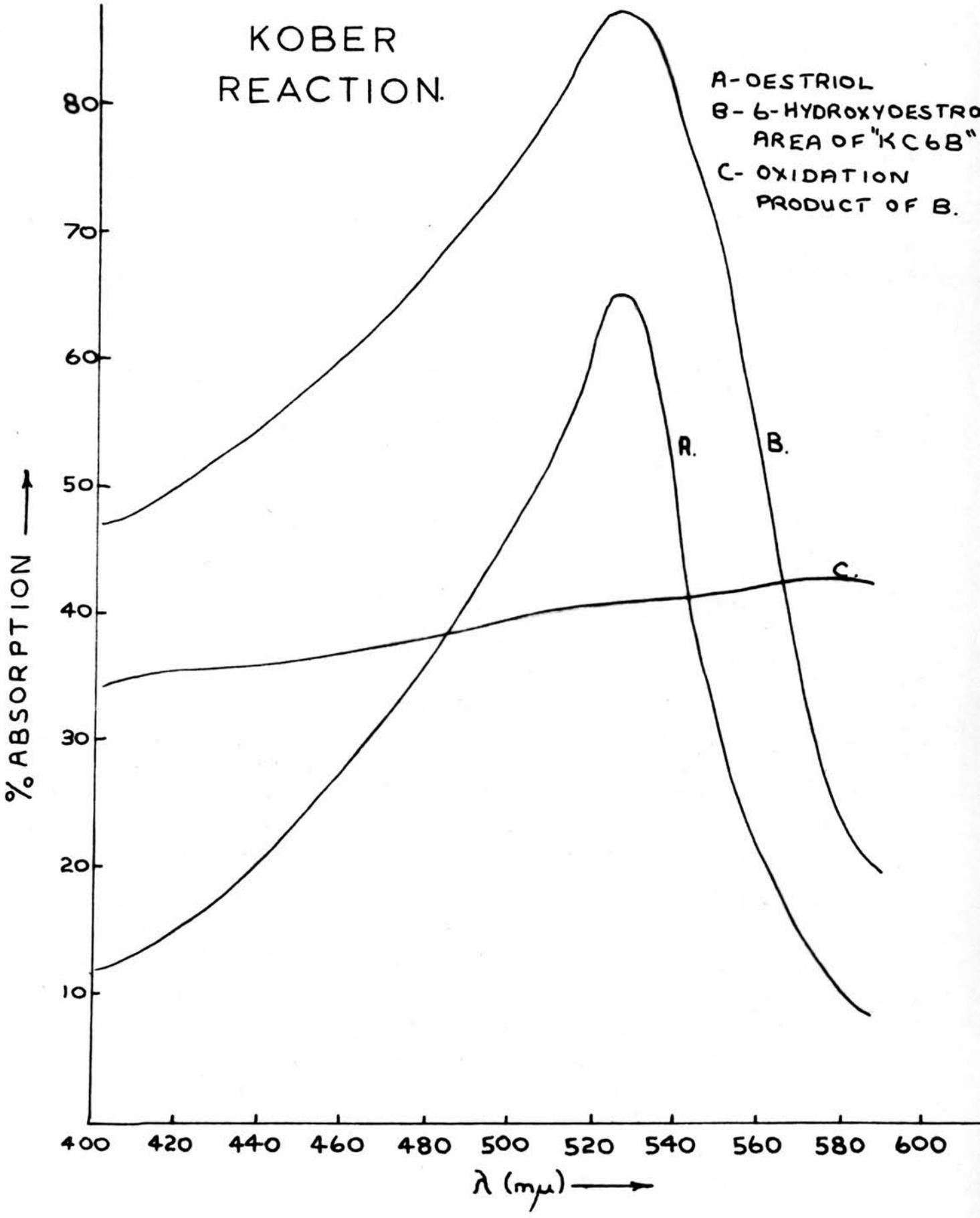

KOBER
REACTION.
A-OESTRIOL
B-6-HYDROXYOESTRO
AREA OF "KC6B"
C- OXIDATION
PRODUCT OF B.
A.
B.
C.
% ABSORPTION
80
70
60
50
40
30
20
10
400
420
440
460
480
500
520
540
560
580
600
λ (mμ)

FIG. 24.

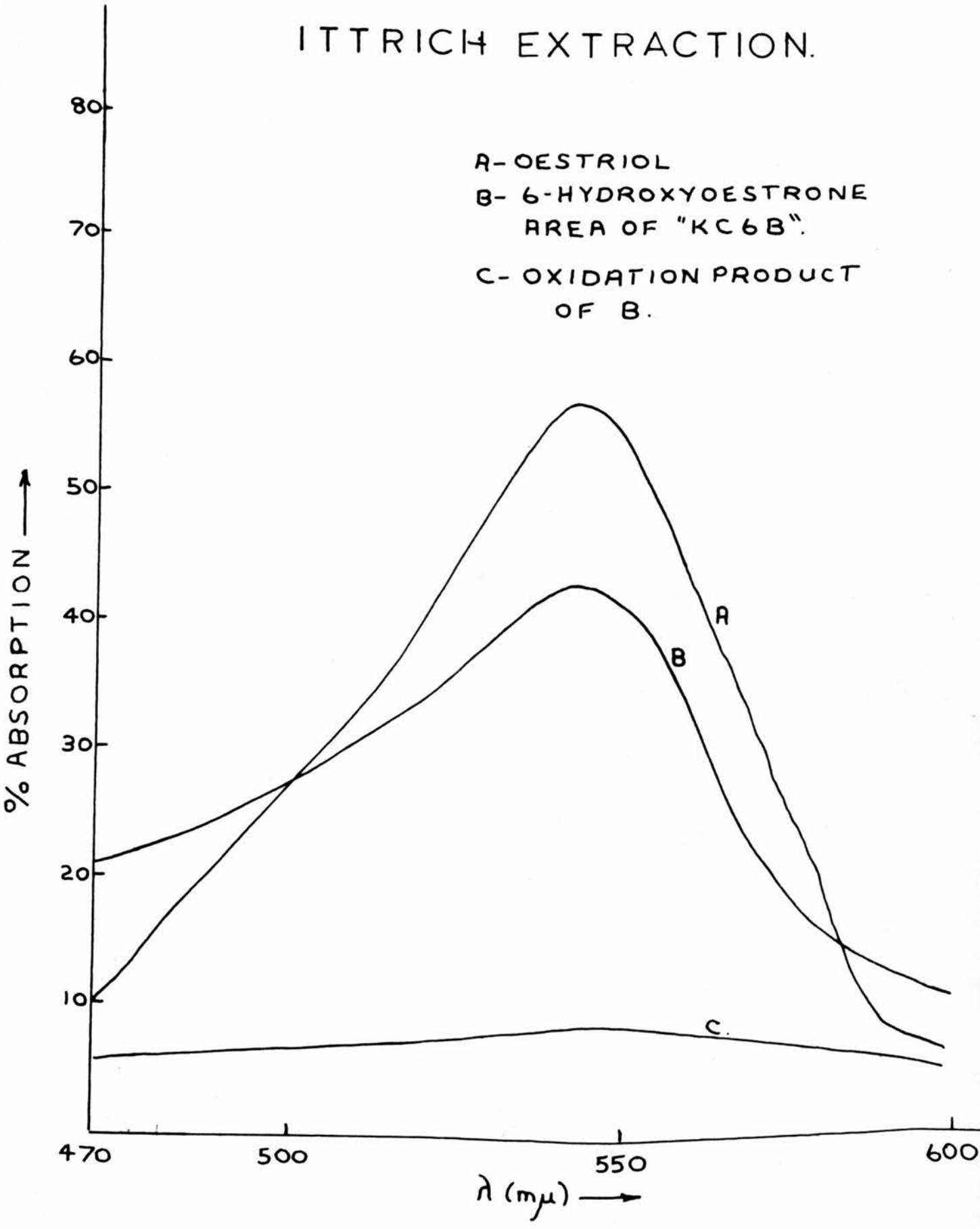

ITTRICH EXTRACTION.
A- OESTRIOL
B- 6-HYDROXYOESTRONE
AREA OF "KC6B".
C- OXIDATION PRODUCT
OF B.
% ABSORPTION
80
70
60
50
40
30
20
10
A
B
C.
470
500
550
600
λ (mμ)

FIG. 25

showed a single absorption maximum at 526 mμ after the Kober reaction and at 540.5 mμ on extraction. The 6α-hydroxyoestrone area solution also showed a single absorption maximum at 521 mμ and 546.5 mμ after the Kober reaction and on extraction respectively. The oxidation product of the 6α-hydroxyoestrone area gave a clear brownish solution having a rather unspecific absorption spectrum after the Kober reaction. On extraction, the solution was indistinguishable from the reagent blank and showed two broad and very ill-defined absorption maxima at 450.5 mμ and 556 mμ.(See Figs. 24 & 25)

Thus the 6α-hydroxyoestrone area from KC-6B is a normal positive Kober chromogen which on oxidation, by a method known to convert 6α-hydroxyoestrone to 6-oxo-oestrone, becomes a negative Kober chromogen. As 6-oxo-oestrone has also been found to be a negative Kober chromogen, this evidence fully supports the postulate that KC-6B is in fact a 6-hydroxyoestrone.

The sulphuric acid spectra of 40 μgs.-quantities of authentic samples of the following oestrogens were examined in the usual way and showed the following absorption maxima:-

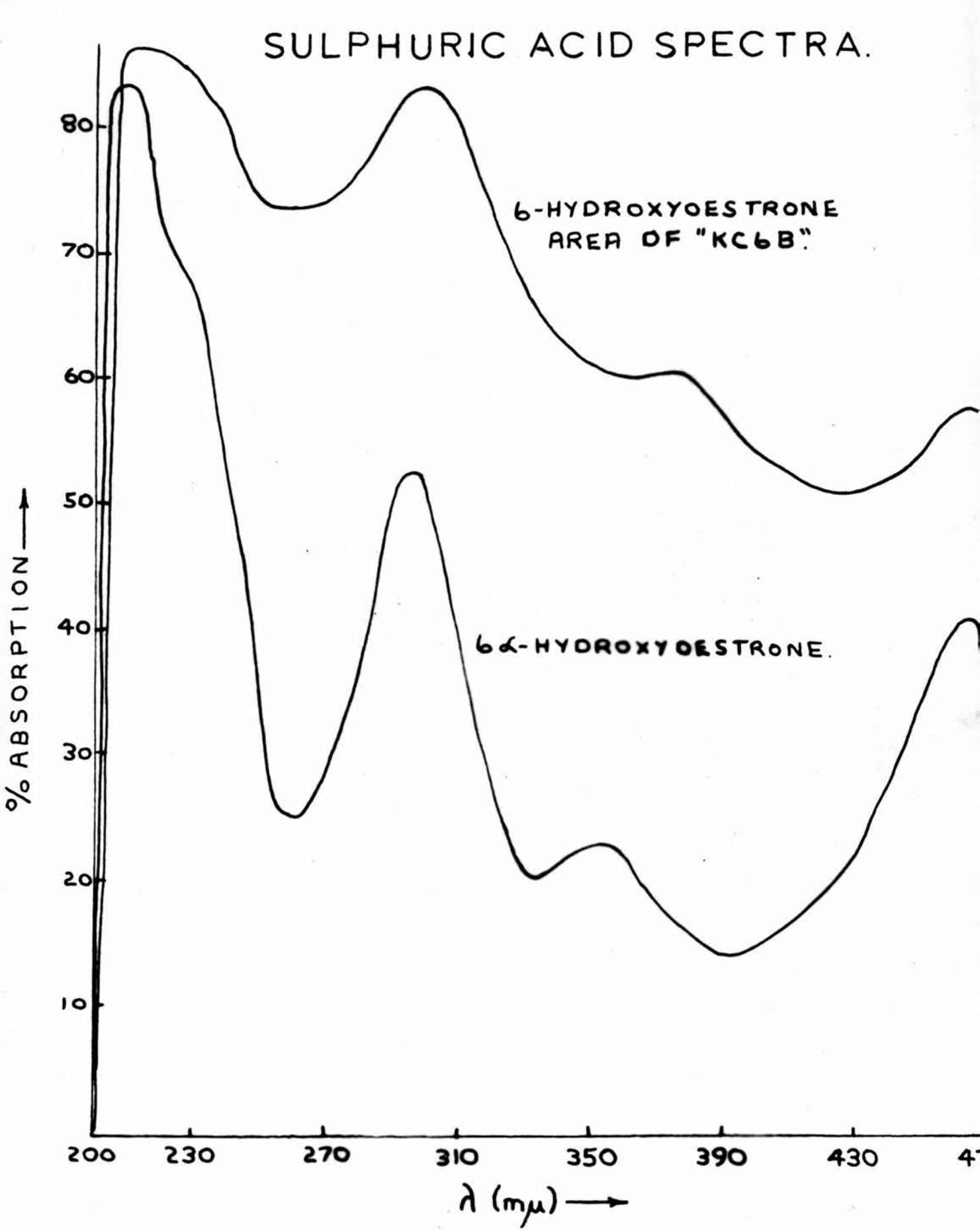

SULPHURIC ACID SPECTRA.
6-HYDROXYOESTRONE
AREA OF "KC6B".
6α-HYDROXYOESTRONE.
% ABSORPTION
80
70
60
50
40
30
20
10
200
230
270
310
350
390
430
λ (mμ)

FIG 26.

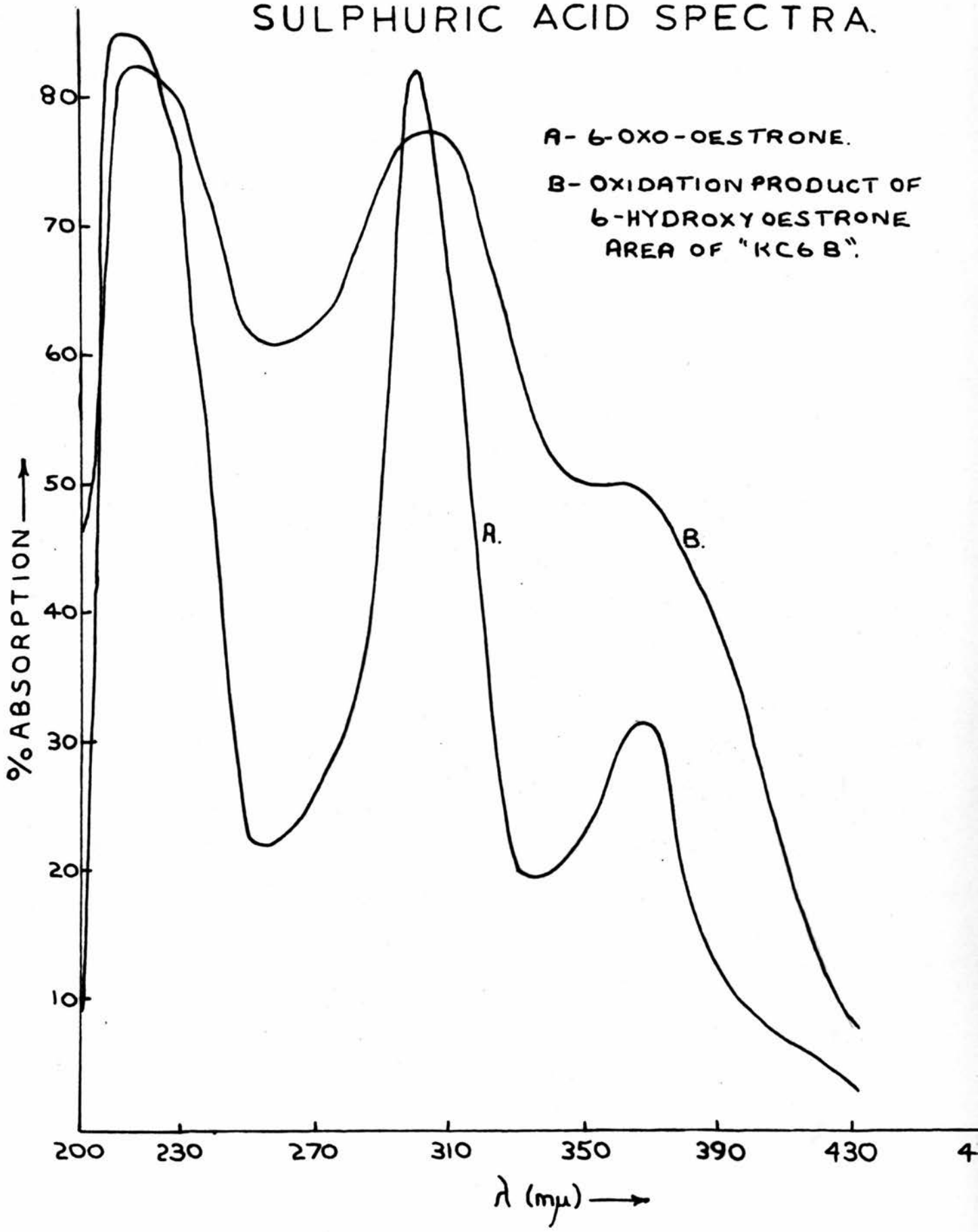

SULPHURIC ACID SPECTRA.
A- 6-OXO-OESTRONE.
B- OXIDATION PRODUCT OF 6-HYDROXY OESTRONE AREA OF "KC6 B".
A.
B.
% ABSORPTION
80
70
60
50
40
30
20
10
200
230
270
310
350
390
430
λ (mμ)

FIG. 27.

Compound	Absorption Maximum (mμ)
Oestriol	232, 306.5, 455.
6α-hydroxyoestradiol-17β	266, 301, 370.5 435, 454.5
6α-hydroxyoestrone	297.5, 355, 467.
6-oxo-oestrone	301, 367.5.

The sulphuric acid spectra of the 6α-hydroxyoestrone area from KC-6B and its oxidation product were also examined together with oestriol as reference, and showed the following absorption maxima:

Compound	Absorption Maximum (mμ)
Oestriol	234.5, 308.5, 458.5.
6α-hydroxyoestrone area from KC-6B	303, 356, 467.
Oxidation product of 6α-hydroxyoestrone area of KC-6B	300.5, 357.

The very close similarities of these spectra with those given by authentic samples further support the suggestion that KC-6B is a 6-hydroxyoestrone and that its oxidation product is 6-oxo-oestrone. (See Figs. 26 & 27)

The evidence obtained in support of this suggestion is summarized below:-

(1) The material has the same mobility as 6α-hydroxyoestrone in the Kushinsky system, in which the resolving power is sufficiently good to effect a clear separation of 6α-hydroxyoestrone and 6-oxo-oestradiol-17β.

(2) It shows a single ultra-violet absorption maximum at 285 mμ in ethanol, identical with that of authentic 6α-hydroxyoestrone.

(3) Its infra-red spectrum in chloroform solution shows absorption bands at 1725 cms.$^{-1}$ and 1600 cms.$^{-1}$ indicating the presence of a five-membered ring ketone and an aromatic ring.

(4) It is a positive Kober chromogen showing a single absorption maximum at 521 mμ and at 546.5 mμ following extraction by the Ittrich method. This conforms with the behaviour of 6α-hydroxyoestrone in this reaction and furthermore the Ittrich extract of the KC-6B material shows no fluorescence c.f. oestriol and 6α-hydroxyoestriol.

(5) The chromogen given with concentrated sulphuric acid shows the same characteristics as 6α-hydroxy-oestrone.

(6) On oxidation by a method which is known to convert 6α-hydroxyoestrone to 6-oxo-oestrone the oxidation

product shows the following characteristics:

(a) The ultra-violet absorption spectrum in ethanol shows absorption maxima at 256.5 mμ and 327 mμ which conforms to the unique spectra given by 6-oxo-oestrogens.

(b) The infra-red spectrum of the oxidation product indicates the presence of a five-membered ring ketone plus a six-membered ring ketone and an aromatic ring.

(c) The oxidation product is a negative Kober chromogen, as is 6-oxo-oestrone.

(d) The sulphuric acid spectrum of the oxidation product is very similar to that of authentic 6-oxo-oestrone.

It is submitted therefore that KC-6B is in fact a 6-hydroxyoestrone.

(6) DETERMINATION OF THE CONFIGURATION OF THE 6-HYDROXY GROUP IN KC-6B

The configuration of the 6-hydroxyl group in the phenolic steroids has been definitely established in a most convincing manner by Breuer, Knuppen and Pangels (1961). These authors separated the α and β isomers of 6-hydroxy-oestradiol-17β by chromatography in the system 5 : 1 chloroform : ethyl acetate/formamide, and it was considered that this system might enable the assignment of a definite configuration to the 6-hydroxyl group in KC-6B.

As a preliminary to investigating KC-6B in this way it was necessary to prepare authentic samples of 6α-hydroxyoestradiol-17β and 6β-hydroxyoestradiol-17β; the latter by catalytic hydrogenation of 6-oxo-oestradiol-17β diacetate with platinum oxide in ethanol.

6-oxo-oestradiol-17β diacetate (10.3 mgs) was dissolved in 1.0 mls. of pure ethanol and 4. 5 mgs. of platinum oxide was added. This solution was shaken for three hours under an atmosphere of hydrogen and was then left standing under an atmosphere of hydrogen for two days. The ethanolic solution was filtered through a small sintered glass funnel to remove platinum oxide and the solution was evaporated to dryness under nitrogen. The residue was then hydrolyzed by

standing overnight at room temperature under an atmosphere of nitrogen with 5 mls of a 5% methanolic solution of potassium hydroxide. The solution was diluted with water, made acid to phenolphthalein with carbon dioxide and was then extracted with ether.

The infra-red spectrum of the extracted residue in chloroform showed the complete absence of any ketone absorption bands indicating that the material had been completely reduced.

The 7.0 mgs. of this residue was then crystallized overnight at 0°C from acetone : benzene to give 4.15 mgs of crystalline material.

6α-Hydroxyoestradiol-17β was prepared by sodium borohydride reduction in methanol of 6-oxo-oestradiol-17β diacetate at room temperature. The reduced material was extracted with ether and the residue hydrolysed overnight at room temperature with 5% methanolic potassium hydroxide, under an atmosphere of nitrogen. This solution was diluted with water and made acid to phenolphthalein with carbon dioxide, before being extracted with ether.

Infra-red spectroscopy of the residue in chloroform against a chloroform blank showed the complete absence of any ketone peaks, indicating that the material had been fully reduced. The 4.0 mgs. of this residue was then

recrystallized from acetone/benzene overnight at 0^0 C : yield 3.50 mgs. of crystalline material.

The 6α-hydroxyoestrone area material from the KC-6B residue was also subjected to sodium borohydride reduction in methanol at room temperature in order to reduce the 17-oxo-group. Infra-red spectroscopy of the extracted reduction product showed the presence of a small absorption band at 1730 cms^{-1}. Although this would appear to indicate an unreduced 17-ketone, the presence of a similar peak in the paper-blank residue makes it of doubtful significance.

A $^1/_{10}$th aliquot of this reduction product was chromatographed against 6α- and 6β-hydroxy-oestradiol-17β's in the system chloroform (5) : ethyl acetate (1)/formamide using Whatman No. 2 paper. This was impregnated with formamide in the usual way and after spotting was equilibrated for one hour and run for three hours. The compounds had hardly moved from the origin, indicating that a much longer running time would be required.

This chromatogram was repeated using various papers and varying conditions with running times of the order of eighteen hours but no separation of 6α- and 6β-hydroxy-oestradiol-17β's was achieved. This was thought to be due to

the marked elongation of the spots and this trouble was traced to the presence of moisture (presumably) in the formamide, as after passing the formamide through a short column of dried alumina, this was overcome. However, there was still no separation of the two epimers under the conditions used.

In a personal communication, Dr. Breuer very kindly gave further details of this chromatographic method and it was found that he used Schleicher and Schüll paper 20436 MgI. It was also interesting to note that he placed a dish of anhydrous calcium chloride in the bottom of the chromatography tank, presumably to absorb moisture.

In view of the difficulty experienced in obtaining the recommended grade of chromatography paper it was decided to investigate the possibility of separating the epimeric 6-hydroxyoestradiol-17β's by other means.

It was considered that the methyl ethers of these compounds might be more readily separable and so the standard solutions of 6α and 6β-hydroxyoestradiol-17β's were methylated in the usual way.

Aliquots of 10 μgs. of each of the methyl ethers were applied to Whatman No. 2 paper in the system 70 : 30 benzene : heptane /60 : 40 methanol : water and after equilibrating overnight the paper was run for four hours. Treatment of the paper with fuming sulphuric acid and examination under ultra-violet light showed that there was no

separation of the methyl ethers.

A second chromatogram was run on Whatman No. 2 paper in the system iso-octane : 100 / methanol : water 80 : 20 for nineteen hours. As the spots had only moved very slightly from the origin no separation was achieved but it appeared that with further movement a separation might be achieved in this type of system.

It was decided to try to speed up the rate of running of the methyl ethers by :

(a) Addition of benzene to the mobile phase.

(b) Use of a "faster" paper i.e. Whatman No. 1 instead of Whatman No. 2.

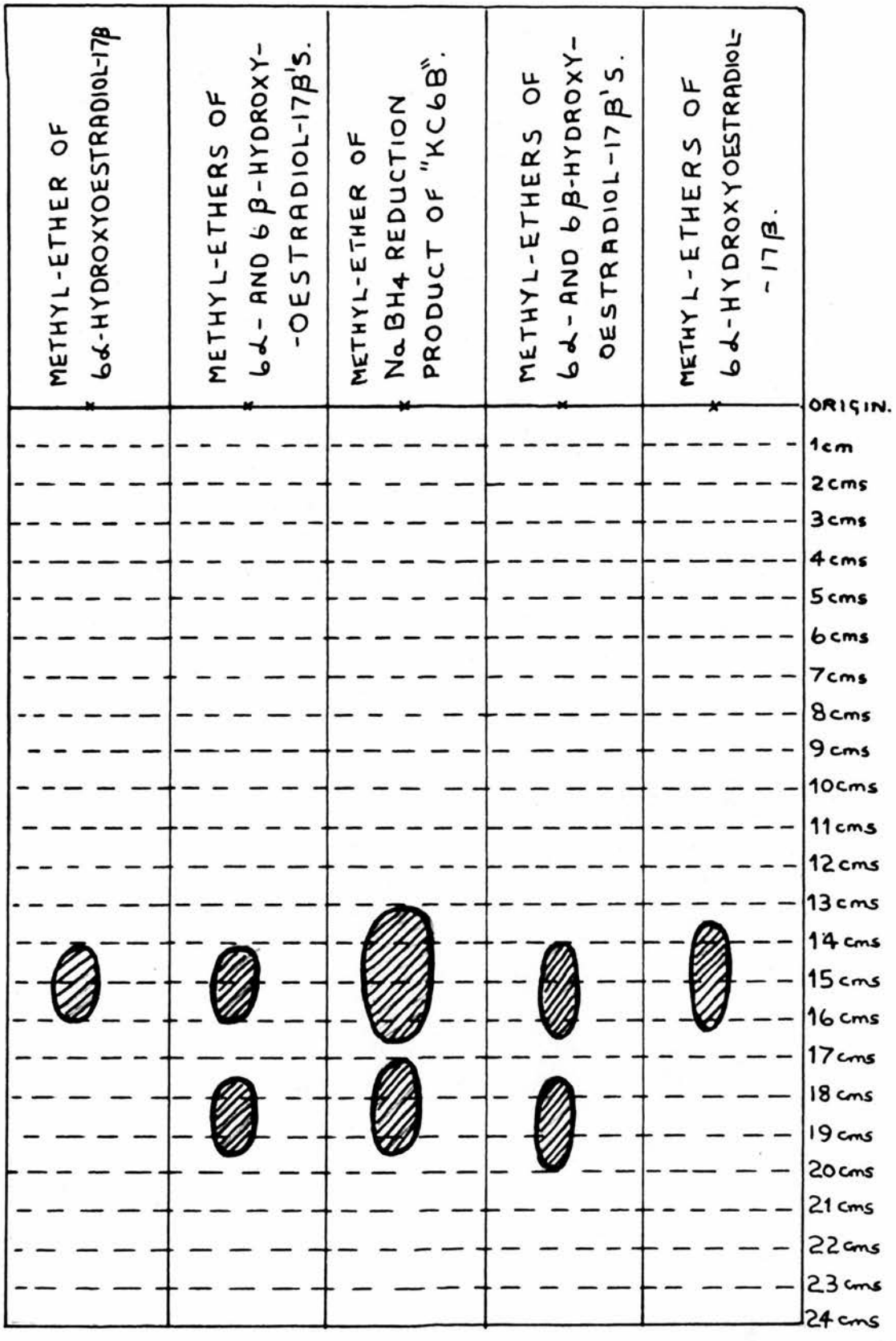
Therefore a tank was set up in the system iso-octane : benzene 70 : 30 /methanol : water 80 : 20. Aliquots of 10 μgs. of each of the methyl ethers were spotted on Whatman No. 1 paper which was then equilibrated for three hours and run for sixteen hours. On development with fuming sulphuric acid, the 6α-hydroxyoestradiol-17β methyl ether gave a single spot from 6.0 to 8.0 cms. from the origin. The 6β-hydroxy-oestradiol-17β gave a single spot from 8.0 cms. to 9.5 cms. from the origin with a "tail" from 8.0 cms. to 8.5 cms. from the origin. The mixed spot of both epimers gave a spot from 6.2 cms to 9.8 cms. from the origin with a marked construction at 8.0 cms. from the origin. It was thought that the "tail" shown by the 6β-epimer might well be due to

contamination with 6α-hydroxyoestradiol-17β as this would be expected from catalytic hydrogenation even after crystallization (Wintersteiner and Moore 1959).

The above chromatogram was then repeated, running for forty-eight hours. This time, a very clear-cut separation of the two epimers was achieved and it was also clearly shown that the 6β-hydroxyoestradiol-17β was contaminated with approximately 10 % of the 6α-epimer. Thus, the 6α-hydroxyoestradiol-17β methyl ether showed a single spot at 14.0 cms. from the origin whilst the 6β-hydroxyoestradiol-17β methyl ether showed a major spot at 17.5 cms. from the origin with a minor spot at 14.0 cms. from the origin. The compound spot of both epimers showed the presence of two strong spots at 14.0 cms. and 17.1 cms. from the origin.

This system therefore gives an excellent separation of the methyl ethers of the epimeric 6-hydroxyoestradiol-17β's.

Approximately 100 μgs. of the sodium borohydride reduction product of the 6α-hydroxyoestrone area from the KC-6B residue was methylated in the usual way. The methyl ether was then run in the system iso-octane : benzene 70 : 30 /methanol : water 80 : 20 on Whatman No. 1 paper for forty-nine hours, against standard methyl ethers of 6α- and 6β-hydroxyoestradiol-17β's.



BENZENE : ISO-OCTANE : METHANOL : WATER :: 30:70:80:20.
STAINED BY FUMING SULPHURIC ACID.

FIG. 28.

After running for forty-nine hours the paper was dipped in fuming sulphuric acid and examined under ultra-violet light, when it was seen that the reduction product of the 6α-hydroxyoestrone area from KC-6B consisted of a mixture of 6α and 6β-hydroxyoestradiol-17β's. As judged from the relative intensities of the spots produced, the ratio of 6α- to the 6β-epimer appeared to be approximately 2 : 1, (See Fig.28)

The presence of both epimers in the 6-hydroxy-oestrone fraction isolated from KC-6B was a most interesting finding, but it seemed possible that one of these might have arisen from the other by epimerization during the course of the isolation and it was decided therefore that this point should be checked as far as possible.

The most obvious point that sprung to mind was the alkali treatment to which the residue had been subjected in order to destroy 18-hydroxyoestrone. Accordingly, two aliquots of 100 μgs.each of 6α-hydroxyoestrone and two aliquots of 100 μgs.each of 6β-hydroxyoestradiol-17β were taken in separate tubes. To one of each pair of tubes was added 10 mls.of N sodium hydroxide and these were allowed to stand at room temperature for two hours. The sodium hydroxide was then neutralized by the addition of 10 mls.of N hydrochloric acid and the solutions were extracted with ether.

The residue from the 6α-hydroxyoestrone tube, and the control 6α-hydroxyoestrone material were then

reduced by sodium borohydride in methanol. All four residues were then methylated in the usual way and the methyl ethers were run on Whatman No. 1 paper in the system 70 : 30 iso-octane : benzene / 80 : 20 methanol : water for 48 hours.

Development of the chromatogram with fuming sulphuric acid and examination under ultra-violet light showed that both epimers were unaffected by this treatment. Thus the NaOH-treated 6α-hydroxyoestrone spot and the control 6α-hydroxyoestrone spot showed only single spots corresponding to a standard solution of the 6α-epimer.

Similarly, both β-hydroxy epimers showed a main spot corresponding to 6β-hydroxyoestradiol-17β standard. These two compounds also showed the presence of a faint spot corresponding to the 6α-epimer, but as this was of equal intensity in both the control and the standard solutions, it was concluded that this material was the impurity present due to the mode of preparation and had not been produced by alkali treatment.

It has been similarly found for example, that whilst oestradiol-17β is the sole product of reduction of oestrone with lithium aluminium hydride (Papineau-Couture, Richardson and Grant, 1949) a mixture of oestradiol-17α and oestradiol-17β is obtained on reduction of oestrone in aqueous potassium hydroxide with Raney Nickel Alloy (Whitman

Wintersteiner and Schwenk, 1937) the former never constituting more than 10 - 20 % of the mixture.

In order to investigate the effect of activated alumina on 6α-hydroxyoestrone, 200 μgs. of 6α-hydroxyoestrone was dissolved in 1% methanol in benzene and applied to a column of 10 gms. of alumina (which had been activated at 100° C *in vacuo* and then deactivated with 3% vol/wt. water) slurried in benzene. The column was eluted with 200 mls. of 1% methanol in benzene and then with 50 mls. of 10% methanol in benzene. The eluate from the latter solvent mixture was evaporated to dryness under nitrogen. The residue was reduced by sodium borohydride in methanol at room temperature and then methylated in the usual way. The product was examined chromatographically in the solvent system 70 : 30 iso-octane : benzene/ 80 : 20 methanol : water as before. This showed that no epimerization of the 6α-hydroxy group had occurred.

When a similar experiment was performed with 6β-hydroxyoestradiol-17β it was found that epimerization had occurred to give *ca.* α : β :: 2 : 1. This experiment was repeated when it was found that epimerization had again occurred but to a much lesser extent to give *ca.* α : β :: 1 : 10. When the same experiment was performed with 6α-hydroxyoestradiol-17β a similar degree of epimerization was noted, giving *ca.* α . β :: 10 : 1.

In order to study the effects of the conditions used in the Girard reaction on the epimerization of 6-hydroxyl groups, 200 µgs. each of 6α-hydroxyoestrone and 6β-hydroxy-oestradiol-17β were taken in two separate tubes. To each tube was added 0.5 gms. Girard reagent T + 1 ml. glacial acetic acid plus 5 mls. of methanol and the solutions were allowed to stand at room temperature overnight. To each tube was added 100 mls. of chilled water containing enough N.- NaOH to neutralize $^{9}/_{10}$ths of the acid and the solutions extracted twice with an equal volume of ether. Each extract was washed twice with one-fifth volumes of 5% sodium bicarbonate solution; twice with one-fifth volumes of water; dried over anhydrous sodium sulphate and evaporated to dryness to give the non-ketonic fractions. The aqueous phases were acidified with 10 mls. of concentrated HCl, allowed to stand for one hour at room temperature and were then extracted as above to give the ketonic fractions.

The ketonic fraction from the 6α-hydroxyoestrone tube was reduced by borohydride in methanol and methylated. The non-ketonic fraction from the 6β-hydroxyoestradiol-17β tube was methylated and both methyl ethers were then chromatographed in the system 70 : 30 iso-octane : benzene/ 80 : 20 methanol : water.

The 6β-hydroxyoestradiol-17β material was unchanged whilst the 6α-hydroxyoestrone fraction showed considerable epimerization to give ca. α : β :: 2 : 3.

In order to investigate whether the epimerization of the latter was due to the concentrated HCl added to disrupt the Girard complex, two separate aliquots of 6α-hydroxy-oestradiol-17β were taken and subjected to the Girard reaction as above. One of these aliquots was extracted in the normal way as for a non-ketonic fraction whilst the other was subjected to the treatment given to a ketonic fraction before being extracted. The former showed some epimerization to give ca. α : β :: 10 : 1 whilst the latter showed a much greater degree of epimerization to give α : β :: 1 : 1 therefore suggesting that in the course of a Girard separation, the greatest degree of epimerization is likely to occur at the stage of disruption of the Girard complex and therefore the ketonic fraction will be more greatly affected than the non-ketonic fraction.

Unfortunately, lack of the appropriate standards did not permit further investigation of this question of epimerization of 6-hydroxy groups and considerable work remains to be done before precise details of this process are known. In particular more detailed studies are necessary to show the effects of activated alumina on such groups and also the conditions employed in the Girard fractionation require further investigation.

From the results obtained it is unfortunately not possible to assign a definite configuration to the 6-hydroxy-

oestrone identified in late pregnancy urine, but it is possible that this may be 6α-hydroxyoestrone which has been epimerized in the numerous Girard fractionations to which this material has been subjected and extracted as the ketonic fraction.

(7) DISCUSSION

One of the first difficulties encountered in the work reported here on the identification of KC-6B was the separation of this material from 18-hydroxyoestrone, which has very similar partition characteristics. Initially this was achieved by paper chromatography using the system chloroform/formamide and the material so obtained yielded evidence which was in agreement with the findings of Loke (1958) and was consistent with the suggestion that KC-6B might be a 6-hydroxyoestrone.

It is of some interest to note that following treatment with N NaOH and alumina chromatography, this material gave a deep-red colour in the Kober reaction, but that the U.V. absorption spectrum of this solution showed a normal absorption maximum at 512 mμ. Breuer, Knuppen, Ortlipp, Pangels and Puck (1960) reported that 6β-hydroxyoestrone, formed by incubation of 6β-hydroxy-Δ^4-androstene-3,17-dione with Ryan's enzyme preparation from human placenta (Ryan 1959) gave a red colour in the Kober reaction. The possible significance of this observation will be discussed later in relation to the configuration of KC-6B.

Apart from evidence which supported the suggestion that KC-6B might be a 6-hydroxyoestrone, this preliminary work yielded valuable information with regard to

the amount of material one might expect to obtain from 1,000 litres of late pregnancy urine and so brought about a re-appraisal of the methods to be employed in identifying this metabolite. In addition, subsequent investigations were made much easier by the fact that a satisfactory method had been found for the synthesis of 6α-hydroxyoestrone (Knuppen and Breuer 1961) and that the configuration of the 6-hydroxyl group in phenolic steroids had been firmly established (Breuer, Knuppen and Pangels 1961). It was thus possible to use synthetic 6α-hydroxyoestrone to test the feasibility of suggested methods for the identification of KC-6B.

The main method used in this identification was based on the alteration of the U.V. absorption spectrum of a 6-hydroxyoestrogen to that of a 6-oxo-oestrogen, following chromic acid oxidation in acetone at 0^0 C. The reasons for the validity of this criterion of identification in the present case have been fully dealt with in Section 11 of this thesis and will not be repeated here. It is sufficient to note that both authentic 6α-hydroxyoestrone and KC-6B gave identical results in this experiment and that they were also identical with regard to their infra-red spectra; chromatographic mobility in a system capable of separating 6α-hydroxyoestrone and 6-oxo-oestradiol-17β; in the Kober reaction and subsequent extraction of the colour produced, by the method of Ittrich (1958) and in their spectral characteristics in concentrated

sulphuric acid. Furthermore, following oxidation of KC-6B with chromic acid at 0^0 C, the product showed exactly the same characteristics as the product obtained by identical treatment with 6α-hydroxyoestrone. Notably the U.V. absorption spectra of both products showed the unique characteristics of a 6-oxo-oestrogen; the infra-red spectrum indicated the presence of a six-membered ring ketone in addition to a five-membered ring ketone, and there was a complete loss of Kober chromogenicity following oxidation. This agreed with the finding that 6-oxo-oestrone is a negative Kober chromogen. It was considered therefore that KC-6B could with some confidence be identified as a 6-hydroxyoestrone.

It is regrettable that the question of the configuration of the hydroxyl group at C-6 in KC-6B could not be definitely ascertained. The apparent finding of both of the epimeric 6-hydroxyoestrones in human pregnancy urine must be very carefully considered and cannot be accepted per se without further conclusive experimental proof. The whole question of configuration of the hydroxyl groups at C-6 in oestrogens was a matter of some conjecture until 1959 and indeed, until that time these groups had been given designations which were ultimately shown to be wrong (Breuer et al 1961).

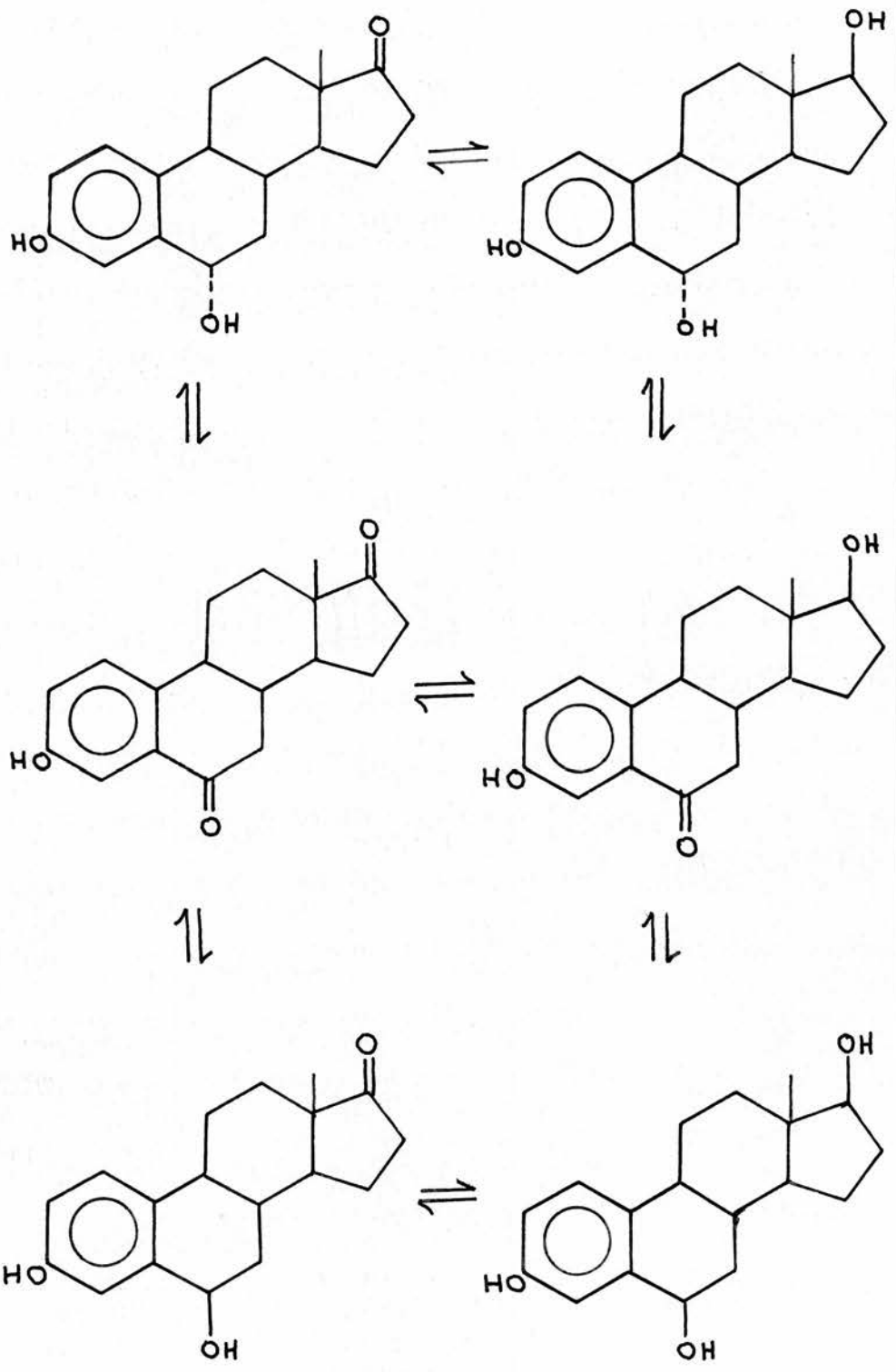
Wintersteiner and Moore (1959) published tentative evidence for the reversal of the configurations which

had hitherto been applied to the 6-hydroxyl groups in oestrogens. Thus the epimer produced by sodium borohydride reduction of 6-oxo-oestradiol-17β they described as 6"α"-hydroxyoestradiol-17β, whilst the epimer produced by catalytic reduction of 6-oxo-oestradiol-17β with platinum oxide in ethanol was described as 6 "β"-hydroxyoestradiol-17β. They suggested that as these designations were tentative, they should be retained in inverted commas but it was suggested that they were probably correct on the following grounds : the epimer formed in good yield on reduction of the ketone with sodium borohydride should be the thermodynamically more stable one and in the present case this would be the 6α-epimer with a quasi-equatorial hydroxyl group. The apparently preferential formation of the 6β-epimer on catalytic reduction might be explained by approach of the catalyst from the relatively less-hindered α-face of the molecule. The designations chosen by these authors were later shown to be correct by Breuer et al (1961) who converted 6α- and 6β-hydroxy- Δ^4-androstene-3,17-diones to the corresponding C-18 phenolic steroids using the aromatizing enzyme preparation from human placenta (Ryan, 1959).

Prior to this convincing proof of the configuration of 6-hydroxy groups in oestrogens, the configuration of such groups in 6-hydroxyoestrogen metabolites was either not stated at all (Breuer, Knuppen and Pangels,

1960) or was arbitrarily assigned by comparison with a 6-hydroxyoestrogen of "known" configuration, using methods which were not known to separate the two epimers (Mueller and Rumney 1957; Bush, Klyne and Short, 1960).

In a recent publication, Breuer et al (1962) have discussed the configuration, biosynthesis and metabolism of 6-hydroxylated oestrogens. They have shown that the microsomal fraction of rat liver contains 6α- and 6β-hydroxylases both of which are capable of metabolizing oestradiol-17β. They suggest that the 6α-hydroxylase is much more active than the 6β-hydroxylase as incubation of oestradiol-17β with rat liver microsomes in the presence of NADPH and molecular oxygen led to formation of approximately five times as much 6α-hydroxyoestradiol-17β as 6β-hydroxy-oestradiol-17β. In addition to 6α- and 6β-hydroxylases, this fraction of rat liver was shown also to contain 6α- and 6β-hydroxyoestrogen dehydrogenases. The overall general metabolism of the 6-hydroxylated oestrogens in rat liver is given by these authors as shown below: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Despite the fact that rat liver contains 6α- and 6β-hydroxylases the validity of the finding of both epimers in the work reported here is still open to considerable doubt in view of the epimerization of 6-hydroxy groups by procedures which were used during the isolation of KC-6B. Such epimerization can occur to quite a marked degree, particularly in the acid conditions employed in the rupture of a ketone-Girard complex. The experiments performed here on pure compounds may not show the full extent to which epimerization can occur as there exists also the possibility of influence by contaminating urinary residues on epimerization under conditions in which the pure compound is stable. Thus in the first batch of KC-6B it was found that following treatment with N-NaOH the material gave a deep-red Kober colour, whereas it had previously given a normal Kober colour. As Breuer et al (1960) reported that 6β-hydroxyoestrone gives a red Kober colour whilst 6α-hydroxyoestrone does not, it seems possible that epimerization may have occurred in this instance.

The question of the configuration of the 6-hydroxy group in KC-6B is one that cannot be ascertained with any degree of certainty in the work reported here. On the one hand we have the apparent existence of both epimers, which is in agreement with Breuer's finding of both 6α- and 6β-hydroxylases in rat liver. On the other hand we have shown that

epimerization can and does occur under conditions used in the isolation of KC-6B. It is possible that both epimers are excreted and that the discrepancy between the rates of 2 : 1 :: α : β reported here, and that of 5: 1 :: α : β reported by Breuer et al (1962) might be due to epimerization, but this suggestion must be treated with considerable reserve until further experimental evidence is available.

SECTION IV

(1) INTRODUCTION

Following the isolation of 18-hydroxyoestrone from human pregnancy urine (Loke, Watson and Marrian, 1957), the question of the mode and site of biosynthesis of this compound presented a very interesting problem. At that time, the only recorded instance of 18-hydroxylation was that of Kahnt, Neher and Wettstein (1955), who showed the hydroxylation of deoxycorticosterone at C-18 after incubation with ox adrenal homogenates.

In the course of experiments to determine whether or not oestrone could be hydroxylated at C-18 by bovine adrenal homogenates in vitro, Loke (1958) obtained some evidence that such a hydroxylation might take place, but the results were not sufficiently conclusive to enable this point to be definitely established.

From the results obtained by Loke it seemed significant that although his small scale experiments yielded evidence for the formation of 18-hydroxyoestrone from oestrone, by incubation with bovine adrenal homogenates, subsequent large-scale experiments provided no evidence for 18-hydroxylation. Examination of the conditions employed in

both sets of experiments showed no gross differences between the two, but it was realised that there must have been some considerable difference in the "freshness" of the material used, due to the difference in numbers of glands required.

In order to obviate the possibility that this was in some way responsible for the difference in Loke's results, it was decided that glands should be incubated one pair at a time, transporting them from the slaughterhouse as soon as possible after the death of the animal. By so doing it was found to be possible to start each incubation within one hour of the animal being killed.

(2) EXPERIMENTAL METHODS

All incubations were carried out in 50 ml. portions of a modified Ca^{++} - ion free Krebs-Ringer phosphate buffer (pH 7.4) which had the following composition, the figures in parentheses denoting final concentrations:-

NaCl (96.0 mM); KCl (4.6 mM); KH_2PO_4 (1,16 mM); $MgSO_4$ (1.16 mM); $NaHCO_3$ (3.48 mM) sodium fumarate (8.21 mM); sodium L-glutamate (4.77 mM); glucose (11.2 mM); nicotinamide (5.92 mM); Na_2HPO_4 (10.8 mM); NaH_2PO_4 (2.7 mM).

Before use, the buffer was placed in a water bath at 37^0C and was gassed for one hour with a 95% oxygen; 5% carbon dioxide gas mixture.

Bovine adrenal glands, obtained within ten to fifteen minutes after slaughter, were transported to the laboratory in a chilled container and there dissected free from adhering fat. Each gland was split longitudinally, the medulla was removed as completely as possible and the cortical tissue was minced in a chilled Latapie mincer, 5 gms of this mince being used for each incubation. Each incubation was started within one hour of the animal being killed.

The minced cortex was added to 50 mls of buffer

and to this was added a solution of 500 μgs. of oestrone in 0.5 mls. of ethanol + 0.04 mls. of a standard solution of [16 - ^{14}C] oestrone in ethanol, from which 3 x 0.005 ml. samples had been plated on to aluminium planchettes at infinite thinness. Radioactivity determinations were made on these samples using the Nuclear-Chicago gas-flow counter having a thin end-window. In a typical experiment in which these samples were each counted twice, it was found that the 0.005 ml. aliquot had a count-rate of 1531± 52 counts/min. Therefore as a total of 0.525 mls. of this solution was added, 1.58×10^5 counts/min were added to the total incubate.

The Nuclear-Chicago gas-flow counter, when fitted with a thin end-window, is claimed by the manufacturers to have an efficiency of approximately 35% for ^{14}C. This was not determined experimentally in the course of the work reported here.

The incubations were carried out for one hour at 37°C under an atmosphere of 95% oxygen : 5% carbon dioxide and at the end of this time each incubation was terminated by addition of 117 ml. of methanol to give a methanol concentration of 70% (v/v). After vigorous shaking the mixture was centrifuged and the supernatant decanted off. The residue was re-extracted with 167 ml. of aqueous 70%

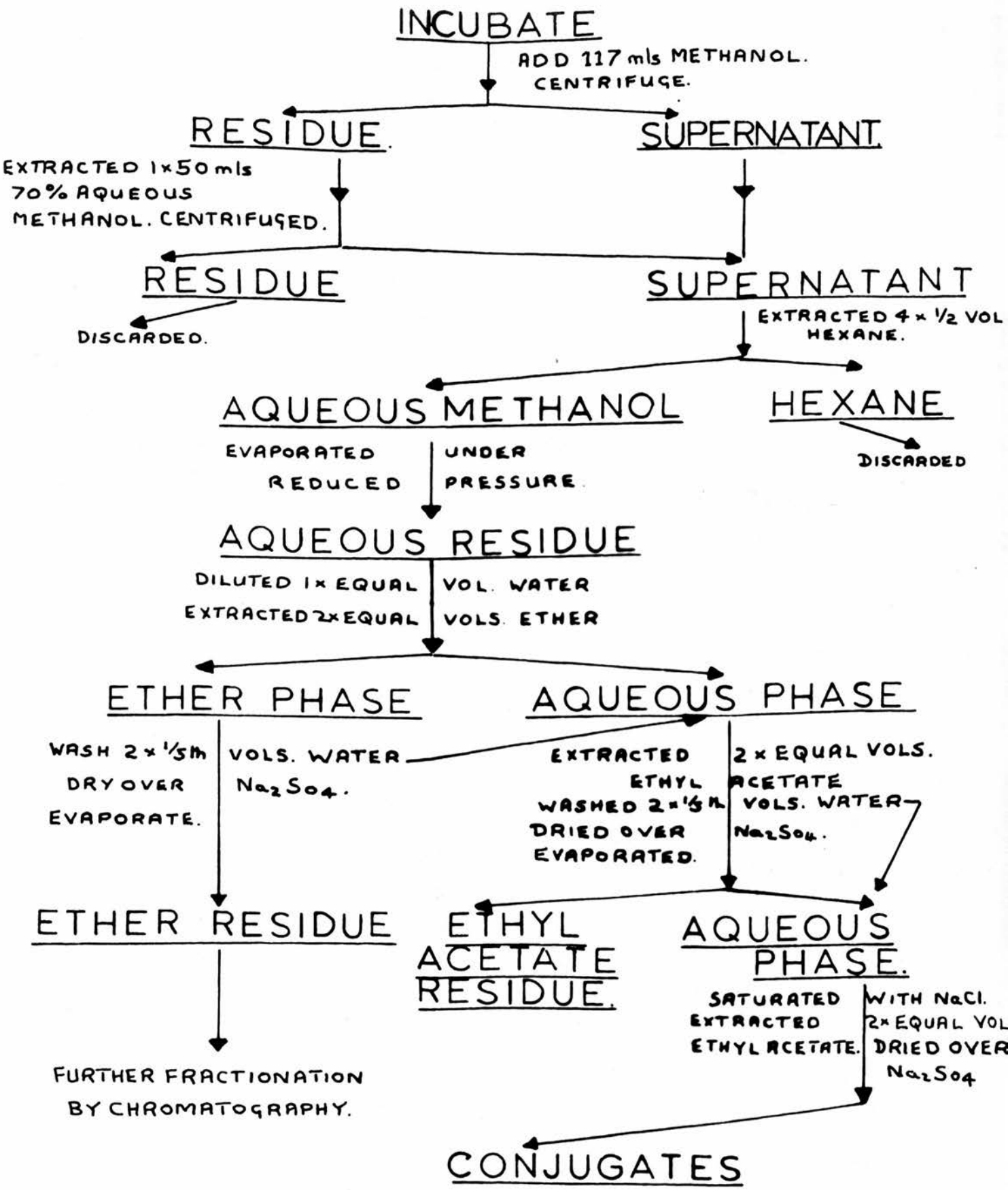

INCUBATE
ADD 117 mls METHANOL.
CENTRIFUGE.
RESIDUE.
SUPERNATANT.
EXTRACTED 1×50 mls
70% AQUEOUS
METHANOL. CENTRIFUGED.
RESIDUE
SUPERNATANT
DISCARDED.
EXTRACTED 4 × 1/2 VOL
HEXANE.
AQUEOUS METHANOL
HEXANE
EVAPORATED
REDUCED
UNDER
PRESSURE.
DISCARDED
AQUEOUS RESIDUE
DILUTED 1× EQUAL
VOL. WATER
EXTRACTED 2× EQUAL
VOLS. ETHER
ETHER PHASE
AQUEOUS PHASE
WASH 2 × 1/5th
VOLS. WATER
DRY OVER
Na2SO4.
EVAPORATE.
EXTRACTED
ETHYL
WASHED 2 × 1/5th
DRIED OVER
EVAPORATED.
2 × EQUAL VOLS.
ACETATE
VOLS. WATER
Na2SO4.
ETHER RESIDUE
ETHYL
ACETATE
RESIDUE.
AQUEOUS
PHASE.
SATURATED
EXTRACTED
ETHYL ACETATE.
WITH NaCl.
2× EQUAL VOL
DRIED OVER
Na2SO4
FURTHER FRACTIONATION
BY CHROMATOGRAPHY.
CONJUGATES

FIG. 29.

(v/v) methanol and the combined supernatants were extracted four times with half-volumes of hexane.

The aqueous methanol phase was evaporated under reduced pressure to remove all methanol and the aqueous residue, diluted with an equal volume of water, was extracted twice with an equal volume of ether. The ether extract was washed twice with small volumes of water, dried over anhydrous sodium sulphate and evaporated to dryness.

The aqueous phase and washings from the ether extraction were combined and extracted twice with equal volumes of ethyl acetate. The extract was evaporated to dryness after washing with water and drying over sodium sulphate.

The aqueous phase and washings from the ethyl acetate extraction were combined, saturated with sodium chloride and extracted twice with equal volumes of ethyl acetate. The extract was dried over anbydrous sodium sulphate without previous water washing, evaporated to dryness, immediately redissolved in methanol to which a few drops of aqueous ammonia had been added and the solution stored at 0^{0}C. This procedure is summarized in Fig. 29.

In each experiment suitable aliquots of each extract were taken and plated at infinite thinness. Radioactivity determinations were made on these samples using the Nuclear-Chicago Autochange gas-flow counter with a thin end-window.

Fractionation of ether extracts was made initially on Celite partition chromatography columns as described by

Givner, Bauld and Vagi (1960), using the solvent system benzene : n-hexane / methanol : water, 50 : 50/ 70 : 30. When 30 mls. of eluate had been collected the mobile phase was changed to 60 : 40 benzene : n-hexane and then after another 30 mls. of eluate had been collected the column was finally eluted with 50 mls of 80 : 20 benzene : n-hexane. By calibration with authentic oestrogen samples, this procedure was found to give three fractions corresponding to oestrone, 16α-hydroxy-oestrone and 6α-hydroxyoestrone respectively. The corresponding fractions from adrenal incubation extracts were designated "oestrone-fraction", "α-ketol fraction" and "KC-6 fraction" respectively.

The separation of incubation extracts into these three fractions was subsequently found to be much quicker and easier by thin-layer chromatography on plates of silica-gel-G (Camlab Ltd., Cambridge), using the solvent system ethyl acetate : cyclohexane 50 : 50 (Lisboa and Diczfalusy 1962). The extract from one incubation was applied in methanol as a streak along the origin line of a silica-gel plate which was then developed by ascending chromatography in the above solvent system. The centre of the plate was masked by a perspex strip whilst the edges were sprayed with Folin and Ciocalteu's reagent and developed in ammonia vapour. The centre portion was then divided according to the positions of

the bands on each edge, the relevant portion of silica-gel scraped off into a porosity-four sintered glass filter, and eluted with methanol. The thin-layer chromatography equipment and silica-gel-G were purchased from Camlab Ltd., Cambridge.

Radioactivity determinations on strips from paper chromatograms were made using the Nuclear-Chicago gas-flow chromatogram scanner fitted with a thin end-window.

Purification of conjugated oestrone fractions from adrenal extracts was by chromatography on columns of Sephadex-G 25 (Pharmacia, Uppsala), eluting with distilled water (Beling 1961) and also by chromatography on columns of Celite-Alumina (Purdy, Engel and Oncley, 1961).

(3) PRELIMINARY EXPERIMENTS

(a) Standardization of Celite Columns.

Celite columns of 12 cms. x 1 cms. were prepared according to Givner, Bauld and Vagi (1960), using the solvent system benzene : hexane 50 : 50 /methanol : water 70 : 30. A mixture of 500 µgs. oestrone + 200 µgs. 16α-hydroxyoestrone + 200 µgs. 6α-hydroxyoestrone in a total volume of 0.9 mls. ethanol was added to 0.5 gms. dry celite which was then stirred thoroughly for five minutes to ensure even distribution of the oestrogens. The celite was dried off in an oven at 90° C to remove all methanol and after cooling, 0.5 mls. of stationary phase was added and stirred in thoroughly to ensure even distribution. Excess mobile phase was then added to give a slurry which was packed on top of the previously prepared column. This was eluted with 30 mls. of 50 : 50 benzene : hexane; 50 mls. of 60 : 40 benzene : hexane; and finally with 50 mls. of 80 : 20 benzene : hexane taking 3.5 ml. fractions.

Each fraction was taken to dryness, dissolved in 3.5 mls. of ethanol and scanned on a Unicam S.P. 700 recording spectrophotometer against an ethanol blank over the range 250 mµ - 320 mµ. The results from six such determinations showed that in each case the appropriate

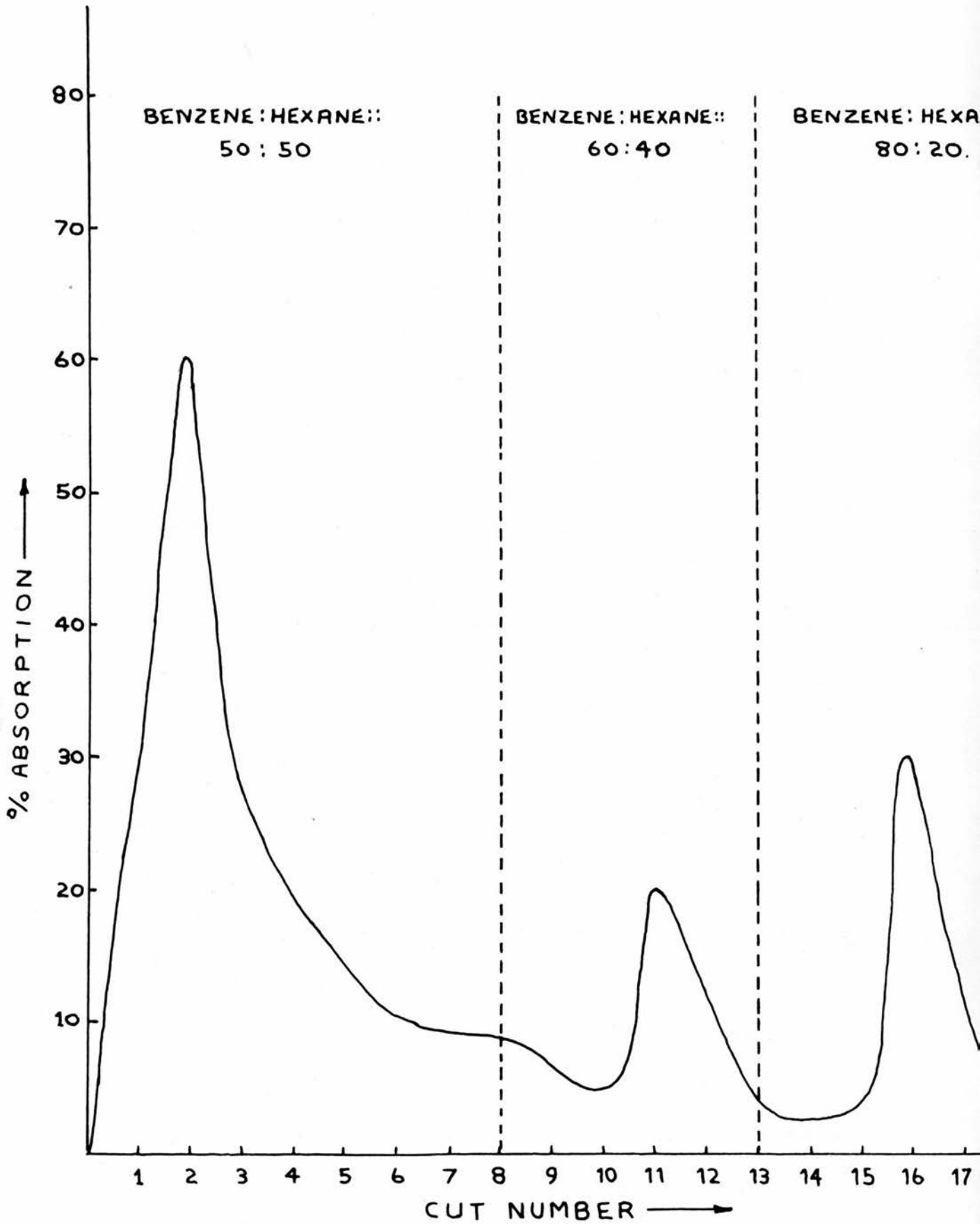

BENZENE : HEXANE ::
50 : 50
BENZENE : HEXANE ::
60 : 40
BENZENE : HEXA
80 : 20.
% ABSORPTION
80
70
60
50
40
30
20
10
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
CUT NUMBER

FIG. 30.

oestrogen had been eluted before change-over to the next mobile phase. A typical separation is shown in Fig. 30.

(b) Standardization of Extraction Procedure.

In order to standardize the extraction procedure and to check the recovery of [16-^{14}C] oestrone obtained, a single experiment was carried out as follows: 5 gms. of adrenal cortex mince, obtained from a fresh cow adrenal was incubated for one hour under an atmosphere of 95% oxygen : 5% CO_2 in 50 mls. of Krebs-Ringer Ca^{++}- free buffer pH 7.4. At the end of this time a solution of 500µgs. of oestrone in 0.5 mls. of ethanol plus 0.04 mls. of a standard solution of [16-^{14}C] oestrone was added to the incubate. This was followed immediately by addition of 117 mls. of methanol to precipitate protein. The solution was centrifuged at 2,000 r.p.m. in an M.S.E. Major refrigerated centrifuge for ten minutes and the supernatant decanted off into a separating funnel, rinsing the bottle and the surface of the residue once with a further 10 mls. of methanol. The residue was shaken vigorously with a further 200 mls. of 70% aq. methanol, recentrifuged and the supernatant taken to dryness <u>in vacuo.</u> After being dissolved in methanol, aliquots of this solution were plated for counting.

The supernatant from the first centrifugation was extracted 4 x 100 mls. of hexane which was discarded.

The aqueous methanolic solution was evaporated in vacuo to remove the methanol and the aqueous residue was diluted with an equal volume of water. This was then extracted 3 x 150 mls of ether which was washed 2 x 100 mls. water, dried over anhydrous sodium sulphate and taken to dryness. The residue was dissolved in methanol and aliquots plated for counting. Radioactivity determinations, made on a Nuclear-Chicago gas-flow counter with a thin end-window, showed that 82% of the added counts were recovered in the final ether extract, whilst a further 4.2% were recovered from the second extraction of the centrifugation residue. It was decided therefore that a double extraction would be made with the 70% aq. methanol, giving a final recovery of the order of 85%.

As the final recovery of radioactivity from each incubation could be checked quite readily it was thought at this stage that little would be gained by performing a series of dummy extraction recovery experiments. The procedure given here was therefore adopted, care being taken to make accurate determinations of the number of counts added to each incubation and the percentage of counts recovered in each individual case.

(c) Determination of Optimum Steroid /Tissue Ratio.

In order to obtain some information on the optimum amount of oestrone : cortex to be used in the incubations, the following experiment was carried out:-

Three portions of 5 gms. of cortex mince were taken in 50 mls. of Krebs-Ringer Ca^{++}-free buffer pH 7.4 and the following amounts of oestrone were added to these;

1) 250 µgs. oestrone + 0.04 mls. standard solution of [16-^{14}C] -oestrone.

2) 500 µgs. oestrone + 0.04 mls. of standard solution of [16-^{14}C] -oestrone.

3) 1000 µgs. oestrone + 0.04 mls. standard solution of [16-^{14}C] -oestrone.

These were incubated and extracted in the usual way, the final ether extracts being chromatographed as described previously on Celite to give "oestrone", "α-ketol" and "KC-6" fractions. The columns were finally stripped with 50 mls. of methanol.

Aliquots of each of these fractions were taken and radioactivity determinations made on them in the Nuclear-Chicago gas-flow counter with a thin end-window. The results of these determinations are summarized in the following table:-

Weight of oestrone incubated with 5 gms. Cortex mince.

	250 µgs. oestrone	500 µgs. oestrone	1000 µgs. oestrone
Total %age of added counts recovered in ether extract.	79.8%	85.6%	84.5%
Total %age of added counts recovered in hexane.	3.29%	3.33%	3.39%
Total	83.09%	88.93%	87.89%
Of total counts added to Celite column, %age recovered in			
1. "Oestrone" Fraction	61.8%	58.0%	62.5%
2. "α-ketol" Fraction	1.26%	2.29%	7.78%
3. "KC-6" Fraction	9.72%	13.88%	5.71%
4. Column Strippings	1.42%	1.93%	1.34%
Total	74.20%	76.10%	77.33%

These results suggest that from the point of view of production of "KC-6", the optimum steroid/tissue ratio is 500 µgs./5 gms, and this was therefore adopted in succeeding experiments.

(d) Comparison of Cortex Mince and Capsule Strippings.

Since Ayres, Hechter, Saba, Simpson and Tait (1957) showed that capsule strippings of ox adrenals, previously shown to be mainly zona glomerulosa tissue (Ayres, Gould, Simpson and Tait, 1956), converted progesterone, deoxycorticosterone and corticosterone to aldosterone (in the course of which 18-hydroxylation was presumably involved) it was decided to investigate the metabolism of the cortex mince in comparison with the capsular stripping material. This investigation was further prompted by the fact that it had been noticed on mincing adrenal cortex in the Latapie mincer that the tough outer capsule of the gland was left behind in the small volume of dead space within the mincer just behind the cutting discs. This was in the form of a sheet of tightly-packed tough material having a white appearance, and it is this which will hereafter be referred to as capsule stripping material.

One pair of adrenal glands obtained immediately after slaughter was taken and the cortex minced as described previously. The capsule stripping material obtained from the dead space within the mincer was taken and minced up as finely as possible using a small pair of dissecting scissors. This mince and that of the cortex tissue were incubated with

oestrone + [16-^{14}C] oestrone and extracted in the usual way. The extracts were subjected to Celite column chromatography and aliquots of each fraction so obtained were plated and radioactivity determinations made as before.

The results of this experiment are given below:-

Fraction (obtained from Celite column)	Cortex Mince (%age of counts applied to column)	Capsule Stripping Material (%age of counts applied to column)
"Oestrone" Fraction	49.65%	75.30%
"a-ketol" Fraction	3.78%	0.645%
"KC-6" Fraction	21.58%	2.35%
Column Stripping Fraction	0.158%	0.592%
Total recovery	75.16%	78.88%

From this experiment it will be seen that the capsular stripping material obtained by the method described here is almost completely in active in metabolizing oestrone. This material was therefore discarded in subsequent experiments.

(e) Investigation of the Effect of "Ageing" on Adrenal Glands.

In order to test the hypothesis advanced in the introduction to this section, that the time factor between death of the animal and incubation of the glands is important due

perhaps to its effect on the inherent NADPH level within the cell, a series of experiments were carried out as follows: pairs of glands obtained from the slaughterhouse were separated, one of each pair being used immediately whilst the other was allowed to stand at room temperature for one hour and was then stored overnight at -18°C before being incubated in the usual way. From radioactivity determinations on the fractions obtained by Celite column chromatography the metabolic activity was compared in fresh and aged glands. The results of a number of such experiments are summarized below:-

	FRESH GLANDS		AGED GLANDS	
Experiment No	"Oestrone" Fraction*	"α-ketol & KC-6" Fraction*	"Oestrone" Fraction*	"α-ketol & KC-6" Fraction*
1.	50.0%	25.0%	69.0%	7.0%
2.	62.0%	39.0%	62.0%	19.0%
3.	46.0%	35.0%	57.0%	47.0%
4.	50.0%	43.0%	45.0%	18.0%
5.	51.0%	44.0%	67.0%	27.0%
Mean	51.8%±5.4	37.2%±6.9	60.0%±8.5	23.0%±13.3

* Percentages quoted are percentage recoveries in each fraction of total counts added to Celite column in each experiment.

From the results shown above it is not possible to say that the fresh glands were significantly more efficient in metabolizing oestrone than the aged glands. However there seems to be a large variation in both sets of glands of the extent to which oestrone is metabolized and from some of these results there is a suggestion that the aged glands might produce a lower yield of "α-ketol + KC-6" fraction than the fresh glands. It is unfortunate that only five such experiments were performed, which were insufficient to establish this suggestion with any statistical significance.

However it was decided that in view of the low recoveries of "α-ketol + KC-6" fraction in some of the aged gland experiments, it would be better to use fresh glands and this decision was followed in the course of subsequent experiments.

(4) SULPHATION OF OESTRONE IN VITRO BY BOVINE ADRENAL TISSUE

In the course of preliminary experiments described in part 3 of this section it was found that recoveries of radioactivity added to the incubates, by ether extraction of the aqueous incubation medium, varied considerably from the figure of 86.2% found in the single incubation recovery experiment. Thus in the first sixteen experiments performed, the percentages of radioactivity recovered by ether extraction had an average value of 54.8%, but the standard deviation was ± 22.2%.

Initially it was assumed that these variations might represent variations in experimental technique in the extraction procedure, being shown up very clearly by the sensitivity of radioactivity determinations. However, when some of the recoveries were found to be extremely low, in the order of 50%, 30% and even 20%, it was felt that there must be some other explanation.

In order to show the relative distribution of the radioactivity, two incubation experiments were carried out in the usual way and aliquots taken of each extract and also of the final aqueous residues. Radioactivity determinations made on these aliquots gave the following results:-

	Incubation I	Incubation II
Ether Extract	28.1%	33.4%
Hexane Extract	2.7%	2.2%
Aqueous Residue	31.5%	31.8%
	62.3%	67.4%

(Figures refer to %ages of counts added to each incubation)

The relatively low total recoveries are in some measure a reflection of the amount of residue found in evaporating the aliquot of aqueous phase, which must have diminished the counts obtained by self absorption.

It was apparent therefore that a very large proportion of the "missing" radioactivity was to be found in the aqueous phase and the next experiment was designed to investigate the extractability of this radioactivity by solvents other than ether. Thus, following the initial ether extraction the aqueous residue was extracted with ethyl acetate and finally with n-butanol. Aliquots of each extract were plated and radioactivity determinations made on them. The radioactivity was found to be distributed as follows:-

Ether Extract	18.8%
Ethyl Acetate Extract	4.3%
n-butanol Extract	56.6%
	79.7%

This was extremely interesting as it showed that the adrenal cortex converted oestrone into a metabolite which was not extractable with ether or ethyl acetate, but which could be extracted with n-butanol, suggesting that it might be some form of conjugate. This suggestion was supported by results obtained on the extractability of the radioactivity of the butanol extract, following solvolysis by the method of Burstein and Lieberman (1958).

The butanol extract was taken to dryness in vacuo and the residue dissolved in 10 mls. of water. To this was added 0.06 mls. of 16N H_2SO_4 plus 2.0 gms. of sodium chloride, and the solution was extracted 1 x 15 mls. ethyl acetate. The extract was incubated at 37°C for 3 hours and was then washed 1 x 5 mls. of 5% aqueous $NaHCO_3$; 2 x 5 mls. water; dried over anhydrous sodium sulphate and taken to dryness. An aliquot of the final extract was plated and counted when it was found that 74.8% of the initial radioactivity remained in the ethyl acetate extract.

Thus the water soluble, n-butanol extractable material was found on solvolysis to become very largely extractable with ethyl acetate. These results very strongly suggested that the unknown material was a conjugated form of oestrone and it was decided to carry out further experiments in order to isolate and identify this.

In order to test the effect of ageing on the

formation of this water-soluble metabolite, a number of experiments were carried out as before, i.e. one of each pair of glands was incubated as soon as possible whilst the other was allowed to stand at room temperature for one hour and then overnight at -18°C. The results of these experiments are given below:-

	FRESH GLANDS			AGED GLANDS		
	%age of counts extracted by			%age of counts extracted by		
	Ether	Ethyl Acetate	n-butanol	Ether	Ethyl Acetate	n-butanol
1.	18.8	4.3	56.6	62	3.3	14.4
2.	33.0	3.0	53.0	57.0	4.0	10.0
3.	53.0	4.0	27.0	76.0	1.0	11.0
4.	58.0	4.0	19.0	66.0	3.0	7.0

From these results it will be seen that in each case, ageing of the gland before incubation resulted in a marked decrease in the amount of water-soluble metabolite produced.

In order to obtain sufficient material for the identification of this metabolite, a series of experiments was carried out as before using fresh glands.

Following the initial ether extraction, the aqueous residue was extracted once with an equal volume of ethyl

acetate. The aqueous phase was then saturated with sodium chloride and extracted twice with an equal volume of ethyl acetate. Aliquots were plated from each extract and counted. The ethyl acetate extracts after sodium chloride saturation were taken to dryness in vacuo and the residue immediately dissolved in methanol to which a few drops of ammonia had been added. These solutions were stored at 0°C. The results of six such experiments carried out on adrenals from four six-year old cows and two young steers are shown in the following table:-

	%age of counts extracted		
Animal	By ether	By ethyl acetate	By ethyl acetate after saturation with NaCl.
Cow	81.7	1.5	20.1
	77.5	5.0	12.8
Cow	77.5	0.9	14.7
	73.7	2.3	20.4
Cow	67.0	4.2	18.8
	94.5	2.7	14.3
Cow	42.8	0.7	44.7
	48.6	1.1	46.1
Steer	77.5	1.5	14.3
	79.6	1.4	11.8
Steer	77.5	1.5	25.6
	71.8	1.9	31.6

The conjugate fractions so obtained were pooled to give a fraction having a total radioactivity of 2.927×10^5 counts/min.

Initial purification of this fraction was made by celite/alumina column chromatography as described by Purdy, Engel and Oncley (1961). Alumina was activated by heating at 100°C in vacuo and the reactivated by shaking well with 3% (v/wt) of water. This was used to prepare 12 gms. of a mixture of 4 : 1 alumina : celite which was added in the dry state to a chromatography column filled with methanol. The powder was added in very small portions to prevent the complete separation of alumina and celite due to density differences. The residue of oestrone conjugate was dissolved in 2 mls. of methanol and one drop of methanolic N-NaOH was added to make it alkaline. This solution was applied to the column which was then eluted with 250 mls. of methanol, 250 mls. of 40% aqueous 0.1N NH_4OH in methanol; and 250 mls. of 10% acetic acid in methanol, to give the sulphate, glucusiduronate and phosphate fractions respectively. Aliquots of each fraction were plated and counted and the results showed the following distribution of radioactivity:-

Sulphate Fraction	94.5% of counts applied
Glucusiduronate Fraction	2.3% " " "
Phosphate Fraction	0% " " "

This very strongly suggests that the conjugate is almost entirely sulphate and the last two fractions were therefore discarded.

Attempts at this stage to obtain evidence for the nature of the conjugate by paper chromatography failed due to heavy contamination of the residues with impurities which caused the spots to spread during equilibration and to streak on running. In an attempt to purify this material further it was partitioned between n-butanol and N-NaOH (c.f. Schachter and Marrian 1938). This removed a considerable quantity of brown material from the butanol solution but on taking this to dryness in vacuo, the residue was still found to be rather "dirty" in appearance.

It was therefore dissolved in methanol and applied to a 12 cms. x 1 cms. column of 8.5 gms. of 3% deactivated alumina slurried in methanol. The column was eluted with methanol (100 mls.) and the total eluate taken to dryness. At this stage the residue appeared to be much cleaner, but further attempted chromatography resulted in spreading of the spots during equilibration. It was noticed when spotting the chromatograms that the residue appeared to contain an almost waxy component which made the application of the spots very difficult.

It was decided therefore to effect a further purification of the conjugate fraction by chromatography on Sephadex-G25 (Pharmacia, Uppsala) eluting with distilled water (Beling 1961). Sephadex-G25 (15 gms.) was covered

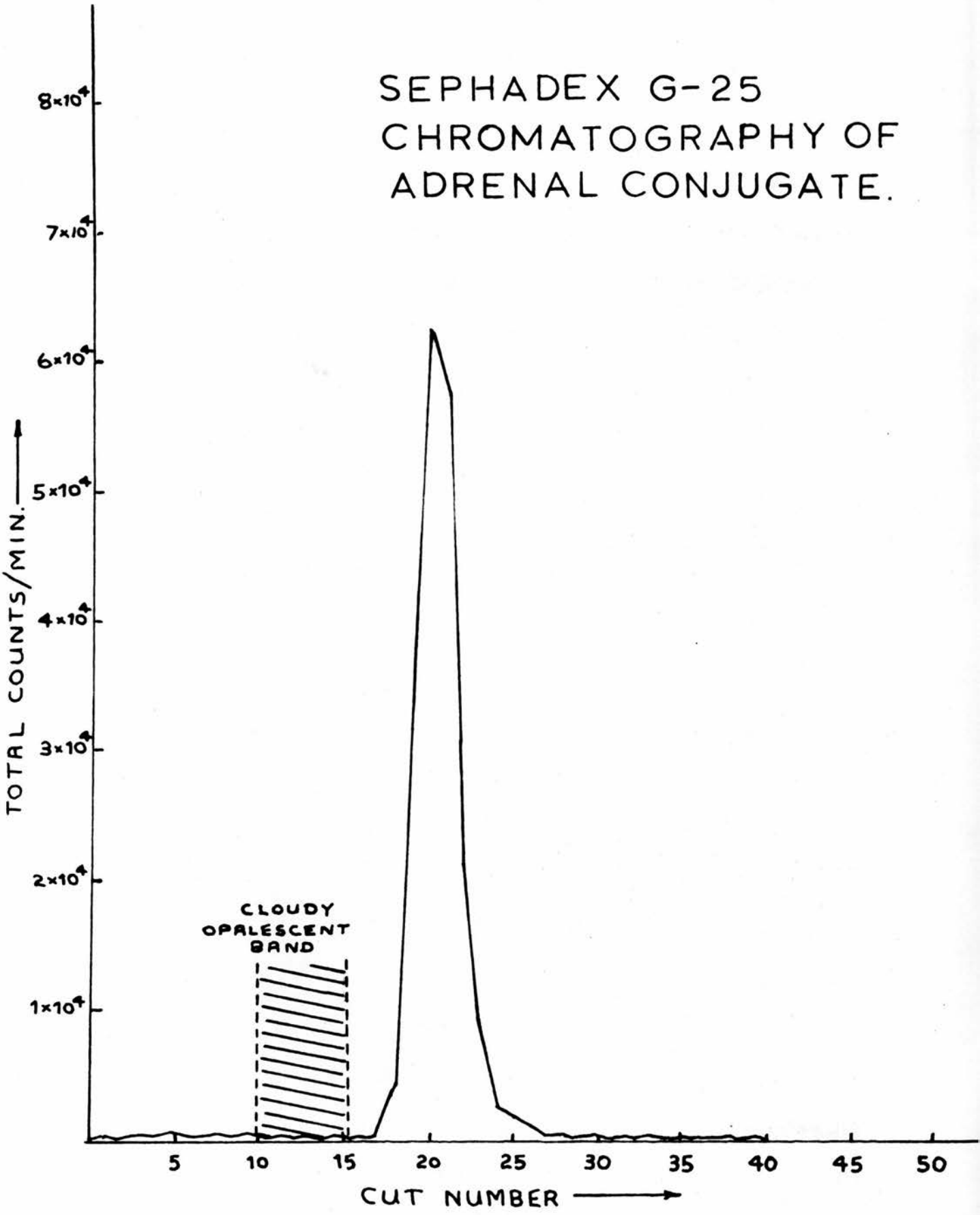

SEPHADEX G-25
CHROMATOGRAPHY OF
ADRENAL CONJUGATE.
8×10⁴
7×10⁴
6×10⁴
5×10⁴
4×10⁴
3×10⁴
2×10⁴
1×10⁴
TOTAL COUNTS/MIN.
CLOUDY
OPALESCENT
BAND
5
10
15
20
25
30
35
40
45
50
CUT NUMBER

FIG. 31.

with distilled water and allowed to stand overnight, when it was used to pack a column 24 cms. x 2 cms. in distilled water. The conjugate residue was dissolved in 10 mls. of distilled water and applied to the column, the eluate being collected immediately. The column was then eluted with distilled water taking 3.5 ml. cuts. Aliquots of 0.035 mls. were plated from each cut for counting. The elution curve obtained is shown in Fig. 31 from which it will be seen that 95.4% of the counts added to the column were eluted as a narrow peak behind a band of cloudy opalescent material. The residue obtained by combining cuts 17 to 33 inclusive, saturating with sodium chloride and extracting with ethyl acetate followed by evaporation, was dissolved in methanol to which a few drops of NH_4OH had been added. Radioactivity determinations made on this solution showed that it contained 1.91×10^5 counts/min or 65% of the radioactivity present in the original crude conjugate solution.

Investigation of this material was continued by paper chromatography on Whatman No. 1 paper in the following systems:-

System 1. 0.2% aqueous ammonia (sp. gr. 0.880): ethyl acetate : n-butanol, 200 : 175 : 25 (Schneider and Lewbart, 1956).

System 2. n-butyl ether : t-butanol : ammonia (sp. gr. 0.880) : water, 100 : 100 : 20 : 180. (Schneider and Lewbart 1959).

Aliquots of the adrenal conjugate fraction, each equivalent to 0.4×10^3 counts /min, were applied to four channels on each of two Whatman No. 1 chromatography papers. Aliquots of authentic oestrone sulphate, each equivalent to 50 µgs, were applied to four separate channels on each of the same two papers. The papers were equilibrated overnight before running in systems 1 and 2 respectively. Pairs of strips were removed from each paper such that one strip contained the adrenal conjugate fraction whilst the adjoining strip contained the authentic oestrone sulphate fraction and these were subjected to the following tests:-

Examination of strips from each system for radioactivity on the Nuclear-Chicago chromatogram scanner using a gas-flow detector with a thin end-window showed that in the channels on which the adrenal conjugate fraction had been run there were main peaks of radioactivity having R_F values of 0.42 and 0.14 in systems 1 and 2 respectively, and lesser peaks having R_F values of about 0.90 and 0.80 respectively in these two systems.

These lesser peaks with the high RF values were almost certainly due to oestrone liberated from the conjugate by hydrolysis before or during chromatography, since

components with nearly identical R_F values in the two systems, which gave positive reactions with the Zimmermann reagent, positive reactions with Folin and Ciocalteu reagent and negative tests for sulphate ions after solvolysis in situ, were detected on strips containing both the adrenal conjugate fraction and authentic oestrone sulphate.

On staining one pair of strips from each of the two systems with Zimmermann reagent (Bush 1961), a main ketonic component with R_F values of 0.37 and 0.14 in systems 1 and 2 respectively was detected in the adrenal conjugate fraction. The corresponding R_F values of the main ketonic component in the authentic oestrone sulphate were 0.37 and 0.15.

On staining with Folin and Ciocalteu reagent by the method of Mitchell and Davies (1954), no phenolic components could be detected in either the adrenal-conjugate fraction or the authentic oestrone sulphate other than that believed to be due to free oestrone.

One pair of strips from each system were then solvolysed in situ by the method of Schneider and Lewbart (1956). A large measuring - cylinder was lined with Whatman 3 mm chromatography paper and a mixture of 180 mls.dioxan + 20 mls.concentrated hydrochloric acid was poured into the bottom of the cylinder. The strips were suspended in the cylinder, care being taken to see that they did not touch the

lining paper, and the top of the cylinder closed by an inverted beaker. After three hours at room temperature, the strips were removed and dried off in an air stream. Staining with Folin and Ciocalteu's reagent now revealed a phenolic component in the adrenal conjugate fraction having R_F values of 0.38 and 0.14 in systems 1 and 2 respectively. In the authentic oestrone sulphate fraction a similar phenolic component was detected having R_F values of 0.38 and 0.12 in these systems.

A second pair of strips from each system were solvolysed in situ as above and were then subjected to the rhodozonic acid test for sulphate ions as described by Schneider and Lewbart (1956). Each strip was dipped through a freshly-prepared solution of 20 mgs. of $BaCl_2$ in 100 mls. of 75% aqueous methanol and was then dried for ten minutes in an air stream. It was then dipped through a freshly-prepared solution of 12 mgs. of the potassium salt of rhodozonic acid dissolved in 15 mls. water + 10 mls ammonia (sp.gr. 0.880) + 25 mls. of absolute ethanol. This causes the strip of paper to assume a pink colouration except in the presence of free sulphate ions, at which positions a yellow spot is obtained. By this method, yellow spots having R_F values of 0.38 and 0.17 in systems 1 and 2 respectively were demonstrated in the adrenal conjugate fraction, and ones with R_F values of 0.44 and 0.19 in the same systems in the authentic oestrone sulphate fraction.

These R_F values are in rather poor agreement with those found in the other tests described above although there is reasonable agreement between the values found for the adrenal conjugate fraction and authentic oestrone sulphate. The reason for this was apparent when it was found that sulphate ions applied to a strip of paper, spread and migrated downwards during a dummy solvolysis procedure, sufficient to displace the centre of the rhodozonic acid-stained spot by as much as 2 cms.

From the evidence obtained so far, there could be no doubt that the adrenal conjugate fraction is in the form of a sulphate and it only remained to identify the oestrogen component. The analogous behaviour of this material with that of authentic oestrone sulphate strongly suggested that it is in fact oestrone sulphate. However since the sulphates of different ketonic oestrogens might not necessarily have greatly differing R_F values in the two solvent systems used, confirmatory evidence was sought for the presence of oestrone.

The main bulk of the adrenal conjugate fraction was solvolysed by the method of Burstein and Lieberman (1958), as described earlier. One-twentieth of the solvolysed material was chromatographed on Whatman No. 2 paper in system B5 of Bush (1952), with oestrone, oestradiol-17β, 16-oxo-oestradiol-17β and 6α-hydroxyoestrone as standards. On scanning with the Nuclear-Chicago

chromatogram scanner using a gas-flow detector with a thin end-window, the solvolysed adrenal conjugate fraction showed a single peak of radioactivity which coincided exactly with the position of authentic oestrone. On spraying with Folin and Ciocalteu's reagent the R_F values of the spots obtained were: - solvolysed adrenal conjugate 0.93; oestrone 0.90 and 0.91; oestradiol-17β, 0.81 and 0.79; 16-oxo-oestradiol-17β, 0.65 and 0.62; 6α-hydroxyoestrone, 0.45 and 0.42.

To the remainder of the solvolysed material was added 209.7 mgs. of authentic oestrone, which had previously been purified by crystallizing twice from ethanol. The mixture was then crystallized three times from ethanol, giving a final yield of 100.6 mgs. of material, crystallizing to a final constant specific activity of 281 counts /min/mg.

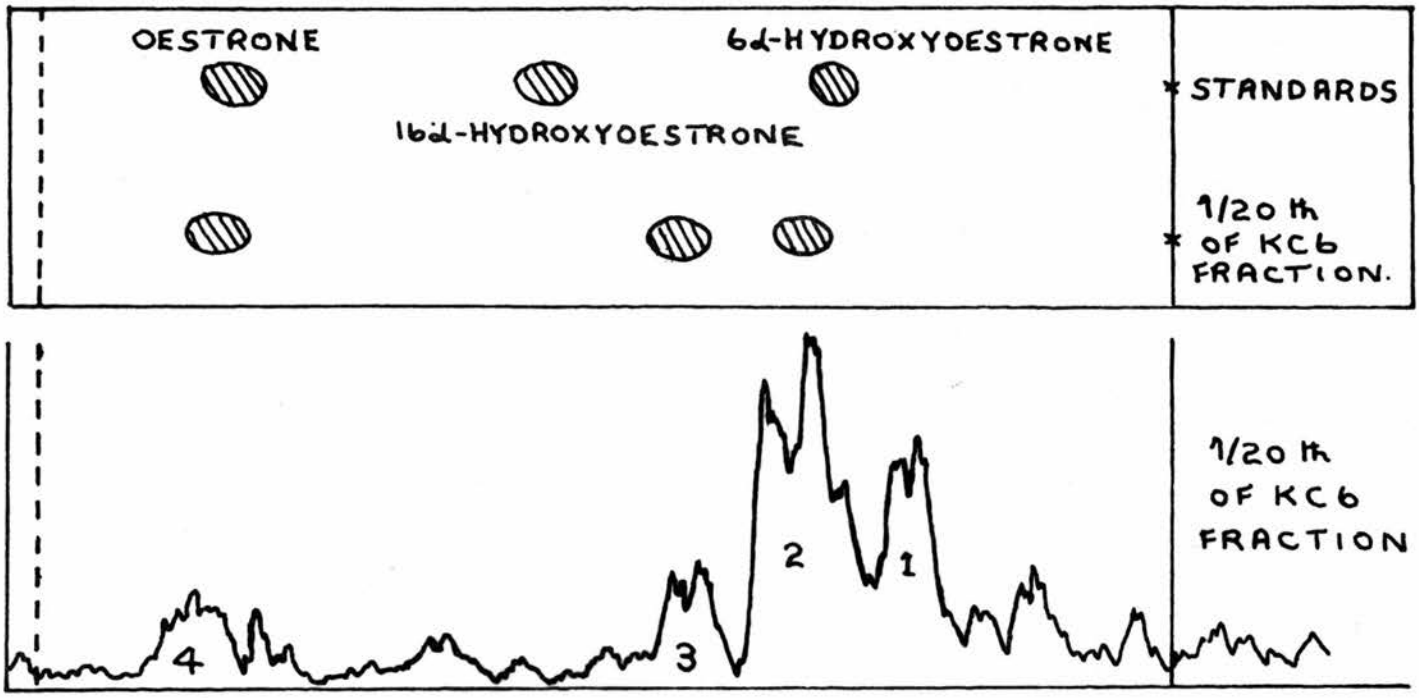
The thrice-crystallized material was then acetylated with 0.5 mls. of acetic anhydride in 0.5 mls. of pyridine at room temperature overnight, to give 115.9 mgs. of crude acetate. This was recrystallized three times from hexane giving a final yield of 40.1 mgs. of material having a constant specific activity of 242 counts/min/mg.

The evidence presented here shows conclusively that the water-soluble material obtained on incubation of oestrone <u>in vitro</u> with bovine adrenal cortex homogenates is oestrone sulphate.

(5) INVESTIGATION OF METABOLITES PRODUCED BY INCUBATION OF OESTRONE WITH OX ADRENAL GLANDS

From results obtained in the preliminary experiments it was found that oestrone was metabolized by bovine adrenal cortex homogenates to ether-extractable products, which could be separated on celite columns into fractions corresponding to the α-ketol and KC-6 fractions of late pregnancy urine. As this work was initiated in an attempt to demonstrate the 18-hydroxylation of oestrone by bovine adrenal cortex, the latter fraction was of particular interest and investigations were carried out on the material obtained from these preliminary experiments.

In one experiment in which [16-C^{14}] oestrone was incubated with cortex from one pair of fresh glands, the ether extractable products of incubation were fractionated on celite columns to give oestrone, α-ketol and KC-6 fractions. The latter represented 19.02% of the radioactivity applied to the incubation and was further investigated by chromatography on Whatman No. 2 paper in the system benzene : methanol : water :: 100 : 50 : 50, both before and after treatment with N-NaOH at room temperature for two hours. The chromatograms were scanned on the Nuclear-Chicago gas-flow chromatogram



BENZENE : METHANOL : WATER :: 100 : 50 : 50.

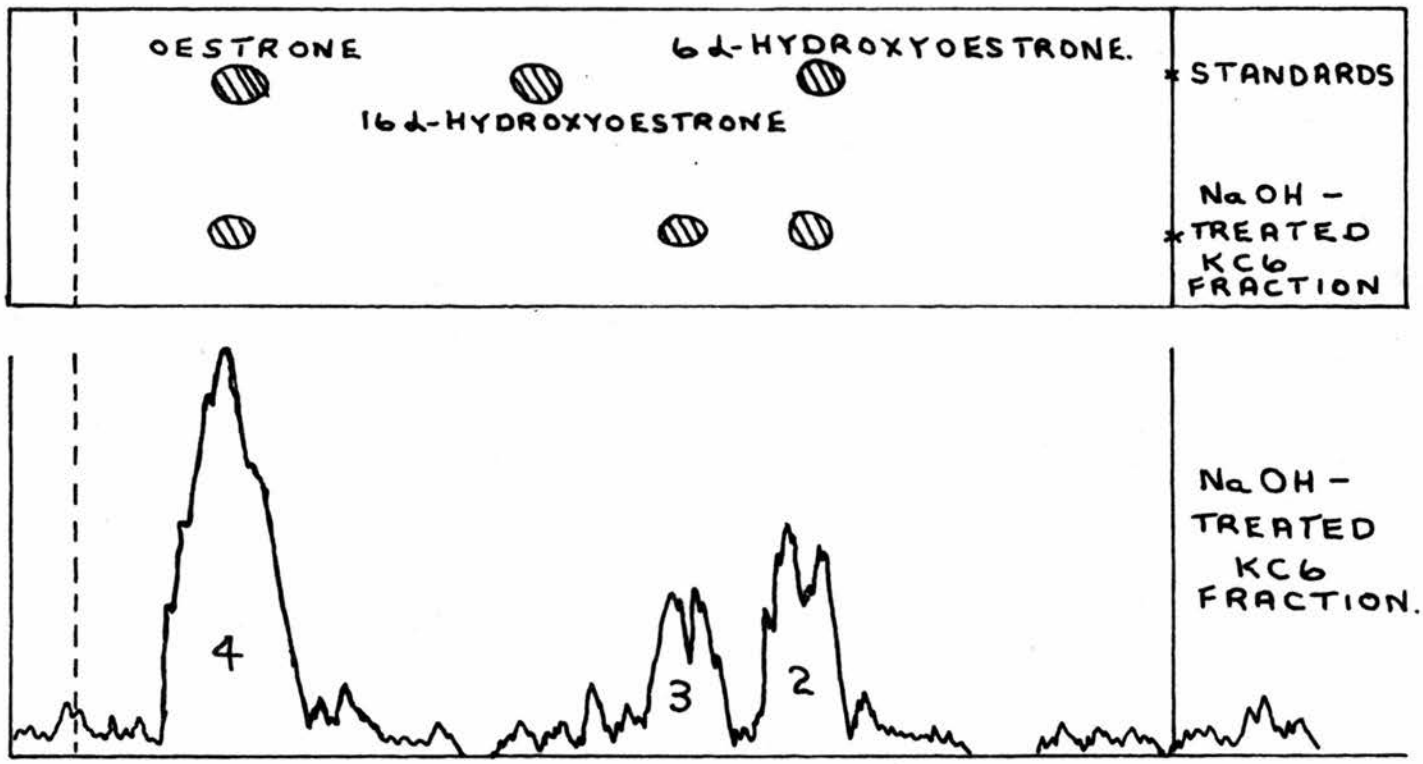


FIG. 32.

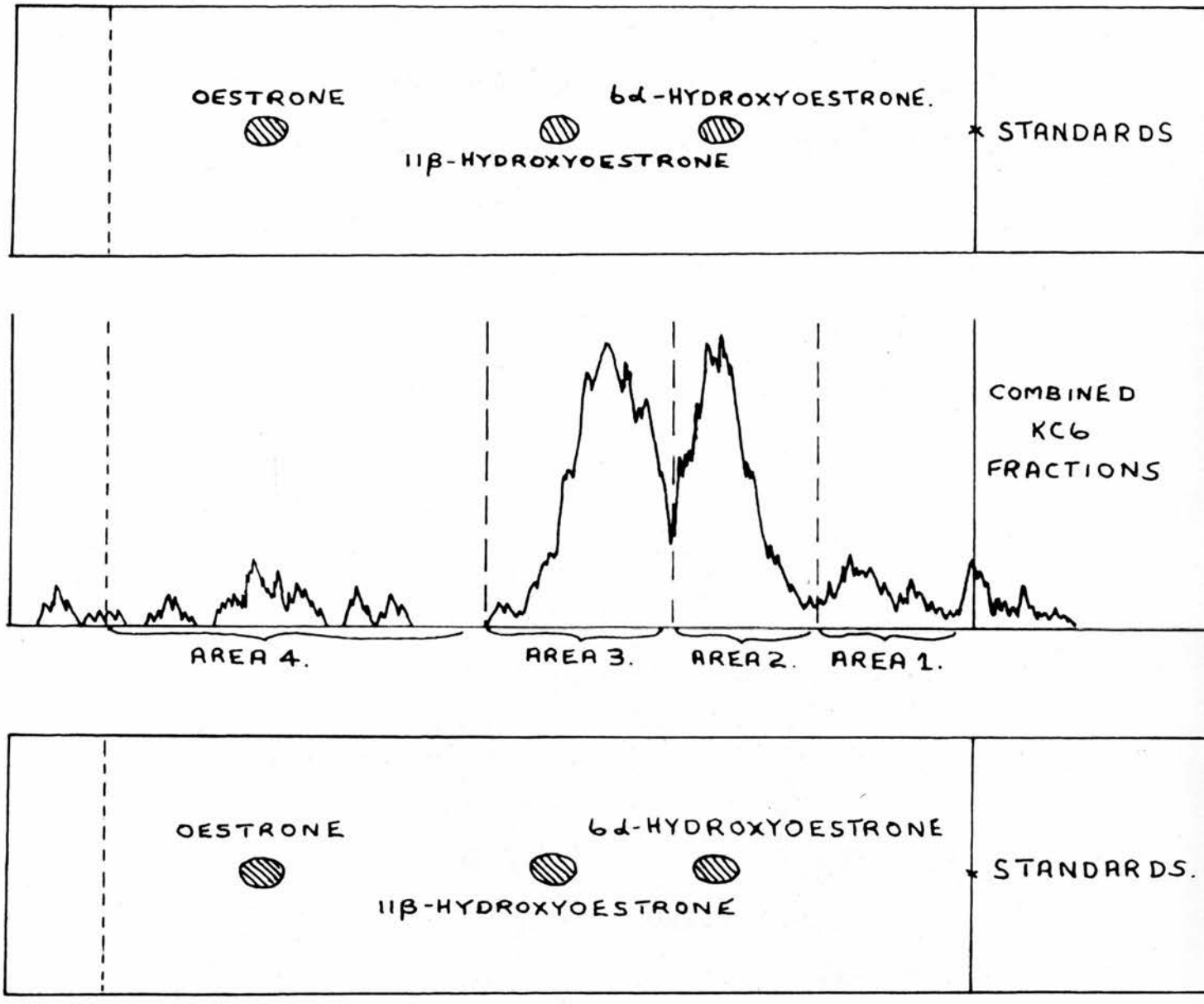
scanner, and the strips were then sprayed with $K_4Fe(CN)_6$/$FeCl_3$ reagent (Barton, Evans and Gardner, 1952). The results of this experiment are summarized in Fig. 32. Two points are of particular interest in this experiment;

(a) the KC-6 fraction contains a metabolite, peak 1, which does not stain with $K_4Fe(CN)_6/FeCl_3$ reagent and which seems to disappear after treatment with N-NaOH.

(b) Peak 2, which is slightly less polar than 6α-hydroxyoestrone is diminished on treatment with N-NaOH, whilst peak 4, having the same mobility as oestrone, is increased. By planimetry, peak 2 decreased by 0.159 units whilst peak 4 increased by 0.149 units.

These results seemed to indicate the presence of 18-hydroxyoestrone in peak 2 as this fraction is slightly less polar than 6α-hydroxyoestrone and shows a loss of radioactivity on treatment with N-NaOH at room temperature, the greater part of this radioactivity appearing in peak 4 which runs as oestrone. Further investigations were therefore made on KC-6 fractions obtained from the preliminary experiments in order to obtain confirmatory evidence for this conclusion.

The fractions corresponding to KC-6 by celite column chromatography from all of the preliminary experiments, were combined and applied as a streak over 2 cms. of the origin of a Whatman No. 2 paper chromatogram. Aliquots of authentic oestrone, 11β-hydroxyoestrone and 6α-hydroxyoestrone were



BENZENE : METHANOL : WATER :: 100:50:50.

FIG. 33.

applied on either side of this central channel and the paper was run in the system benzene : methanol : water :: 100 : 50 : 50. The strips containing the authentic standards were sprayed with $K_4Fe(CN)_6/FeCl_3$ reagent whilst the centre strip was scanned on the Nuclear-Chicago gas-flow chromatogram scanner. This strip was divided into four areas as shown in Fig. 33 and after eluting with methanol, an aliquot of each of these areas was re-run on Whatman No. 2 paper in the system benzene : methanol : water :: 100 : 50 : 50.

On scanning these strips it was found that Area 1 showed a small peak of radioactivity remaining at the origin; Area 2 showed a strong peak of radioactivity having approximately the same mobility as 6α-hydroxyoestrone; Area 3 showed a strong peak of radioactivity less polar than 6α-hydroxyoestrone; and Area 4 showed a small peak of radioactivity having the same mobility as oestrone.

The remainder of each fraction was subjected to formaldehyde determinations as described by Loke, Marrian and Watson, (1959) and the optical densities of the formaldehyde colours so obtained were compared with a previously constructed calibration graph. This showed that on treatment with N-NaOH for two hours at room temperature;

Area 1 gave 3.98 μgs HCHO

Area 2 gave 1.67 μgs HCHO

Area 3 gave 0.51 μgs HCHO

Area 4 gave 1.28 μgs HCHO

An aliquot of the NaOH-treated Area 2 material was run in the system benzene : methanol : water :: 100 : 50 : 50 on Whatman No. 2 paper. Radioactivity determinations indicated that 31.2% of the original material remained in the same position whilst 64.2% appeared in a peak running as oestrone. Calculations from the amount of radioactivity appearing in the "oestrone fraction" after NaOH treatment, indicated that the original material should have contained c.a. 3.6 µgs. of 18-hydroxyoestrone. However, from the formaldehyde determinations the figure should be 15.94 µgs. of 18-hydroxy-oestrone. These two calculations are grossly different and therefore it cannot be said that Area 2 contains 18-hydroxy-oestrone. From the results obtained in the formaldehyde determination experiment it is obvious that such determinations are of very little value on any material which has not been highly purified. Thus Areas 1 and 4 also appear to contain considerable quantities of formaldehydogenic material but from their chromatographic mobilities they could not contain 18-hydroxyoestrone.

A further series of thirty-two incubations were carried out in the usual way, incubating 5 gms. of adrenal cortex (within one hour of the animal being killed) with 500 µgs. of oestrone containing oestrone [16-C^{14}]. Extractions were made from the incubation solution as described for the preliminary experiments and the final ether extract was then

subjected to a Girard fractionation, the ketonic fraction being further separated into phenolic and non-phenolic fractions. By this fractionation, 88.5% of the radioactivity in the initial ether extract passed into the ketonic fraction, whilst the final ketonic phenolic fraction represented 69.1% of the radioactivity in the ketonic fraction. A $^{1}/_{100}$th aliquot of the ketonic phenolic fraction was chromatographed on a thin-layer silica-gel G plate by ascending chromatography in the system ethyl acetate : n-hexane : ethanol :: 80 : 15 : 5, against aliquots of authentic oestrone, 11β-hydroxyoestrone, 16α-hydroxyoestrone, 18-hydroxyoestrone and 6α-hydroxyoestrone. After developing with Folin and Ciocalteu's reagent and ammonia vapour, the spots in the ketonic phenolic fraction were eluted separately with aqueous methanol and the eluates plated for counting. The R_F values and appropriate percentage recoveries of radioactivity are shown below:-

Compound	R_F value		%age of applied radioactivity
oestrone	0.65		-
	0.68		-
16α-hydroxyoestrone	0.60		-
11β-hydroxyoestrone	0.60		-
18 -hydroxyoestrone	0.53		-
6α- hydroxyoestrone	0.50		-
$^{1}/_{100}$th ketonic phenolic fraction	0.66		60.5%
	0.59		3.9%
	0.52		6.4%
	0.00		1.7%
		Total	72.5%

Before proceeding further with this material, a series of investigations were carried out in order to try to find a chromatographic system giving an adequate separation of 16α-hydroxyoestrone and 11β-hydroxyoestrone. Eventually it was found that this could be achieved on a thin-layer silica-gel G plate by ascending development, running three times consecutively in the system monochlorobenzene : ethyl acetate 50 : 50. The solvent front was allowed to rise for 10 cms, the plate then being removed from the tank and the solvent allowed to evaporate. This was repeated twice more before staining the plate with Folin and Ciocalteu's reagent. Such a chromatogram showed that a compound spot of 11β-hydroxyoestrone and 16α-hydroxyoestrone could be readily separated into two distinct spots having R_F values of 0.73 and 0.82 respectively.

The ketonic phenolic fraction obtained from the series of thirty-two incubations was then subjected to fractionation on a thin-layer silica-gel G plate, running three times in the system ethyl acetate : monochlorobenzene 50 : 50. On either side of the central area containing the ketonic phenolic residue, standards of oestrone, 16α-hydroxyoestrone, 11β-hydroxyoestrone, 18-hydroxyoestrone and 6α-hydroxyoestrone were run and detected by Folin and Ciocalteu's reagent. The central portion was then divided

into six areas, corresponding to these five standards plus the origin area and these were eluted separately with ethanol on porosity-4 sintered disc filtration units. Aliquots of each of these eluates were then run as separate spots on a silica-gel G plate developing three times in the above system as before, with the same standards on either side. This showed that Area 3 had the same mobility as 18-hydroxyoestrone and was relatively pure being contaminated with only a small proportion of oestrone-like material. In order to remove this, the whole eluate was chromatographed on a silica-gel G plate, developing once in the ethyl acetate : monochlorobenzene : 50 : 50 system with standards of oestrone and 18-hydroxyoestrone on either side. The area corresponding to 18-hydroxyoestrone was eluted with ethanol, the eluate taken to dryness under nitrogen and dissolved in 10 mls. of ethanol. Two separate aliquots were taken from this solution, added to 2 mls. of N-NaOH and allowed to sit at room temperature for two hours and four hours respectively, whilst a third aliquot was spotted directly on to a Whatman No. 1 chromatography paper. The treatment with 2 mls. of N-NaOH for two hours at room temperature was also carried out on 20 µgs. of 18-hydroxyoestrone, whilst 20 µgs. of untreated 18-hydroxyoestrone was spotted on a separate channel of the same paper. At the end of the appropriate times, the

alkaline solutions were brought to pH 2 by addition of N HCl, extracted with 1 x 10 mls ether which was then washed 1 x 3 mls 5% $NaHCO_3$ solution, 2 x 3 mls water, dried over anhydrous sodium sulphate and taken to dryness. These residues were then spotted in separate channels on the above paper which was equilibrated overnight in the system benzene : methanol : water :: 100 : 50 : 50 and was then run for two hours. The spots were developed by spraying with Folin and Ciocalteu's reagent and exposing to ammonia vapour and the appropriate channels were scanned on the Nuclear-Chicago integrating gas-flow chromatogram scanner having a thin end-window. The results of this experiment are shown below:-

Material	R_F of spot after spraying with Folin & Ciocalteu's reagent	R_F of radioactive peak	Quantitation of counts under peak
18-hydroxyoestrone	0.375	-	-
18-hydroxyoestrone) after 2 hrs. N-NaOH)	0.370 0.810	- -	- -
Area 3 residue	0.288	0.288	1910 counts
Area 3 residue) after 2 hrs. N-NaOH)	0.302	0.291	1920 counts
Area 3 residue) after 4 hrs. N-NaOH)	0.294	0.287	1360 counts
Oestrone	0.815	-	-

From these results it would appear that the Area 3 material is not in fact 18-hydroxyoestrone as even four hours alkali-treatment does not produce any oestrone-like material and furthermore the untreated Area 3 residue is slightly more polar than 18-hydroxyoestrone in the system benzene : methanol : water :: 100 : 50 : 50. In order to obviate the possibility that any 18-hydroxyoestrone in the Area 3 material might have been destroyed in the process of working up, it was decided to submit authentic 18-hydroxy-oestrone to Girard separation and phenolic fractionation under the same conditions as used for the adrenal extract. Subsequent chromatography in the system benzene : methanol : water :: 100 : 50 : 50 showed that the 18-hydroxyoestrone had not been altered at all by this treatment. It would appear therefore that the negative result given by this series of experiments is in fact a true result.

It was decided to repeat this experiment, but instead of fractionating by Girard and phenolic separations, the ether extract from the adrenal incubations was subjected to a six-funnel counter-current distribution in the system benzene : hexane : 80 : 20 :: ethanol : water : 50 : 50 using 50 mls of each phase per funnel. In this system oestrone had been shown to have a partition coefficient of 9.8 whilst 16α-hydroxyoestrone has a partition coefficient of 0.95

(Watson and Marrian, 1955). Calculations showed that Funnel 1 of such a distribution should contain 60.2% oestrone + 2.74% 16α-hydroxyoestrone, whilst Funnels 2 - 6 combined should contain 38.6% oestrone + 97.3% 16α-hydroxyoestrone. As a preliminary experiment with the ether extract from a number of sham adrenal incubations had shown that the greater part of the gummy residue remained in Funnel 1, it was hoped by this process to remove the greater part of the oestrone plus the gummy material without subjecting the metabolites to any extreme conditions. Accordingly a further twenty incubations were performed and ether extracts were obtained as described previously. This yielded 38.9 mgs.of ether extractable residue, which following a six-funnel counter-current distribution in the system benzene : hexane : 80 : 20 :: ethanol : water 50 : 50, was distributed as 24.6 mgs. in Funnel 1 residue and 14.6 mgs. in Funnels 2 - 6 residue. Radioactivity determinations showed that of the total counts applied to the incubations 65.7% were recovered in the ether extract, 21.1% being recovered on extracting the aqueous residue with ethyl acetate after saturating with sodium chloride. In the above counter-current distribution it was found that 46.6% of the counts in the ether extract were recovered from Funnel 1 residue, whilst 57.9% were recovered from Funnels 2 - 6 residue.

The 14.6 mgs.of Funnels 2 - 6 residue was chromatographed as a streak on a thin-layer plate in the system 50 : 50 ethyl acetate : monochlorobenzene, developing three times consecutively. The residue was divided into three fractions corresponding in chromatographic mobility to "oestrone", "α-ketol + KC-6", and "residual origin area". These fractions after elution contained respectively 52%, 40.6% and 0.3% of the counts in the original residue.

Following chromatography of the "α-ketol" + KC-6" fraction on Whatman No. 1 paper in the system benzene : methanol : water :: 100 : 50 : 50, and scanning of the paper on the Nuclear-Chicago gas-flow chromatogram scanner fitted with a thin end-window, it was found to contain a considerable proportion of material having the same mobility as oestrone, plus a major radioactive fraction which appeared to contain two main peaks, both of which were more polar than 16α-hydroxyoestrone. The latter fraction was eluted with methanol and re-run in the same system, which on scanning showed that the oestrone-like material had been completely removed. Treatment of this "KC-6-like" fraction with chilled N/10 NaOH for 5 min and subsequent chromatography as above showed that it was completely unaffected. The material was therefore again eluted with methanol and treated with 10 mls N-NaOH at room temperature for 2 hrs;

chromatography and scanning as before showed that this treatment too was without effect.

This result therefore precludes the possibility that this fraction might contain 18-hydroxyoestrone, as this compound is known to be degraded under such conditions to 18-noroestrone which has the same chromatographic mobility as oestrone in this system. This, together with the result of the previous experiment suggests that 18-hydroxyoestrone is not a product of the metabolism of oestrone by the adrenal cortex.

In view of the reported isolation of 11β-hydroxyoestrone and 16α-hydroxyoestrone, following incubation of oestrone with adrenal cortical tissue (Knuppen and Breuer, 1962), the ketonic phenolic residue obtained from the ether extract of a series of thirty-two incubations was examined for the presence of these compounds. This residue was fractionated by chromatography on a thin layer silica-gel G plate, running this three times in the system 50 : 50 :: ethyl acetate : monochlorobenzene, to give six areas corresponding in chromatographic mobility to standards of oestrone, 16α-hydroxyoestrone, 11β-hydroxyoestrone, 6α-hydroxyoestrone, 18-hydroxyoestrone and origin-line area. Following elution, subsequent chromatography of an aliquot of each of these extracts showed that Area 4 contained two spots - a major spot having the same R_F as 11β-hydroxyoestrone plus a minor spot running as oestrone. Area 5 also showed the presence

of two spots - a major spot running as oestrone plus a minor spot having the same R_F as 16α-hydroxyoestrone.

The Area 4 residue was freed from oestrone-like material by chromatography on a thin-layer silica-gel G plate, running once in the system 50 : 50 :: ethyl acetate : monochlorobenzene. The area corresponding to 11β-hydroxyoestrone was eluted with methanol and the eluate added to 67.1 mgs.of 11β-hydroxyoestrone (synthesized by Mr. H. Roderick, Imperial Cancer Research Fund, after Magerlein and Hogg, 1958) which had been recrystallized once from ethyl acetate and twice from methanol, m. pt. 254 - 255°. This material was recrystallized twice from methanol and twice from ethyl acetate and was then acetylated and re-crystallized three times from methanol. Radioactivity determinations were made at each stage and the results are summarized below:-

Free Compound	1st crystallization	2nd crystallization	3rd crystallization	4th crystallization
Solvent	ethyl acetate	methanol	ethyl acetate	methanol
cpm/mg. of crystals	55	33	27	13
cpm/mg. of mother liquors	257	655	26	30
Total counts in crystalline fraction	2200	656	398	209
Total counts in mother liquors	5873	989	312	153
Total	8073	1645	710	362
	mgs.	mgs.	mgs.	mgs.
Weight of crystals	39.95	19.70	14.75	16.10
Weight of mother liquors	22.85	19.20	1.20	5.10
Total	62.80	38.90	15.95	21.20
Acetate				
Solvent	methanol	methanol	methanol	methanol
cpm/mg. of crystals	566	346	0	-
cpm/mg. of mother liquors	1118	934	521	-
Total counts in crystals	17100	6100	0	-
Total counts in mother liquors	21400	11900	4300	-
Total	38500	18000	4300	-
	mgs.	mgs.	mgs.	
Weight of crystals	30.25	17.70	9.0	-
Weight of mother liquors	19.15	12.80	8.25	
Total	49.40	30.50	17.25	

From these results it would appear that the metabolite having the same R_F value as 11β-hydroxyoestrone on thin layer chromatography after three consecutive runs in the system 50 : 50 :: ethyl acetate : monochlorobenzene is not actually 11β-hydroxyoestrone. However, in view of the fact that the amount of 11β-hydroxyoestrone available was limited and therefore that this material was not fully authenticated, together with the small amount of metabolite available, this question would require more rigorous examination before refuting the suggestion that 11β-hydroxyoestrone is a product of the incubation of oestrone with adrenal cortical tissue.

(6) DISCUSSION

The work reported in this section was undertaken with a view to proving whether or not 18-hydroxyoestrone is a product of incubation of oestrone with adrenal cortical tissue. As indicated previously, it was suggested from the work of Loke (1958) on this topic, that the ageing of the tissue used, due to delay between death of the animal and incubation of the tissue, might be a critical factor in the formation of 18-hydroxyoestrone, in providing a sufficiently high intracellular level of NADPH to enable 18-hydroxylation to take place. This would be particularly more important in whole-cell tissue minces than in experiments using homogenates with added co-factors, because in the former case, the NADPH although not auto-oxidisable to any great extent within the cell will not be replaced, once used, in non-fresh tissue as its production from NADP requires a functional carbohydrate metabolism which will presumably cease sometime after death due to lack of substrate.

As it has been established that NADPH is required for 11β-hydroxylation of neutral steroids (Grant, 1956; Sweat and Lipscomb, 1955) and as Loke (1958) found an increased yield of KC-6-like material when NADP was added to cortex homogenates in small-scale experiments, it

was thought to be likely that this difference in "freshness" of material might account for the difference found by Loke between his small-scale and large-scale experiments. It was surprising therefore that the experiments on production of KC-6-like material in fresh as opposed to aged glands failed to demonstrate any statistically significant difference between the two types. However it must be borne in mind that the term KC-6 -like material is rather vague and that one really requires to compare the production of 18-hydroxyoestrone. This lack of precision is indicated by the rather high standard error in percentage recoveries of radioactivity in each fraction in this experiment, and particularly so in those of the "KC-6" fraction of the aged glands. As only five such experiments were performed it was not possible to get a statistically significant result in the face of such large individual variations but the results of individual experiments did tend to suggest that the "fresh" tissue was more efficient at metabolizing oestrone than the "aged" tissue. However this difference is not nearly so great as expected and this raises doubts as to the validity of the argument put forward concerning the effect of "ageing" on intracellular NADPH levels and/or the involvement of NADPH in 18-hydroxylation. It almost appears that the effect suggests merely a falling off in efficiency of metabolism which one would naturally expect some twenty-four hours after death.

The actual results obtained from the investigation on the production of 18-hydroxyoestrone from oestrone by adrenal cortical tissue are conflicting. On the one hand, the results of two series of large-scale incubations which were processed in different ways in order to obviate destruction of any 18-hydroxyoestrone, show definitely that no alkali-labile material was present; on the other hand, in preliminary small-scale experiments it was equally definitely shown that material was produced, which was of the approximate chromatographic mobility of 18-hydroxyoestrone and which on alkali treatment yielded material of the same chromatographic mobility as oestrone. This question will be dealt with more fully in the final discussion at the end of this thesis.

The second rather surprising result of these investigations is the failure to identify 11β-hydroxyoestrone by reverse isotope dilution. From the results obtained in this work it appeared that the radioactive metabolite was very closely similar to the synthetic 11β-hydroxyoestrone as the counts /min/mg. of the free compound fell very slowly on successive crystallizations. This close similarity is further supported by the fact that the radioactive metabolite and the synthetic 11β-hydroxyoestrone showed the same chromatographic mobility on a silica-gel G plate run three times consecutively in the system 50 : 50 :: ethyl acetate : monochlorobenzene, a procedure which effects a separation of 18-

hydroxyoestrone and 6α-hydroxyoestrone. The most obvious possibility is that the reverse isotope dilution material might have contained both epimeric forms of 11-hydroxyoestrone; this possibility will be discussed more fully in the final discussion section.

The most interesting result obtained from the investigation of the metabolism of oestrone by adrenal cortex tissue is possibly the finding that this tissue is capable of conjugating oestrone to a considerable extent as the sulphate. The identification of this conjugate presented no serious difficulties, using the established chromatographic techniques of Schneider and Lewbart, (1956,1959) but particular reference must be made to the extremely useful procedure of Beling (1961) for chromatography of the crude sulphate on columns of Sephadex-G25 eluting with distilled water. By this technique it was possible to obtain a much cleaner fraction, which permitted the use of paper chromatography in helping to identify the compound. This had been found difficult hitherto due to the presence of a contaminant in the extract which caused the spots to streak rather badly on running. Beling (1961) has already advocated the use of such columns in the determination of urinary oestrogens and it is obvious that they will be particularly useful in conjugation studies. This rather unexpected finding is made more interesting in

the light of the report of Purdy, Engel and Oncley (1961), that oestrone sulphate is the major circulating form of oestrogen in the blood and has led to a re-appraisal of the tendency to regard steroid sulphates solely as "detoxication products".

SECTION V

DISCUSSION

The work described in this thesis has dealt with two major aspects of the biochemistry of oestrogens - the isolation and identification of a minor oestrogen derivative from human late pregnancy urine, and the investigation of the *in vitro* metabolism of oestrone with bovine adrenal preparations.

One of the major difficulties facing the worker in the field of oestrogen biochemistry is the fact that these compounds, on account of their high physiological activity, occur in nature in very small amounts, and one is therefore dealing with relatively small concentrations of material. This was undoubtedly the greatest obstacle to the work reported in Section III of this thesis i.e. the identification of "KC-6B" from human pregnancy urine, in which the situation was further exasperated by the fact that this compound was only a very minor urinary oestrogen derivative from the quantitative point of view. Thus in the initial work on this problem, the processing of one thousand litres of human late pregnancy urine, yielded approximately four mgs.of impure material. This fact therefore precluded the use of the "classical" techniques of obtaining the material in a pure crystalline form and identifying it by melting point determinations, formation of derivatives and elementary analyses. Furthermore, due

to the difficulty of separating "KC-6B" from 18-hydroxy-oestrone by the paper chromatographic systems available, it was obvious that the partition characteristics of KC-6B in such systems were unlikely to be of significant value as criteria for the identification of this compound. However, the problem was simplified to some extent by the fact that Loke (1958), from preliminary investigations on "KC-6B", obtained information which led to the postulate that it might be a 6-hydroxyoestrone and also by the fact that Longwell and Wintersteiner (1940) had evolved a satisfactory method for the introduction of a 6-keto group into the oestrogen molecule.

Although it was then a relatively easy matter to synthesize some of these 6-oxygenated oestrogen derivatives, the synthesis of 6-hydroxyoestrone itself was a much more formidable task, necessitating the reduction of the 6-keto group whilst keeping a free 17-keto group. Due to the greater reactivity of the former, it was extremely difficult to protect the 17-keto group by formation of a derivative which would have enabled one to reduce the 6-keto group with sodium borohydride. In the work reported in Section 11, this was attempted by formation of an enol acetate derivative at the 16,17 position but from the results obtained it was abundantly clear that formation of such a group must have taken place preferentially at the 6,7 position. Knuppen and Breuer (1961) adopted a different approach to this problem and utilized the greater

reactivity (enhanced by the presence of an acetoxy group at C3) to benzoylate the former by the Schotten-Baumann reaction, which then allowed them to oxidize the C-17 hydroxyl group and thus eventually to obtain 6α-hydroxyoestrone. With the availability of authentic 6α-hydroxyoestrone it was then possible to obtain direct comparisons with "KC-6B" in chromatographic systems, in the Kober reaction, and in U.V. absorption spectra, both in ethanol and in concentrated sulphuric acid. It was also possible to utilize the authentic material in order to devise methods for the identification of "KC-6B", and the reasons for believing these methods to be unequivocal have been discussed fully in Section 111 of this thesis. It has also been shown in this section that it is not possible to assign to the 6-hydroxyoestrone identified from human late pregnancy urine, any definite configuration. It is of interest however, in view of the apparent finding of both epimers, that Breuer, Knuppen and Pangels (1961) have shown that rat liver contains a 6α-hydroxylase and a 6β-hydroxylase, both of which attack phenolic steroids. These authors have also shown that both rat liver and human liver also contain 6α- and 6β-hydroxysteroid dehydrogenases. It is tempting therefore to speculate that the finding of both epimers in human late pregnancy urine is indicative of the presence of both hydroxylases in human liver, but in view of the work reported here on the epimerization of 6-hydroxy-

steroids by procedures which were used in the isolation of "KC-6B", such speculations must be treated with considerable reserve until they are proved by further experimental evidence.

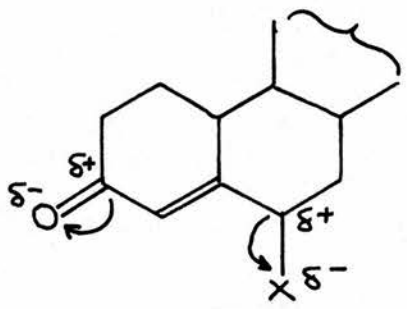
The question of the physiological significance of the 6-oxygenated oestrogens is one which is extremely difficult to assess from the information available at present. From the relatively minute quantity of 6-hydroxyoestrone in human pregnancy urine, one is tempted to dismiss such compounds immediately as being of no significance whatsoever, but this raises the interesting question as to whether or not urinary levels are a measure of physiological importance. If one assumes that in its physiologically active form the hormone exerts its specific action and is then altered, either by virtue of its action or by a process of degradation before being excreted, then the more abundant urinary metabolites might be regarded merely as "degradation products" of the physiological hormone and therefore as unimportant. Doubtless the truth lies somewhere between these two extremes, but the wide distribution of 6-hydroxylases and 6-hydroxysteroid dehydrogenases which are capable of attacking phenolic steroids suggests that 6-oxygenated oestrogen derivatives might play some important part in the biochemistry of oestrogens. Such enzymes have been shown to occur in mouse liver (Mueller and Rumney, 1957) rat liver (Breuer, Knuppen and Pangels, 1961) human liver (Breuer et al. 1961)

and of particular interest is the reported isolation of 6"α"-hydroxyoestradiol-17β from the follicular fluid of the mare (Bush, Klyne and Short 1960).

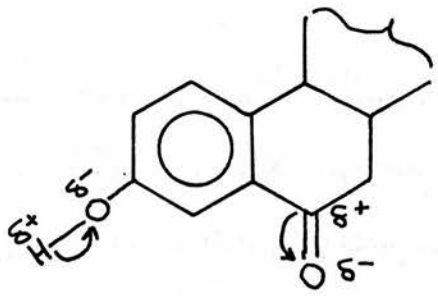
Examination of the characteristics of 6-oxygenated oestrogen derivatives, reported in Section 11, reveals that these compounds exhibited the unusual property of being considerably more soluble in aqueous media than corresponding derivatives bearing an oxygen function in some other part of the molecule. This was attributed to the unique position of the 6-oxygen function in being adjacent to and in conjugation with the unsaturated benzenoid A-ring and it seems possible that if the 6-oxygenated oestrogens have in fact some special physiological significance, then this might be related to this rather unique property. Indeed it would be surprising if anything which affected the C-3 hydroxyl group of oestrogens, in such a marked manner as a 6-oxygen function appears to, were to be without some effect on the physiological activity of these steroids. Thus it is well established that in naturally occurring steroid hormones and in almost all active hormone analogues, an oxygen function at C-3 and an oxygen function or acetyl side chain at C-17 is required for physiological activity. In particular, from studies on methylated oestrogen-derivatives, Ringold (1961) has come to the conclusion that for optimal oestrogenic activity, a planar aromatic ring A in which the phenolic hydroxyl group is free for some type of interaction is

required. It would seem therefore that the effects of substituting electronegative groups at C-6 in the oestrogens would be worthy of some study, but no information seems to be available on this point. The only available information concerns 6-halogenated corticoids and progestational compounds in which there seems generally to be an increase in hormonal activity (c.f. 6α-fluorotestosterone which is about 40% as active as testosterone; Bowers and Ringold, 1958). At first sight this increase in hormonal activity on introduction of an electronegative group at C-6 seems encouraging in speculating that 6-oxo-oestrogens might have some special importance, but closer examination of work by Ringold, et al (1959) and Bowers et al (1959) suggests that "electronegativity" of the 6-substituted group is unimportant in the enhancement of activity of such compounds. In studying the activity as oral progestins of 6α-halogen substituted-17α-acetoxy-progesterone derivatives, it was found that these compounds exceeded the activity of the parent compound by a factor of from 15 to 40 times, depending on the halogen substituted at C-6. However, if the electronegativity of the 6-substituent and its transmitted effect on the Δ^4-3 ketone system is of importance, it should be possible to establish a biological activity gradient of either $F > Cl > Br > H > CH_3$ or $CH_3 > H > Br > Cl > F$ depending on which of these groups is substituted at C-6. It was found that neither order could be established, and the high

activity of these compounds was attributed to a lower rate of inactivation. However, it does not necessarily follow that this work which shows that electronegativity of 6-substituted groups is unimportant in progestins, can be taken to mean that this is also true for oestrogens, for the "active site" being considered in the two groups of compounds are totally different. Thus in the progestins we have a Δ^4-3 ketone system in which the 3-ketone group is itself of an electronegative character, and introduction of a second electronegative group in the 6-position will oppose the electronegativity of the 3-ketone group, by transmission of the effect through the conjugated double bond at C 4-5:-



This does not affect the conclusion of Ringold, that relative electronegativity is of no importance in the enhanced activity of these compounds, but it does emphasise that one cannot arbitrarily assign similar conclusions to the oestrogens, in which activity seems to require a free hydroxyl group at C-3, and in which the introduction of an electronegative group at C-6 would almost certainly increase the degree of dissociation of the 3-hydroxyl:-



Some evidence in favour of this suggestion is furnished by the greatly increased water-solubility of 6-oxygenated oestrogens over oestrogens possessing a ketone group at other positions, whilst a 6-hydroxyl group has only the same effect as a hydroxyl group in any other position. It would therefore be extremely interesting to pursue this investigation with other oestrogen derivatives substituted with electronegative groups at C-6.

Mention has already been made of the work on oestrogens methylated in various positions in Ring A (Ringold 1961) which showed that in each case a profound decrease in oestrogenic activity resulted (as determined in the mouse, with uterine weight increase following injection as the criterion of activity). The much greater decrease in activity of the C-2 and C-4 methyl oestrogens over C-1 and C-5 methyl derivatives indicated that oestrogenic activity is extremely susceptible to steric interaction with the C-3 phenolic hydroxyl group.

The actual mechanism by which the C-3 phenolic hydroxyl group is involved in oestrogenic action is of course as yet unknown but it has been implicated in a number of

proposed mechanisms of oestrogenic action, particularly in hypotheses which involve the binding of oestrogens to enzymes, e.g. (Krupka and Laidler (1960) have suggested a mechanism for the enzymatic oxidation of oestradiol-17β by NAD which involves the binding of oestradiol-17β to an active site on the enzyme by hydrogen bonding involving the 3-hydroxyl group. In such a case, the enhanced dissociation of the 3-hydroxyl group due to the presence of a 6-oxo group might lead to stronger hydrogen bonding and therefore greater affinity for the enzyme. Similar conclusions might be drawn for other proposals concerning the binding of oestrogens to proteins and sub-cellular components, but until more precise information is available on the part played in such processes by the 3-hydroxyl group there is little of value to be gained in speculating on the possible effects of increasing the dissociation of this group.

From the evidence available at the present time therefore, it is impossible to assign to the 6-oxygenated oestrogens any particular physiological significance and if one attaches any importance to urinary concentrations as a measure of physiological importance then they can be dismissed as unimportant. However, in view of the occurrence of 6α- and 6β-hydroxylases and the corresponding 6-hydroxysteroid dehydrogenases in the livers of a number of species, and the rather unique nature of 6-oxo oestrogens, particularly

with regard to their effect on the 3-hydroxy group, it would be extremely interesting to investigate the possible physiological role of these compounds.

In Section IV of this thesis the results of an investigation into the possible formation of 18-hydroxyoestrone by bovine adrenal tissue were presented, and it was shown to be not possible to state unequivocally that 18-hydroxyoestrone is not formed. Although the last two sets of experiments performed show conclusively that no 18-hydroxyoestrone was present, initial small-scale experiments indicated the formation of an alkali-labile component which was degraded to a product having a similar chromatographic mobility to oestrone. This discrepancy, which is similar to that found by Loke (1958) is very difficult to explain and the suggestion made in the initial stages of this work, that "ageing" of the tissue used might be a critical factor due to intracellular $NADPH_2$ levels, seems to be without foundation. Thus the comparison of "fresh" and "aged" glands failed to show any statistically significant difference between them in the production of "KC-6-like" material and the slight difference found was suggestive merely of a falling off in general enzyme activity with increasing "agedness" rather than of a critical metabolic difference. That bovine adrenal cortex contains an 18-hydroxylase is shown by the work of Kahnt, Neher and Wettstein (1955) on the formation of 18-hydroxycortexone from cortexone, and that this hydroxylase is

capable of attacking oestrone is suggested by the results of the small-scale experiments, both in the work reported here and in that of Loke (1958). If this is so, then the failure to isolate 18-hydroxyoestrone from adrenal cortex incubation experiments suggests either some variability in the glands themselves or some variability in the experimental technique employed. Although this latter criticism is true in the work of Loke (1958), in which the conditions between his small-scale and large-scale experiments were not the same, this possibility is excluded by the work reported in Section IV in which the conditions of incubation and initial extraction were constant throughout. The only variability was in the methods of fractionation of the ether extract, and these were shown to be without effect on 18-hydroxyoestrone. The other alternative, i.e. variability in the glands used is one which might well repay further investigation.

The adrenal cortex of the adult animal consists of concentric layers of cells divided into three main zones - the glomerulosa, fasciculata and reticularis. The problem of correlating function with structure in the adrenal cortex has given rise to conflicting theories; that the zones and their secretions are interrelated in varying degrees and are inter-dependent, or that the zones, once formed are independent and have distinctly different steroid secretions. Evidence

has been presented that the latter concept is the correct one (Ayres, 1960). Thus by incubation of ox-adrenal slices Ayres' group obtained direct evidence that there was a preferential production of steroid between the zones of the cortex, (Ayres, Gould, Simpson and Tait, 1956) aldosterone being preferentially produced by the zona glomerulosa and hydrocortisone by the zona fasciculata, (the reticularis and fasciculata zones cannot be differentiated in the ox). The results of further work in the ox (Ayres, Hechter, Saba, Simpson and Tait, 1956) indicate that the 11β-hydroxylating system is equally divided per gram of tissue, between the two zones whilst the 17α-hydroxylating system is in the fasciculata only and the 18-oxidase system is confined to the zona glomerulosa. This therefore brings us to the realization that as the zona glomerulosa is immediately below the capsule layer it is probable that it was lost in the present experiments, unless it was a reasonably thick layer, as the capsule remained inside the Latapie mincer. This, together with the fact that there appears to be a seasonal variation in the thickness of the zona glomerulosa in the ox adrenal (Ayres, personal communication) offers an explanation of the variability of the results obtained in these different experiments. Against this explanation however is the result of one experiment (not reported in Section IV) in which the capsular material, on

incubation with (16-^{14}C) oestrone, failed to show any alkali-labile material produced in the "KC-6" fraction isolated by thin-layer chromatography.

The presence of an 18-oxidase system which is limited to the zona glomerulosa of the ox-adrenal has been amply demonstrated and the suggestion that this might be capable of attacking phenolic steroids has been partly suggested by results of some preliminary experiments, both in the work reported here and in that of Loke (1958). Further investigation of this problem would be made easier if a satisfactory method could be found for the synthesis of 18-hydroxyoestrone, as this would enable the use of reverse isotope dilution techniques in the identification of this metabolite. Unfortunately at the present time, no such method exists.

One of the most interesting results of the work reported in Section IV was that of the isolation and identification of oestrone sulphate following the incubation of oestrone with bovine adrenal cortex tissue. This finding is of particular interest both from the implication of the adrenal cortex as a possible oestrogen-secreting tissue, and from the increased interest which has recently been focussed on oestrogen-conjugates in general and sulphates in particular with the suggestion that the latter compounds might play some fundamental role in the biochemistry of oestrogens.

The evidence for production of oestrogens by adrenal tissue is mainly indirect in the human but Beall (1940) has isolated oestrone from bovine adrenal tissue, furnishing direct evidence in this species for adrenal oestrogen-synthesis. The indirect evidence for adrenal oestrogen production has recently been the subject of an excellent review by Engel (1962) who has summarized the evidence as follows:-

1) The occurrence of oestrogens in the urine of males and females following castration.

2) The disappearance of oestrogens from the urine of gonadectomized-adrenalectomized patients.

3) The increased excretion of oestrogens in some adrenocortical tumours and adrenocortical hyperplasia.

4) The decline in oestrogen excretion levels after removal of the tumour.

5) A decline in oestrogen excretion levels in patients with adrenal hyperplasia following suppressive corticosteroid therapy.

6) The increased excretion of oestrogens after ACTH therapy.

7) The failure of ACTH to exert this effect in adrenalectomized patients.

This evidence would suggest that the adrenal is a site of primary production of oestrogens but as West Damast , Sarrs and Pearson (1956) have shown that there is an

increased excretion of oestrogens in the urine of castrate - adrenalectomized patients treated with testosterone, and as in the evidence sited above there is a general parallelism between oestrogen excretion and the excretion of C-19 steroids, the possibility exists that the urinary oestrogen arises from aromatization of adrenal C-19 compounds in some other tissue - perhaps the liver. Thus the evidence for production of oestrogens in the human adrenal is not yet conclusive, as it is for bovine adrenals. However, it is extremely interesting to note that since this work was completed Adams (1963) has shown that cell-free extracts of human female adrenal tissue are capable of conjugating oestrone as well as androgens but not corticoids as their sulphates. In this respect therefore the human adrenal cortex is identical with bovine adrenal cortex.

Until the presentation of the work reported here, and that of Adams (1963), enzymes capable of converting steroids to their sulphate esters had only been found in mammalian liver (Roy, 1960), and the presence of such enzymes in tissue which is possibly capable of oestrogen production poses the question as to their biological significance. The work of Purdy, Engel and Oncley (1961) in which it was shown that oestrone sulphate is the main form of circulating oestrogen in human blood, indicates that such compounds are of immediate importance as the transport form of oestrogens, and that this might be true of other steroids has been

shown by the fact that sulphuric acid conjugates of 17-keto-steroids are by far the major form of 17-ketosteroids in plasma (Ceresa and Cravetto, 1958). However some evidence is also available that oestrogen sulphates might play some more fundamental role in the biochemistry of these compounds other than that of being the means of transport. Mason and Gullekson (1959) and (1960) have shown that the disulphates of oestradiol-17β and diethylstilboestrol were found to inhibit the kynurenine transaminase system of rat kidney at concentrations as low as 5×10^{-7}M. Oestrone sulphate and pregnanediol glucuronide inhibited only at much higher concentrations whilst the free compounds oestradiol, oestrone and diethylstilboestrol were not inhibitory even in saturated solutions. Further investigation of the inhibition revealed that it was due to competition between the oestrogen sulphates and the coenzyme pyridoxal phosphate for the apoenzyme. This work is extremely interesting and is worthy of further consideration. Although the precise role of kynurenine transaminase is not known, the importance of pyridoxal phosphate as a coenzyme in reactions involving transamination and decarboxylation of amino acids is well known and it is therefore of considerable interest that oestrogen disulphates can inhibit such reactions. From this point of view it would be interesting to examine the effects of

such compounds on other coenzymes - particularly the thiamine pyrophosphates and lipoic acid coenzymes involved in the relationships between pyruvate and the citric acid cycle. Effective actions by oestrogen disulphates at such points could be of fundamental importance in cellular metabolism. The second interesting point that arises from this work, is the concentration of 5×10^{-7}M at which these steroids inhibited, as this falls within the range of what is now regarded as being the "physiological level" of oestrogens. The fact that oestrone sulphate inhibited at only much higher levels of concentration also seems to be significant; if the disulphate is the physiological form of the oestrogens, then this would agree with the known greater relative potency of oestradiol-17β over oestrone, which can of course only form the monosulphate. It is also significant that the free compounds, oestrone and oestradiol-17β were not inhibitory even in saturated solutions.

In considering the possible implications of sulphate conjugates in the biochemistry of oestrogens, the separation of two separate steroid sulphokinases by Nose and Lipmann (1957) suggests a possible metabolic rule of such enzymes. It has been shown that formation of steroid sulphates takes place by transfer of sulphate from an "active sulphate" intermediate, adenosine 3'-phosphate-5'-phosphosulphate (PAPS), (Bernstein and McGilvery 1952); (De Meio, Wizerkaniuk and Schriebman, 1955); (Hilz and Lipmann 1955);

(Segal 1956); (Robbins and Lipmann 1957); (Wilson and Bandurski 1956). The process consists of three reactions, catalyzed by the enzymes ATP-sulphurylase, APS-kinase and steroid sulphokinase respectively:-

$$ATP + SO_4 \rightleftharpoons APS + PP \quad (1)$$

$$APS \quad ATP \rightleftharpoons PAPS \quad (2)$$

$$PAPS + \text{steroid} \rightleftharpoons PAP + \text{steroid sulphate} \quad (3)$$

Nose and Lipmann (1958) by a combination of alumina gel absorption and ammonium sulphate fractionation showed that the sulphate transfer to phenols and steroids from PAPS was attributable to different sulphokinases. Further electrophoresis enabled them to separate the steroid sulphokinases into two fractions which reacted with DHA and oestrone respectively, and although they were not able to obtain further clear-cut separations, they did obtain indications that the DHA-fraction consisted of a number of fractions showing varying degrees of activity with DHA, pregnenolone and androsterone. This work suggests that there might exist a series of steroid sulphokinases, each showing a reasonably high degree of specificity. In addition it is interesting to note from the work of Adams (1963) that there might exist also a tissue specificity in that certain sulphokinases are present in a particular tissue whilst others are absent. Thus Adams found that human adrenal tissue conjugated oestrogens and androgens

as the sulphates but did not conjugate corticoids.

These observations offer exciting possibilities for further work on the possible physiological significance of steroid sulphates and it is hoped to investigate certain aspects of this problem in the near future.

APPENDIX 1

INVESTIGATION OF KC-6A FRACTION

The 27.8 mgs of KC-6A fraction was subjected to a "pipestem" leaching at 0°C from 0.4 mls.of chloroform, to give 13.4 mls.of pale, buff-coloured solid. After a second pipestem leaching at 0°C from 0.25 mls.of chloroform, 12.2 mgs.of whitish solid material was obtained.

For purposes of comparison with the parent material, in the Kober reaction and on chromatography in the system chloroform /formamide, 390 µgs.of this was reduced with sodium borohydride in methanol. In the Kober reaction, the unreduced material gave a normal Kober colour having an absorption maximum at 512 mµ, the intensity of the colour at this wavelength being 22.75% of that given by an equivalent weight of oestriol. The reduced material also gave a normal Kober colour having an absorption maximum at 512 mµ, but this showed an increase in intensity of 178% as compared with the unreduced material.

In the system chloroform/formamide, the parent material showed a single spot less polar than 16-epi-oestriol. The reduced material showed two spots; a main spot having the same mobility as the parent material plus a second very weak spot just less polar than oestriol. The latter suggests

that the material is contaminated slightly with KC-6B material.

In order to determine the quantity of formaldehyde liberated on treatment with alkali, 57.5 μgs. of the KC-6A solid residue was treated by the method used by Loke, Marrian and Watson (1959). It was found that this yielded 112% of the expected amount of formaldehyde. Chromatography in the system chloroform/formamide of the residue extracted from this reaction medium showed the presence of material of approximately the same polarity as oestrone.

The mother liquors from the two pipestem crystallizations of this KC-6A material were leached from the pipestems by hot methanol, and filtered into a small weighed tube, giving 13.7 mgs. of residue. Some of this was again reduced with sodium borohydride in methanol for comparison with the unreduced residue. On chromatography in the system chloroform/formamide the latter showed a faint spot less polar than 16-epi-oestriol. The intensity of this spot was only ∽ 10% of that expected from the weight of material applied to the paper. The reduced material showed a spot of similar intensity which was just less polar than oestriol, plus a very faint spot of the same polarity as the original material. From this it would appear that the mother liquors contain mainly KC-6B plus a slight trace of KC-6A and that there is only ∽ 10% of the gross weight present as KC-6B.

The results of this investigation are in complete agreement with the findings of Loke, Marrian and Watson (1959) and although this material has not been fully characterized it seems reasonably certain that it is in fact 18-hydroxyoestrone.

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PUBLICATIONS

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The Partial Synthesis of 6-Oxo-Oestriol and 6'α'-Hydroxyoestriol

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The Partial Synthesis of 6-Oxo-Oestriol and 6'α'-Hydroxyoestriol

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Mueller & Rumney (1957) showed that when [16-^{14}C]oestradiol-17β was incubated under aerobic conditions with mouse-liver microsomes and reduced triphosphopyridine nucleotide a number of radioactive metabolic products more 'polar' than oestradiol-17β were formed. One of these proved to be identical with 6-oxo-oestradiol-17β (Longwell & Wintersteiner, 1940); a second was shown to be identical with an authentic preparation of a 6-hydroxyoestradiol-17β supplied by Dr O. Wintersteiner which was described as '6β-hydroxyestradiol-17β'; while a third was identified as '6β-hydroxyestrone' since on reduction with sodium borohydride it yielded '6β-hydroxyestradiol-17β' and on oxidation it yielded 6-oxo-oestrone. Since the publication of this work Wintersteiner & Moore (1959) have given reasons for believing that the 6-hydroxyl group in the 6-hydroxyoestradiol-17β which is formed by metal-hydride reduction of 6-oxo-oestradiol-17β may have a quasi-equatorial configuration; accordingly these authors suggest that the reference compound supplied to Mueller & Rumney should be tentatively described as 6'α'-hydroxyoestradiol-17β. Breuer, Nocke & Knuppen (1958, 1959) have detected what are apparently the same 6-hydroxy oestrogen metabolites as were described by Mueller & Rumney among the products formed on incubating rat-liver slices with oestradiol-17β or oestrone.

No 6-oxo or 6-hydroxy oestrogen metabolites have yet been shown with certainty to occur in human urine, but unpublished and as yet incomplete work by K. H. Loke and G. F. Marrian has suggested that the Kober chromogen in the urine of pregnant women, which was designated KC-6B and which is similar in its chromatographic behaviour to 18-hydroxyoestrone in the Bush-type solvent systems used (Loke, Watson & Marrian, 1957; Loke, Marrian & Watson, 1959), may be a 6-hydroxyoestrone. The possibility that human urine might contain 6-oxo and 6-hydroxy derivatives of oestriol was therefore considered. Accordingly, as a preliminary to examining the urine of pregnant women for these compounds, 6-oxo-oestriol and 6'α'-hydroxyoestriol (terminology of Wintersteiner & Moore, 1959) were prepared from oestriol by the methods used for the preparation of 6-oxo-oestradiol-17β and 6'α'-hydroxyoestradiol-17β from oestradiol-17β by Longwell & Wintersteiner (1940) and Wintersteiner & Moore (1959).

EXPERIMENTAL AND RESULTS

Methods

Melting points of acetates were determined on a microscope hot-stage: those of the unacetylated compounds were determined in sealed evacuated capillaries. All melting points were determined on the same thermometer and are uncorrected for emergent stem.

Samples for C and H analysis, optical-rotation determinations and determinations of ultraviolet spectra were dried to constant weight at 100° *in vacuo*.

Optical rotations were determined in a 0·5 dm. tube with a diameter of 2·5 mm.

6-*Oxo-oestriol triacetate*

Crude oestriol (1·96 g.), prepared from late-pregnancy urine, was acetylated with acetic anhydride and anhydrous pyridine at room temperature for 24 hr., and the product recrystallized once from hexane. The recrystallized oestriol triacetate (1·87 g.) was dissolved in 6·5 ml. of acetic acid, and to this solution was added 1·7 g. of CrO_3 dissolved in 9·7 ml. of acetic acid and 1·4 ml. of water. After standing at room temperature for 24 hr. the excess of CrO_3 was reduced by the addition of 2 ml. of ethanol, and, after dilution with 200 ml. of water, the mixture was extracted with ether. The extract was washed with 50 ml. portions of saturated aq. $NaHCO_3$ until the washings showed a pink colour, then with 50 ml. of aq. 5 % (w/v) Na_2CO_3 saturated with $NaHCO_3$, and finally with 50 ml. of water. After being dried over Na_2SO_4 the ether solution of the non-acidic oxidation products was evaporated to dryness.

By treatment of this material (1·00 g.) with trimethylammonium hydrazide chloride in ethanolic acetic acid overnight at room temperature the ketonic fraction was obtained in the usual way. After reacetylation of the product, decolorization with charcoal in methanolic solution and recrystallization from methanol, 0·24 g. of crystalline material, m.p. 135–138°, was obtained.

This product was combined with 0·49 g. of similarly prepared material, and after crystallization again from methanol 0·57 g. of crystals, m.p.

137–139°, $[\alpha]_D^{16.5} - 41°$ in ethanol (*c*, 0·493), was obtained (Found: C, 67·3; H, 6·5. Calc. for $C_{24}H_{28}O_7$: C, 67·3; H, 6·6 %).

The ultraviolet-absorption spectrum in ethanolic solution showed maxima at 249 mμ (ε 9150) and 298 mμ (ε 2260) and closely resembled that given for 6-oxo-oestradiol-17β diacetate by Longwell & Wintersteiner (1940).

6-Oxo-oestriol

6-Oxo-oestriol triacetate (110 mg.) was hydrolysed with 2·0 ml. of 20 % (w/v) KOH in methanol for 24 hr. at room temperature in an atmosphere of N_2. After dilution with 30 ml. of water the solution was acidified to phenolphthalein with CO_2 and extracted three times with 10 ml. portions of ethyl acetate. After two washings with 5 ml. portions of water the extract was dried over Na_2SO_4 and evaporated to dryness. Crystallization of the product twice from methanol–benzene yielded 39 mg. of crystals, m.p. 240–242° (slight yellowing) and $[\alpha]_D^{16} - 7°$ in ethanol (*c*, 0·504) (Found: C, 71·5; H, 7·1. Calc. for $C_{18}H_{22}O_4$: C, 71·5; H, 7·3 %).

The ultraviolet-absorption spectrum in ethanolic solution showed maxima at 257 mμ (ε 7895) and 325 mμ (ε 3052) and closely resembled that given for 6-oxo-oestradiol-17β by Longwell & Wintersteiner (1940).

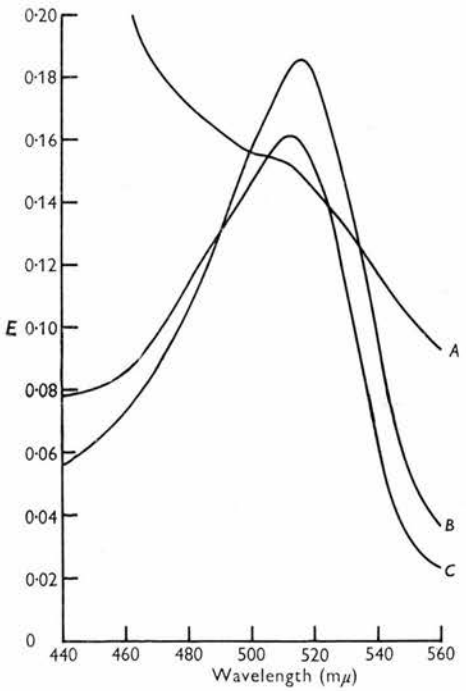

Fig. 1. Kober colour reactions on: *A*, 60·0 μg. of 6-oxo-oestriol; *B*, 4·03 μg. of 6'α'-hydroxyoestriol; *C*, 4·06 μg. of oestriol. Final vol. of reaction mixture, 3·3 ml.

The Kober reaction carried out by the method of Brown (1952) and Bauld (1954), with the former's 'oestriol reagent', was essentially negative (Fig. 1*A*).

6'α'-Hydroxyoestriol

A solution of 84·5 mg. of 6-oxo-oestriol in 8·0 ml. of methanol was added dropwise to a solution of 70·7 mg. of $NaBH_4$ in 6·5 ml. of methanol. After standing for 2 hr. at room temperature the solution was acidified to litmus with 10 % (w/v) aq. acetic acid and evaporated nearly to dryness at room temperature under an air stream. To the residue was added 3 ml. of water; the suspended crystals were filtered off with suction, washed with water and dried *in vacuo*.

Some difficulty was experienced in crystallizing the product, but eventually it was found that by evaporating a solution in 50 % (v/v) aq. methanol slowly to just under half the original volume well-formed needles separated on cooling at room temperature. These were filtered off with suction, washed with water and dried *in vacuo*. The recrystallized product had m.p. 242–245° with evolution of gas (change of form at 140–150°) and $[\alpha]_D^{14} + 84°$ in ethanol (*c*, 0·498) (Found: C, 71·4; H, 7·6. Calc. for $C_{18}H_{24}O_4$: C, 71·0; H, 7·9 %).

The ultraviolet-absorption spectrum in ethanolic solution showed a single maximum at 282 mμ (ε 2415) and closely resembled those of oestradiol-17β, oestrone and oestriol.

In the Kober reaction the spectral characteristics of the colour produced were normal (Fig. 1, *B*), but the extinction at the absorption maximum was only 63 % of that given by the same weight of oestriol under the same conditions.

6'α'-Hydroxyoestriol tetra-acetate

About 40 mg. of recrystallized 6'α'-hydroxyoestriol (m.p. 244–248°) was acetylated in the usual way with acetic anhydride and pyridine at room temperature for 24 hr. The crude acetate was purified by filtering in benzene solution through a short column of alumina (acid-washed; activated at 100°) and crystallizing twice from methanol. The product had m.p. 159–161° and $[\alpha]_D^{16} - 12°$ in ethanol (*c*, 0·506) (Found: C, 66·2; H, 6·8. Calc. for $C_{26}H_{32}O_8$: C, 66·1; H, 6·8 %).

Partition of oestriol, 6-oxo-oestriol and 6'α'-hydroxyoestriol between organic solvents and water

A chance observation made when 6-oxo-oestriol was first prepared by hydrolysis of its acetate suggested that this compound might be much less readily extracted from aqueous solution by ether than oestriol. In view of this the partition of oestriol, 6-oxo-oestriol and 6'α'-hydroxyoestriol in the systems ether–water and ethyl acetate–water

Table 1. *Partition of oestriol, 6-oxo-oestriol and 6'α'-hydroxyoestriol in the systems ether–water and ethyl acetate–water*

$$K = \left(\frac{\text{concn. in organic phase}}{\text{concn. in aqueous phase}}\right)$$

	Ether–water	Ethyl acetate–water
Oestriol	7·7	24
6-Oxo-oestriol	0·66	5·6
6'α'-Hydroxyoestriol	0·12	0·90

was determined quantitatively in the following way:

The steroid (100–300 μg.) spread in a thin film on the inside of a flask was shaken at 25° with 10 ml. of each phase (pre-equilibrated) until all had dissolved. The mixture was then transferred to a separating funnel and, after separation of the phases, 6·0 ml. of each was removed and evaporated to dryness in a vacuum desiccator. Each residue was dissolved in 3·0 ml. of ethanol and the concentration of steroid determined from the extinction of the solution at 281 mμ (oestriol and 6'α'-hydroxyoestriol) or 325 mμ (6-oxo-oestriol). The results (Table 1) show that the substitution of either an oxo or an 'α'-hydroxyl group at C-6 in oestriol has a surprisingly great effect in increasing the relative solubility in water.

DISCUSSION

Although neither 6-oxo-oestriol nor 6'α'-hydroxyoestriol has yet been shown to be present in the urine of pregnant women it is by no means improbable that they, or other highly oxygenated oestrogen derivatives with similar distribution characteristics in the systems ether–water and ethyl acetate–water, may be metabolic products of the oestrogenic hormone. In studies on the metabolism of radioactive oestrogens *in vivo* or *in vitro* it may therefore be dangerous to draw any conclusions about the nature of the metabolites formed (whether 'free', conjugated, or degraded to acidic substances) on the basis of the extractability of radioactive products from aqueous media by arbitrarily fixed proportions of ether or of other organic solvents (cf. Beer & Gallagher, 1955; Valcourt, Thayer, Doisy, Elliott & Doisy, 1955; Jellinck, 1959).

SUMMARY

1. 6-Oxo-oestriol, 6'α'-hydroxyoestriol and their acetates have been prepared from oestriol by methods based on those of Longwell & Wintersteiner (1940) and Wintersteiner & Moore (1959) for the preparation of 6-oxo-oestradiol-17β and 6'α'-hydroxyoestradiol-17β from oestradiol-17β.

2. Determinations of the distribution of 6-oxo-oestriol, 6'α'-hydroxyoestriol and oestriol in the systems ether–water and ethyl acetate–water showed that the substitution of an oxo or hydroxy group at C-6 in the oestriol molecule has a very great effect in increasing the relative solubility of these derivatives in water.

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Sulphation of Oestrone *in vitro* by Bovine Adrenal Tissue

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In a preliminary paper on the isolation of 18-hydroxyoestrone from the urine of pregnant women, Loke, Watson & Marrian (1957) referred to experiments which suggested that this oestrone metabolite was formed in small yield on incubating oestrone with bovine-adrenal homogenates. Subsequent work by Loke (1958), in which attempts were made to isolate 18-hydroxyoestrone from such homogenates, gave inconclusive results. Accordingly it was decided to reinvestigate the metabolism *in vitro* of oestrone by bovine-adrenal preparations by using [16-^{14}C]oestrone as substrate.

In preliminary experiments in which [16-^{14}C]-oestrone was incubated with coarse minces of fresh bovine adrenocortical tissue, the recovery of the added radioactivity in ether or ethyl acetate extracts of the partially worked up incubation mixtures was very variable and sometimes as low as about 30%. Further investigation showed that the greater part of the unaccounted-for radioactivity could be extracted from aqueous media by butan-1-ol or, more conveniently, by ethyl acetate after saturation of the aqueous phase with sodium chloride. Subsequently this oestrone metabolite was fully characterized as oestrone sulphate.

Purdy, Engel & Oncley (1961) have shown that, after the administration of ^{14}C-labelled oestradiol to non-pregnant women, the radioactivity in the plasma was largely present in oestrone sulphate. They also found that in the plasma of pregnant women the molar concentration of oestrone sulphate was about four times that of unconjugated oestrone. On the basis of these findings they concluded that 'estrone sulphate is an important circulating estrogen in the human'. The present finding that the bovine adrenal cortex which, like the adrenal cortex in some other species, may be an oestrogen-secreting organ, can conjugate oestrone with sulphate is therefore of interest.

EXPERIMENTAL AND RESULTS

Materials

The Celite 535 (Johns Manville and Co. Ltd., London) used for column chromatography was purified by standing overnight in conc. HCl, washing with water until the washings were free from Cl^- ions and drying at 110°. The Al_2O_3 (Savory and Moore Ltd., London) used in column chromatography was activated by heating at 100° under reduced pressure and then deactivated by the addition of 3% (by wt.) of water. Sephadex-G 25 was purchased from Pharmacia, Uppsala, Sweden. Unwashed Whatman no. 1 paper was used for paper chromatography.

Oestrone was obtained from Schering A.-G., Berlin, Germany. In the incubation experiments it was used without further purification. For the reverse isotope-dilution experiment it was recrystallized twice from ethanol. [16-^{14}C]Oestrone of activity 12·1 mc/mg. was purchased from The Radiochemical Centre, Amersham, Bucks., and was purified on a Celite column, with methanol–water (7:3, v/v) as stationary phase and benzene–hexane (1:1, v/v) as mobile phase. Sodium oestrone sulphate was obtained from the Medical Research Council's Steroid Reference Collection through the courtesy of Professor W. Klyne (Westfield College). It was purified by passage through a Celite–Al_2O_3 column by the method of Purdy *et al.* (1961).

All solvents were purified before use by distillation.

Incubation procedure

All incubations were carried out in 50 ml. portions of a modified Ca^{2+} ion-free Krebs–Ringer phosphate buffer having the following composition (final concentrations in parentheses): NaCl (96·0 mM); KCl (4·6 mM); KH_2PO_4 (1·16 mM); $MgSO_4$ (1·16 mM); $NaHCO_3$ (3·48 mM); sodium fumarate (8·21 mM); sodium L-glutamate (4·77 mM); glucose (11·2 mM); nicotinamide (5·92 mM); Na_2HPO_4 (10·8 mM); NaH_2PO_4 (2·7 mM). Before use the incubation medium was placed in a water bath at 37° and was gassed for 1 hr. with O_2+CO_2 (95:5).

Pairs of adrenal glands, obtained from animals within a few minutes after slaughter, were transported to the Laboratory in a chilled container, dissected free from fat and cut open longitudinally. The medullae were removed as completely as possible, and the cortical tissue was minced in a chilled Latapie mincer. For each incubation 5 g. of mince was used.

To each incubation mixture was added 500 μg. of oestrone in 0·5 ml. of ethanol and 0·04 ml. of a standard solution of [16-^{14}C]oestrone in ethanol corresponding to an accurately known rate of about $1·5 \times 10^5$ counts/min.; the incubations were carried out for 1 hr. at 37° in an atmosphere of O_2+CO_2 (95:5).

All incubations were commenced within 1 hr. of the death of the animal. Preliminary experiments, not reported in detail, showed that the conjugation of oestrone was greatly diminished when adrenals were used which had been left at room temperature for a few hours and then stored overnight at −20°. Presumably this was due to destruction of ATP which is involved in the synthesis of 'active sulphate' (Robbins & Lipmann, 1956).

Extraction and fractionation of incubation mixtures

Each incubation was terminated by the addition of 117 ml. of methanol so as to give a methanol concentration of 70% (v/v). After vigorous shaking the mixture was centrifuged for 10 min. and the supernatant decanted off. The residue was re-extracted with 167 ml. of aq. 70% (v/v) methanol, and the combined supernatants were washed four times with 0·5 vol. of hexane.

The aqueous-methanol phase was evaporated under reduced pressure to remove all methanol, and the aqueous residue diluted with an equal volume of water and extracted twice with equal volumes of ether. The ether extract was washed twice with small volumes of water, dried over Na_2SO_4 and evaporated to dryness.

The aqueous phase and washings from the ether extraction were combined and extracted twice with equal volumes of ethyl acetate. The extract was evaporated to dryness after washing with water and drying over Na_2SO_4.

The aqueous phase and washings from the ethyl acetate extraction were combined, saturated with NaCl and extracted twice with equal volumes of ethyl acetate. The extract was dried over Na_2SO_4 without previous washing, evaporated to dryness, immediately redissolved in methanol to which a few drops of aqueous ammonia had been added and the solution stored at 0°.

In each experiment suitable samples of the ether extract, of the ethyl acetate extract and of the ethyl acetate extract after saturation with NaCl were plated, and the radioactivity was determined at infinite thinness by using the Nuclear–Chicago Autochange gas-flow counter with a thin end-window.

The results of six such experiments carried out in duplicate on the adrenals of four 6-year-old cows and two young steers are shown in Table 1. In each case extraction with ethyl acetate after the initial ether extraction removed only small additional proportions of the added radioactivity. However, further extraction with ethyl acetate after saturation of the aqueous phase with sodium chloride removed the greater part of the remaining radioactivity; at this stage of the work the assumption was made that this fraction of the radioactivity represented an oestrogen-conjugate fraction.

Table 1. *Incubation of bovine-adrenocortical-minces with* [16-^{14}C]*oestrone and the extractability of the radioactive metabolic products from aqueous media*

Experimental details are given in the text.

	Percentage of added radioactivity extracted		
Animal	By ether	By ethyl acetate	By ethyl acetate after addition of NaCl
Cow	81·7	1·5	20·1
	77·5	5·0	12·8
Cow	77·5	0·9	14·7
	73·7	2·3	20·4
Cow	67·0	4·2	18·8
	94·5	2·7	14·3
Cow	42·8	0·7	44·7
	48·6	1·1	46·1
Steer	77·5	1·5	14·3
	79·6	1·4	11·8
Steer	77·5	1·5	25·6
	71·8	1·9	31·6

Purification of the oestrogen-conjugate fraction

The combined oestrogen-conjugate fraction from the above experiments, having a total radioactivity of $2·927 \times 10^5$ counts/min., was chromatographed on a Celite–Al_2O_3 column as described by Purdy *et al.* (1961) for the separation of oestrogen sulphates, glucosiduronates and phosphates. The sulphate fraction contained 94·5% of the radioactivity applied to the column.

An attempt to characterize the conjugate at this stage by paper chromatography failed because of heavy contamination with impurities that caused the spots to spread during the equilibration period and to streak on running.

Further purification was effected by partition between butan-1-ol and N-NaOH (cf. Schachter & Marrian, 1936, 1938), passage through an Al_2O_3 column in methanolic solution, and finally by fractionation on a column of Sephadex-G 25 with water as eluent as described by Beling (1961). In the last-named procedure 94·5% of the radioactivity applied to the column was eluted in a narrow band just behind a band of cloudy opalescent material. The oestrogen-conjugate fraction was recovered from the aqueous eluate from the column by extraction with ethyl acetate after saturation with NaCl and contained radioactivity equivalent to $1·91 \times 10^5$ counts/min. or 65% of that in the combined crude oestrogen-conjugate fraction.

Provisional identification of the oestrogen-conjugate fraction as oestrone sulphate by paper chromatography

Portions of the purified oestrogen-conjugate fraction, each containing radioactivity equivalent to $0·4 \times 10^3$ counts/min., were chromatographed on paper at 25° with the aq. ammonia (sp.gr. 0·88)–ethyl acetate–butan-1-ol system of Schneider & Lewbart (1956), referred to below as system 1, and the aq. ammonia (sp.gr. 0·88)–dibutyl ether–2-methylpropan-2-ol system of Schneider & Lewbart (1959), referred to below as system 2. On each paper authentic oestrone sulphate (100 μg.) was run as a standard.

Examination of paper chromatograms for radioactive areas on the Nuclear–Chicago Actigraph Chromatogram Scanner by using a gas-flow detector with a thin end-window showed that in the channels on which the oestrogen-conjugate fraction had been run there were main peaks of radioactivity having R_F values of 0·42 and 0·14 in systems 1 and 2 respectively, and lesser peaks having R_F values of about 0·90 and 0·80 respectively in these two systems.

These lesser peaks with the high R_F values were almost certainly due to oestrone liberated from the conjugate by hydrolysis before or during chromatography, since components with nearly identical

R_F values in the two systems respectively, which gave positive reactions with the Zimmermann reagent, positive reactions with the Folin–Ciocalteu reagent and negative tests for SO_4^{2-} ions after solvolysis *in situ* (see below), were detected on chromatograms of both the oestrogen-conjugate fraction and the authentic oestrone sulphate.

On staining chromatograms with the Zimmermann reagent by the method of Bush (1961) a main ketonic component with R_F values of 0·37 and 0·14 in systems 1 and 2 respectively was detected in the oestrogen-conjugate fraction. The corresponding R_F values for the main ketonic component in the authentic oestrone sulphate were 0·37 and 0·15.

On staining with the Folin–Ciocalteu reagent by the method of Mitchell & Davies (1954) no phenolic components could be detected in either the oestrogen-conjugate fraction or the authentic oestrone sulphate other than that believed to be free oestrone (see above). However, after solvolysis *in situ* by the dioxan–hydrochloric acid method of Schneider & Lewbart (1956), a phenolic component with R_F values of 0·38 and 0·14 in systems 1 and 2 respectively was detected in the oestrogen-conjugate fraction, and one with R_F values of 0·38 and 0·12 in the same two systems in the authentic oestrone sulphate.

After solvolysis *in situ* (see above) and by using the rhodozonic acid test for SO_4^{2-} ions as described by Schneider & Lewbart (1956), yellow spots with R_F values of 0·38 and 0·17 in systems 1 and 2 respectively appeared on the oestrogen-conjugate channels and ones with R_F values of 0·44 and 0·19 in the same two systems in the oestrone sulphate channels. These R_F values do not agree very well with those found in the other tests described above, although there was reasonably good agreement between the values for the oestrogen conjugate and the authentic oestrone sulphate in both systems. The reason for poor agreement of these R_F values with those obtained by other tests became apparent when it was found that SO_4^{2-} ions applied to a chromatogram paper spread and migrated downwards during a dummy solvolysis procedure sufficient to displace the centre of the rhodozonic acid-stained spot by as much as 2 cm. In view of this, and since, as described above, the oestrogen conjugate was eluted from a Celite–alumina column in the oestrogen sulphate fraction, there can be no doubt that the conjugate is a sulphate.

Identification of the oestrogen in the conjugate as oestrone

The paper-chromatographic tests described above provided strong evidence that the oestrogen conjugate is oestrone sulphate. However, since the sulphates of different ketonic oestrogens might not necessarily have greatly differing R_F values in the two solvent systems employed, confirmatory evidence was sought for the presence of oestrone.

The main bulk of the oestrogen-conjugate fraction was solvolysed by the method of Burstein & Lieberman (1958) as follows: After dissolution of the fraction in 10 ml. of water, 2 g. of NaCl and 0·06 ml. of 16 N-H_2SO_4 were added and the solution was extracted with 10 ml. of ethyl acetate. The extract was maintained at 37° for 3 hr., washed once with 5 ml. of aq. 5% (w/v) $NaHCO_3$, twice with 3 ml. of water, dried over Na_2SO_4 and evaporated to dryness.

One-twentieth of the dried material was chromatographed on paper at 25° in 'system B 5' of Bush (1952) with oestrone, oestradiol-17β, 16-oxo-oestradiol-17β and 6α-hydroxyoestrone as standards. The R_F values were as follows: unknown, 0·93; oestrone, 0·90 and 0·91; oestradiol-17β, 0·81 and 0·79; 16-oxo-oestradiol-17β, 0·65 and 0·62; 6α-hydroxyoestrone, 0·45 and 0·42.

To the remainder of the solvolysed material was added authentic oestrone (209·7 mg.), and the mixture was crystallized three times from ethanol. The yield after the third crystallization was 100·6 mg. After each crystallization portions of the material were plated and the radioactivity was determined in the Nuclear–Chicago Autochanger gas-flow counter. The counts/min./mg. after the three crystallizations were respectively 278, 308 and 263.

The thrice-crystallized material was then acetylated in the usual way with acetic anhydride and pyridine, and the crude acetate weighing 115·9 mg. was crystallized three times from hexane, yielding 40·1 mg. of final product. The counts/min./mg. after the three crystallizations were respectively 224, 245 and 256.

The paper-chromatographic evidence and that from the reverse isotope-dilution experiments shows conclusively that the oestrogen in the conjugate was indeed oestrone.

SUMMARY

1. Incubation of bovine-adrenocortical minces under aerobic conditions with [16-^{14}C]oestrone gave variable yields of a water-soluble radioactive oestrone metabolite, not extractable from aqueous solution by ether or by ethyl acetate, but extractable by the latter after saturation of the aqueous phase with sodium chloride, or by butan-1-ol.

2. On chromatography on Celite–alumina columns by the method of Purdy *et al.* (1961) the metabolite was eluted in the oestrogen sulphate fraction.

3. By paper chromatography in two systems devised by Schneider & Lewbart (1956, 1959) for

steroid sulphates, and by using tests for keto groups, free phenolic groups and SO_4^{2-} ions before and after solvolysis *in situ*, the reactions and R_F values of the metabolite were found to resemble closely those of authentic oestrone sulphate.

4. Solvolysis of the oestrone metabolite gave a product which was identified as oestrone by paper chromatography in the 'system B 5' of Bush (1952) and by reverse isotope dilution.

5. The above evidence shows that the oestrone metabolite was oestrone sulphate.

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