THE CHEMICAL DETERMINATION OF OESTROGENS IN URINE

by

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I. GENERAL INTRODUCTION

I. GENERAL INTRODUCTION

The chemical determination of oestrogens in the urine of human subjects involves the separate problems of hydrolysis of the conjugated forms, purification and separation of oestriol, oestrone and oestradiol-17β, and the estimation of the purified substances by chemical or physico-chemical means. Previous studies carried out in this Department (Stevenson and Marrian, 1947; Marrian, 1948; Clayton, 1949) resulted in accurate procedures for the acid hydrolysis of the oestrogen conjugates, the initial extraction of the free oestrogens, and the separation of oestriol from the oestrone-oestradiol-17β fraction. In addition their results gave a clear indication of the difficulties which arise in connection with the losses of oestrogen, and interference with production of colour in the Kober reaction in the presence of urinary and solvent contaminants. The investigations reported in this thesis are a direct continuation of this earlier work.

Initially attention was directed to the development of procedures for the partial purification of the urinary extracts. This was facilitated by the classification/

classification of possible urinary contaminants into broad general groups. For example it was to be expected that oestriol, obtained by water-washing a benzene solution of the bicarbonate-washed ether extract, would be contaminated by a group of hydrophilic substances. That these would include compounds of acidic, phenolic, neutral, and basic reaction, was to be anticipated, and was, in fact, demonstrated by qualitative tests. In this way simple procedures were readily devised for removing large proportions of these contaminants without loss of oestriol.

For the final purification of the oestrogen fractions, and for the separation of oestrone and oestradiol, it became clear early in the course of the investigation that it would be most convenient to employ an application of the differential counter-current principle. The same conclusion was reached by all other groups engaged on the problem and four different applications have been used. Since no comparative studies of the different methods of separation of oestrogens have as yet been carried out, it is impossible to be dogmatic as to which is the best procedure. In any event it is inevitable that there will be a wide degree of individual preference based on such criteria as the previous experience of the particular operator, convenience of operation, and the/

the facilities available. It is considered that a general discussion of the advantages and disadvantages, inherent in the various applications, would serve a useful purpose in relating the methods developed in this study to those of other investigators.

Stimmel (1946) used adsorption chromatography on alumina for the separation and partial purification of oestrone, oestradiol-17β,and oestriol. The method described was characterized by convenience of use, but a definite loss of oestriol occurred and inadequate purification was achieved. Moreover, other workers have been unable to confirm his results (cf. Swyer, 1952), possibly because of variations in alumina (cf. Bates, 1952). Engel (1950) gave a very clear exposition of the theoretical and practical limitations to the use of adsorption chromatography in quantitative work. On the other hand, Brown (1952c), using deactivated alumina and moist eluants, successfully separated and purified the oestrogens in urinary extracts after methylation. The zero elution zone of 3-6 ml. between the methyl ethers of oestrone and oestradiol-17β was not as large as might be desired and 'the exact details of elution of the column depended upon the activity of the alumina'. The advantages accruing from the ease of preparation of the columns were to some extent offset by the incorporation/

incorporation of the methylation procedure. It is possible that the practice of applying the water-washed light petroleum and benzene extracts directly to the column may necessitate careful temperature control at this stage of the process. The method has subsequently been considerably improved, and has been used for some months now with satisfactory results.

Engel and his co-workers (1950) employed counter-current liquid-liquid extraction trains, operating discontinuously, for the separation of oestradiol, oestrone, and oestriol. From the standpoints of reproducibility and accuracy, the method is unquestionably superior to all other procedures as is evident from the agreement of observed with theoretical findings, and from the fact that the reported results have been completely confirmed (Diczfalusy, 1953). The elaborate apparatus required and the labour involved in the combination of the various fractions may, however, preclude the adoption of the method for routine assays. Moreover, complete separation of the oestrogens from urinary contaminants has not been achieved (cf. Slaunwhite et al., 1953). Migeon (1953), by carrying out further redistributions, has been able to obtain complete purification of the separated oestrogens but, in so doing, has markedly lengthened the procedure.

Paper/

Paper partition chromatography has been applied to the problem of separation and purification of the natural oestrogens in extracts of urine and tissue (Heftman, 1950; Mitchell, 1952; Axelrod, 1953). The writer's experience with this technique has been limited to qualitative work in connection with selection of suitable solvent systems, so that it is not possible for him to give a fair assessment of the method. It is probable that the choice between partition chromatography on paper and on columns will largely be a matter of personal preference. The inconvenience of elution of the solutes from the paper is offset by the convenience of development of the chromatogram. It is possible that this will be the method of choice for the separation of quantities less than a few micrograms.

In the present investigation partition chromatograms, with Celite 535 as the inert support, have been used for the separation and purification of oestrone and oestradiol-17β. Oestriol is separated by partition between benzene and water in separating funnels and finally purified by a partition chromatogram. The effects of variations in the chromatographic procedure have been investigated in some detail/

detail and it is claimed that when these are carefully controlled the method is entirely satisfactory from the standpoints of accuracy and reproducibility.

It is interesting to compare the results reported here with those obtained simultaneously but independently by Braunsberg and her co-workers (Swyer and Braunsberg, 1951; Braunsberg, Stern and Swyer, 1952). The same solvent system (benzene-aqueous sodium hydroxide) was used in both cases but in the present investigation the dimensions of the column, amount of stationary phase, and percolation rate of the mobile phase, were adjusted to give an R value for the fastest moving oestrogen (oestrone) suitable for the separation of this substance from a group of neutral and basic contaminants in urinary extracts. Under these conditions the retention volume of oestradiol-17β was inconveniently large and the elution of oestriol from the column was rendered virtually impossible. In view of the satisfactory purification of oestrone, however, the conditions for the chromatogram were adopted. Oestradiol-17β was removed by the technique of fractional elution, and oestriol was separated from the other two by a preliminary partition into water from benzene in separating funnels, as was advised by Clayton (1949). This procedure gave complete separation of all three oestrogens, a zero elution zone/

zone of at least 20 ml. between oestrone and oestradiol-17β, and maximum efficiency in the chromatographic purification. Ten hours were required for the separation but the chromatograms required no further attention after the change of solvent at the fourth hour.

It was found, however, that an additional chromatogram was required for the final purification of the separated oestriol and obviously, therefore, the method would be greatly simplified if all three oestrogens were separated and purified on the one chromatogram as recommended by Braunsberg and her co-workers (1952). In this method the phenolic fraction of urine was leached with small quantities of mobile phase (benzene phase) and applied to the column. Oestrone and oestradiol-17β were successively eluted with this mobile phase. The residual urine fraction (containing most of the oestriol) was then transferred to the column with carbon tetrachloride-butanol and oestriol was eluted with this solvent. The procedure is obviously simple but also unlikely to be very efficient. There is likely to be incomplete separation of oestrone from the group of nitrogenous impurities of the phenolic fraction and the zero elution/

elution zone between oestrone and oestradiol is small. Some of the oestriol is added to the column in the initial benzene leaching and the remaining portion is applied to the chromatogram at a time when its characteristics are rapidly changing due to the change of the mobile phase. One would therefore expect that all fractions would be contaminated by urinary components and that this is so has been demonstrated by Mitchell (1953, personal communication). Bitman and Sykes (1953) noted gross contamination of the oestriol fraction and reported that it was lessened by the substitution of displacement analysis for the fractional elution technique. In their modification all three oestrogens are applied to the column by leaching with benzene, and, after the elution of oestrone and oestradiol-17β with benzene, the pH of the stationary phase is decreased by blowing CO_2 into the bottom of the column. It is obvious that this procedure will disrupt the bands on the column and cause gross irregularities in the subsequent elution of oestriol with benzene. The procedure is also open to the criticism that benzene in the amounts used would not be effective in the quantitative transference of oestriol to the column. It is the writer's belief that/

that the wide difference between the distribution of oestriol and that of the other two oestrogens in the usual solvent systems prevents their separation and purification on one partition chromatogram.

In this investigation the purified oestrogens have been estimated by the Kober reaction. It was felt that the specificity of the pink colour developed with oestrogens, which permitted the complete differentiation from non-specific residual urinary or solvent impurities by means of correction formulae, was a distinct advantage over the more sensitive but less specific fluorescence reaction. Moreover, it was hoped that the previous extensive investigations of this reaction would permit the choice of conditions of maximal stability.

Throughout this study difficulties have been encountered at every stage in repeating the work of previous investigators. This was most pronounced in connection with the colour reaction. As is evident from the literature, the Kober reaction has been extensively modified since it was originally described in 1931. In fact in almost every case when an investigator began work on the quantitative estimation of oestrogens, the first step was a modification of the colour reaction (cf. Cohen and Marrian, 1934; Venning/

Venning et al., 1937; Bachman, 1939a; Szego and Samuels, 1940, 1943; Brown, 1952b). It is possible that the development of the numerous modifications was due to the lack of detail in the published reports or to the incomplete investigation of the conditions necessary for maximal production of colour in each case. The present investigation has been concerned mainly with the hydroquinone-aqueous sulphuric acid method of Brown (1952b). It has been found that this reaction is influenced by the diameter of the tube used, by the stirring technique, by the degree of illumination present within the laboratory, and by the type of analytical reagent grade sulphuric acid used. Moreover, it has been demonstrated that the reagent recommended for the determination of oestriol is unsatisfactory in the presence of solvent residues. If the existence of these factors had been appreciated and mentioned in the published reports, a great deal of time would have been saved in the present study.

At an early stage of the investigation of the chromatographic procedures, inconsistencies in the elution patterns began to appear. For one trained in adsorption chromatography, it came as a distinct surprise to find the profound effect on elution patterns/

patterns of minor changes in both the pH and the amount of stationary phase, of variations in rates of flow of the mobile phase, and in particle size and method of packing. Failure to realize the importance of these variables at the outset of the investigation has resulted in very considerable delays. It is hoped that conditions have now been sufficiently well standardized to permit other workers to duplicate the results reported here.

The final, and perhaps the major, source of difficulty in the development of the complete method has been the relative ease with which oestrogens can be altered by mild treatment with urinary or solvent contaminants. The investigation of this factor was not possible until the other difficulties were eliminated. It was found that recoveries of oestrogens, added to aqueous mineral acid or to hydrolysed urine, were often very irregular even with the use of chromatograms whose efficiency was proven by the demonstration of zero elution zones and quantitative recovery, and with the use of a colour reaction proven to be unaltered by the presence of urinary or solvent residues. The losses encountered were on occasion as high as 40-60% and thus could not be entirely due to manipulative errors. When one considers/

considers that measurements are being made at dilutions of one in a hundred million, it is clear that destruction of oestrogen, almost infinitesimal from the absolute standpoint, can assume very significant proportions under these conditions. Methods of oestrogen assay at the present time are operating at a level of dilution where the slight browning often noticed by the earlier workers during isolation studies in transferring processes, can invalidate an assay.

It has been found that oestriol can be heated in a stream of air with aldehyde-containing ethanol without loss. In the presence of the residue obtained from the distillation of peroxide-free (vanadium pentoxide) ether, destruction occurs unless the ethanol is aldehyde free. This type of reaction, which appears to be oxidative is the main potential source of error in oestrogen assays. Carrying out all distillations in an atmosphere of nitrogen has been found to be ineffective. To obviate the danger, the incorporation of an effective anti-oxidant is required. Hydroquinone has been used for this purpose and in addition all solvents have been thoroughly purified.

II. COLORIMETRIC ESTIMATION OF OESTRONE, OESTRADIOL-17β, AND OESTRIOL.

A. Survey of Previous Literature.

1. Kober Reaction and its Modifications.

In 1931 Kober found that heating oestrone with concentrated sulphuric acid gave a yellow compound with greenish fluorescence, which on subsequent dilution with water and reheating gave rise to a pink colour. The Kober reaction is thus a two-stage reaction in which the first stage is the formation of a yellow colour on heating with concentrated sulphuric acid, the second stage the conversion of yellow to pink on heating with diluted sulphuric acid.

Kober also noted that the pink colour could be discharged by neutralization or oxidation. He originally employed phenol to quench the fluorescence of the final product in order to facilitate visual colorimetry.

Cohen and Marrian (1934) adapted Kober's findings to the development of a quantitative method of estimation. They found that maximum intensity of final colour was obtained by a reagent consisting of 3.6 parts of phenol to 5.6 parts of sulphuric acid (by volume). Introduction of 5% of water in the reagent decreased the intensity markedly and also increased the time of heating required to reach maximum intensity. They also found that their conditions for the second-

stage/

stage heating found to be optimal for pure oestrogens caused fading of the final product in the presence of solvent and urine residues. It is highly significant to the results of the present investigation that this defect was corrected by raising the sulphuric acid concentration of the second stage.

Cartland et al. (1935) heated phenol with sulphuric acid and diluted the resulting phenolsulphonic acid with 2 volumes of sulphuric acid. After the second heating stage the solution was further diluted before colorimetry. The authors do not state whether any change occurs in this step. Pincus and co-workers (1936) also used a phenolsulphonic acid reagent and noted marked variation in the intensity obtained when using different reagents, as well as the occasional appearance of turbidity in the final coloured product.

With the introduction of a suitable photoelectric colorimeter (Evelyn, 1936), Venning and her co-workers (1937) investigated the Kober reaction in detail. They used a reagent prepared by mixing phenol and sulphuric acid in the amounts found to be optimal by Cohen and Marrian (1934), the utmost care being taken during admixture to prevent rise in temperature. This reagent then, at least when freshly made up, contained a considerable amount of free phenol. Optimal conditions were established for the first and second heating stages/

stages, and dilution to a low sulphuric acid concentration prior to colorimetry was recommended. The authors stressed the necessity for rigid control of heating and cooling times and for stirring. No mention was made of the necessity for selection of reagents, but Venning subsequently (1952) pointed out that the conditions varied with different batches of sulphuric acid.

Kober in 1938 substituted β-naphthol for phenol as the reducing agent. He found that traces of water (less than 5%) improved the intensity of the colour produced, as did ageing of the reagent especially if exposed to sunlight; moreover he showed that minute traces of acetone residues interfered with the development of the colour. Bachman (1939a) using a reagent containing an excess of sulphuric acid relative to phenol was able to combine the first and second stages into one heating step at 150°C., but found that increased colour was developed from oestriol on standing at room temperature after final dilution for colorimetry. He stressed that fading of the pink complex could occur during this stage but maintained that with the standardized procedure the reagent was stable for months, at least in the determination of the pure oestrogens. Szego and Samuels (1940, 1943) altered the usual procedure somewhat by heating an ethanolic solution of oestrone or oestradiol-17β with concentrated sulphuric/

sulphuric acid in the first stage and adding thiocol (potassium guaiacol sulphonate) in aqueous solution prior to the second-stage. Under these conditions they obtained virtually no colour with oestriol, although a greater intensity was produced when the phenolic salt was present in the first stage of the reaction. These authors pointed out that the coloured compounds did not obey Beer's law completely at higher concentrations. Umberger and Curtis (1949) investigated the reaction of various oestrogens with concentrated sulphuric acid in the absence of phenols and found the behaviour to be qualitatively similar to the findings of other workers; it is significant, however, that their paper included no demonstration of a pink colour with oestriol.

Marlow (1950), using a freshly prepared solution of β-naphthol in sulphuric acid, investigated the specific groups responsible for the development of the pink colour in a two-stage Kober reaction. He used concentrated and 50% (v + v) sulphuric acid for his first and second stages respectively, with no further dilution for colorimetry. He demonstrated the following requirements for the production of the typical pink colour by steroids:

a) Aromatic ring A. (He did not try corticoids).
b) Intact ring D.
c) Oxygenation at position 17.
d) /

d) Position 16 either free, alcoholic, or ketonic with the exception that the 16, 17 diketone was inactive.

Haenni in 1950, using a phenolsulphonic acid reagent containing excess phenol, found that traces of ferric ion in the reagent increased the intensity of the final colour obtained with oestradiol-17β and aided the conversion of the yellow to pink. Cohen and Bates had shown in 1947 that pink colours of maximal intensity could be produced in the absence of phenol. They however used ethanol as a solvent for the oestrogens during the colour reaction, and Brown (1952a) demonstrated conclusively that the ethanol was replacing phenol as a reducing agent. Bates in the discussion on this paper contested this point, but it is significant that he referred to oestrone and not oestriol. Brown (1952b) reinvestigated the Kober reaction completely, and proposed the use of hydroquinone dissolved in aqueous sulphuric acid as a reagent. He found that there was an optimal ratio of sulphuric acid to water for each of the three oestrogens examined, viz. 76%, 66%, and 60% (v/v) for oestriol, oestrone, and oestradiol-17β respectively. As has already been noted, Kober (1938) had already shown that rigid exclusion of water from the first stage of the reaction was not desirable. The reaction when hydroquinone was used appeared to be ideal from the standpoint of stability, and the rigid precautions/

precautions of the previous methods in regard to heating times and stirring were stated to be unnecessary.

2. Other Colorimetric Procedures.

David (1934) found that oestriol after heating with concentrated sulphuric acid and reheating with dilute sulphuric acid in the presence of arsenic acid, gave a blue colour. This reaction has only half the sensitivity of the Kober (1931) reaction. He also noted turbidity of the solution with urine extracts. Pincus and Zahl (1937) used diazotized sulphanilic acid; Pincus et al. (1936) found a pink colour developed by oestrone and oestradiol-17β on heating in a zinc acetic acid mixture with the addition of benzoyl chloride. Pincus (1951) strongly recommended the use of an antimony trichloride reagent. Bachman (1939) produced a satisfactory and specific colour reaction for oestriol using p-phenol sulphonic acid in 85% phosphoric acid; the colour obtained was violet pink, absorbing maximally at 540 mμ.

B. Difficulties Encountered With the Phenolsulphonic Acid Reaction.

In the voluminous literature on the Kober reaction which has been cited above one can find almost no references to difficulties that may arise. On the contrary, the impression is obtained of a perfectly satisfactory/

satisfactory, robust and stable colour reaction. The fact that so many modifications have been produced renders this highly unlikely, and Clayton (1949) working in this laboratory had clearly shown that solvent residues can interfere with the development of the colour from oestriol. In the early stages of the present investigation a series of experiments was carried out to test the stability of the phenol-sulphonic acid reaction.

Oestriol was dissolved in purified ethyl acetate (Type 1, Appendix 1), and 175 ml. aliquots containing 46 µg. of oestriol were treated as shown in Table 1, the residue remaining after distillation being transferred to test tubes by ethanol 3 x 3 ml. The ethanol was removed in a current of air. The colour reaction was carried out based on the method of Venning et al. (1937) and fully described in Appendix II.(p. 6).

Table 1. Variability obtained with phenolsulphonic acid reaction in presence of solvent residues.

	Optical Density	
Ethyl acetate solution distilled to dryness	0.042 0.069	0.083 0.065
Ethyl acetate solution, washed 4 x 1/8 vol. water, distilled	0.117 0.118	0.113 0.104
Ethyl acetate solution, washed 1 x 1/8 vol. 8% $NaHCO_3$, 4 x 1/8 vol. water, distilled	0.138 0.163	0.100 0.124
46 µg. of oestriol, dry residue untreated	0.191 0.187	

That the difficulties encountered above were merely due to incomplete transfer was ruled out by the failure to obtain a Kober reaction on the 'residue' from further ethanol washings of the distillation flasks. Similar depressions of colour were obtained whether distillation was carried out at atmospheric or reduced pressure, whether the ethanol was removed in a current of air or nitrogen, and if methanol was substituted for ethanol as the solvent for transfer. The depression was more marked with ethyl acetate from one manufacturer (McFarlan & Co.) than from another (Griffin & Tatlock). These results suggested that some non-volatile impurity formed during the heating of ethyl acetate was interfering with the development of colour. This suspicion was confirmed by using a modification of the Kober reaction suggested by Brown (1952c), in which 2 ml. of water rather than 3 ml. are added in the second stage; the results are shown in Table 2.

Table 2. Stability of modified phenolsulphonic acid reaction to solvent residues.

	OD
a) Dry residue of oestriol (54μg.)	0.225 0.225
b) 175 ml. of ethyl acetate distilled from 54μg. oestriol at atm. pressure	0.218 0.218
c) 175 ml. of ethyl acetate distilled from 54μg. oestriol at reduced pressure	0.226 0.227

It will be seen that this slight change has caused a considerable improvement in the amount of oestriol/

oestriol recovered after this simple treatment. This suggests that the apparent loss shown in Table 1 is not due to destruction of oestriol but results from interference with colour development in the presence of solvent residues. Cohen and Marrian (1934) were able to overcome the action of solvent residues in interference with the colour reaction by a similar method of increasing the sulphuric acid concentration of the second stage. The colour obtained in the present experiments with the standards was an orange pink,rather than the true pink of the Venning modification (1937), indicating incomplete conversion of yellow to pink and resultant freedom from fading.

At this stage of the investigation the author was finding greater losses of oestrogen in the presence of solvent residues than Brown (1952c). It subsequently developed that his technician was carrying through the colour reaction by a rigid time-procedure in which colorimetry was carried out within 1-2 minutes of the development of the colour. This would of course minimize any difficulties due to fading and is the probable explanation of the discrepancy between the findings of the two laboratories.

By using the modified Kober reaction it was found that oestrogen in solution in ether sometimes gave after distillation, residues which developed a higher optical/

optical density than the untreated standards, e.g.

Table 3. Behaviour of the phenolsulphonic acid reaction in the presence of ether residues.

	O.D.	O.D.after fading	Corr.O.D.
21.5µg. untreated oestriol	0.092	0	0.092
21.5µg. oestriol after distⁿ of 150 ml.ether	0.100 0.094	0.037 0.028	0.063 0.066
20µg. untreated oestrone	0.095 0.099	0 0.002	0.095 0.097
20µg. oestrone after distⁿ of 150 ml. ether	0.130 0.118	0.044 0.039	0.086 0.079

These coloured solutions were faded according to the technique of Stevenson and Marrian (1947) by being reheated for an additional 1½ hours in a boiling water bath, then reread, and the true oestrogen content obtained by difference between the two optical densities. This technique showed, as an additional defect in the colour reaction, that solvent residues not only may depress the pink coloured products, but also may contribute a non-specific component capable of considerable absorption with the D_{604} filter. Thus falsely high values can be obtained by measuring the optical density of the prefaded colours.

C. Difficulties Encountered in Using Hydroquinone Reagents.

1. General Discussion.

At this stage of the investigation it became apparent that the phenolsulphonic acid reaction as described/

described in Appendix II (p. 6) was completely unsatisfactory as a means of measurement of small quantities of oestrogen in the presence of relatively large amounts of solvent residues. It was decided by Professor Marrian that Mr Brown working in the Clinical Endocrine Research Unit should investigate the necessary modifications of the colour reaction, and that I should proceed to a study of the purification procedures for the urinary oestrogens described elsewhere in this thesis. When a new variation of the Kober reaction using hydroquinone in place of phenol had been produced (Brown, 1952b), I adopted this new procedure, the technique of which is fully described in Appendix II (p. 7). The remainder of this section sets forth the results thus obtained.

The procedure has been applied over the course of two years to the measurement of oestrogens in the presence of solvent and urine residues, and in its original form has been found to be unsatisfactory. Evidence has been obtained of three different types of depression of colour and it is proposed to discuss each of these in turn. This reaction, like all other modifications of the original Kober method (1931), is a two-stage reaction in which the first stage is the formation of a yellow coloured compound with a greenish fluorescence, followed by a conversion to a pink non-fluorescent/

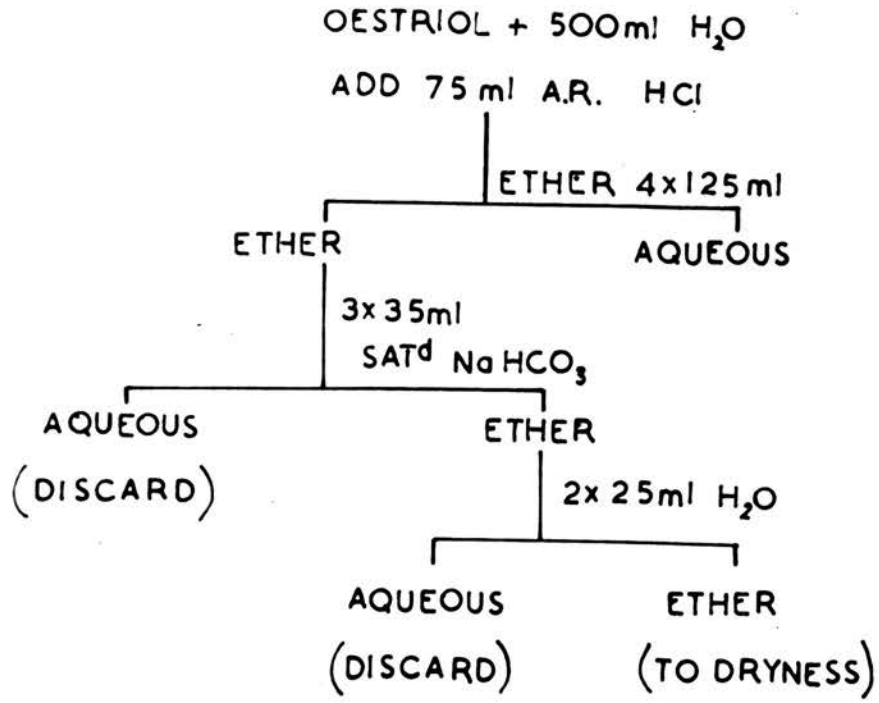
fluorescent compound on reheating in less concentrated H_2SO_4. This pink colour can itself be faded on heating at low concentrations of H_2SO_4. There are thus three possible forms of interference with the colour produced, viz.:-

1) Failure to form the initial yellow coloured complex (Type I Inhibition).
2) Failure to convert the yellow colour fully to the pink colour (Type II Inhibition).
3) Instability of the pink colour (Fading).

2. Type I Inhibition

Type I is peculiar to oestriol. For many years there has been an apparent contradiction with regard to the ease with which this substance undergoes the Kober reaction. Marrian (1938), supported by Bachman (1939),pointed out how readily this compound could be converted into a pink derivative under conditions of mild oxidation which did not affect oestrone or oestradiol-17β. On the other hand in the Kober reaction and its modifications, oestriol requires a longer heating time or higher concentration of H_2SO_4(Cartland *et al.*, 1935;Szego and Samuels,1940;Brown,1952b).Moreover,it has been shown (Brown,1952b) that in the case of oestriol but not oestrone, the absence of an effective reducing agent in the first stage of the reaction prevents formation of the red coloured product in the second stage/

FIG. EXTRACTION OF OESTRIOL FROM AQUEOUS MINERAL ACID.



stage.

		O.D.
50μg. oestriol	No hydroquinone present	0.116
	2% hydroquinone in reagent	0.289

I have confirmed this finding and in addition shown that the type of sulphuric acid used is of importance.

	Hopkins & Williams, AR. H_2SO_4	O.D.
25μg. oestriol	No hydroquinone present	0.070
	Hydroquinone present	0.202
	B.D.H. AR. H_2SO_4	
	No hydroquinone present	0.058

It therefore appears that oestriol when heated with sulphuric acid in the absence of reducing agents forms a yellow coloured complex which does not readily undergo the second stage of the Kober reaction. Oestrone on the other hand needs no reducing agent. (Bates' (1952) use of oestrone, therefore, to show that ethanol is not acting as the required reducing agent, is not justified).

The first indication of difficulties came in an investigation of the initial extraction of oestriol from aqueous mineral acid with ether. This is the first step of the purification procedure and for the pure solution recovery experiments which concern us here involves the steps shown on the facing page. On investigating this step yields of 82, 83, 81,90, 91, 95% were obtained in a group of experiments. Simple mechanical loss was eliminated as the cause by back extractions/

extractions and carrying out colour reactions on the glassware. In addition to the apparent loss of oestrogen, these results were highly variable. The first insight into the complexity of the problem was obtained when three filters D_{604} (green), D_{601} (violet) D_{606} (yellow) were used to read the colour, and a simple correction applied as developed by Brown (1952c), viz:

$$D_{corrected} = \frac{4D_{604} - 3D_{606} - D_{601}}{3.8}$$

The results obtained with this colour correction on oestriol taken through this extraction procedure are shown below.

Table 4. Effect of ether residues on development of colour with oestriol in terms of the colour correction.

	D_{604}	D_{601}	D_{606}	$D_{corr.}$	$D_{corr.}$ % Recovery
Untreated oestriol controls	0.175	0.025	0	0.175	
	0.175	0.025	0	0.175	
Extracted oestriol	0.185	0.070	0.034	0.150	86
	0.180	0.085	0.032	0.142	81
	0.172	0.070	0.025	0.143	82

This explained the variability of the results but also enhanced the problem by showing loss on every occasion. Depression of colour by urine residues was also demonstrated. It was found that increase of the hydroquinone concentration from 2 to 5% improved the yields. The addition of 0.2 ml. ethanol at the start of the colour reaction had a similar effect. This method was in fact used in the earlier recovery experiments/

experiments reported in the section on extraction, but was not entirely acceptable due to variation in batches of alcohol even after extensive purification, including treatment with meta-phenylenediamine. By a completely empirical approach 0.1 ml. of m-cresol was found to be a more suitable stabilizing agent. The efficiency of this agent is exemplified in the experiments listed in Table 5.

Table 5. Effect of addition of m-cresol to hydroquinone reagent in overcoming depression by solvent residues.

Expt.	Cresol in Reagent	% Recovery $D_{corr.}$
a	+	94, 86
	-	61
b	+	87
	-	57

These results are typical of a large series of experiments, more examples of which are given in Appendix IV, p. 16-18.

With the effectiveness of m-cresol thus established, it seemed desirable to determine its mode of action, so that a simpler method of achieving the same end could be used. This further investigation was also necessitated by the fact that very frequently turbid solutions were obtained using the m-cresol or ethanol stabilized hydroquinone reaction, but never with the pure reagent. This turbidity could be filtered from the final solution by a porosity 3 or 4 sinter glass funnel, or taken care of by the colour correction formula/

formula; but the first method is cumbersome and the second a source of error. It was believed that the depression of colour seen with solvent residues was due to their content of oxidizing agent, which seemed to overcome the effectiveness of the reducing properties of hydroquinone and allow Type I inhibition to occur. Darkening of the reagent in the cold on the addition of hydrogen peroxide in small amounts can readily be demonstrated. Various agents were added to the reagent in an attempt to prevent this darkening, and of a large number tried only m-cresol was effective. Reagents containing the other cresols darkened on addition of hydrogen peroxide, but the colour obtained differed from that obtained with the pure reagent and was typical of the respective quinones. It was felt that m-cresol was behaving as an antioxidant to the hydroquinone and not being able to form a quinone itself did not darken but prevented the darkening due to oxidation of the hydroquinone. On the other hand it was almost inconceivable that even in concentrated H_2SO_4 solutions m-cresol was a stronger reducing agent than hydroquinone. The explanation came when it was found that m-cresol, to be effective, had to be added to the 2% HQ 76% (v/v) sulphuric acid reagent immediately before the colour reaction. In other words, standing the m-cresol in sulphuric acid removed/

removed the antioxidant powers. The obvious change to occur would of course be sulphonation. This directed attention to the precise composition of the so-called hydroquinone/sulphuric acid reagent of Brown (1952b). Pinnow (1915, 1917) studied the monosulphonation of hydroquinone at temperatures from 50-100°C. using 10-14 M-H_2SO_4, and from his results one would expect hydroquinone to be almost completely sulphonated by 76% (v/v) sulphuric acid either by heating at 100°C. for twenty minutes or by prolonged standing at room temperature.

His results relative to the present investigation were confirmed as follows: 50 ml. of reagent prepared by dissolving 2 g. of hydroquinone in 100 ml. of 76% (v/v) H_2SO_4 under the conditions listed below, were diluted to 1/3 volume with water slowly and with efficient cooling. The solution was extracted 4 x 1/3 volumes of ether, washed with 1 x 1/10 volume of water, 2 x 1/15 volumes 8% sodium bicarbonate, and 3 x 1/100 volumes of water. The ether was distilled to dryness and the weight of the residue taken as an index of the free hydroquinone content of the reagent.

Table 6. Effect of method of preparation and ageing on the free hydroquinone content of the reagent.

Age of Reagent	Temperature during solution	Free Hydroquinone content
24 hr.	20°C.	908 mg./50 ml.
3 weeks	20°C.	14 mg./50 ml.
24 hr.	30-40°C.	557 mg./50 ml.
24 hr.	90-100°C.	25 mg./50 ml.

Table 7. Effect of method of preparation and ageing of reagent in inhibition of colour production by solvent residues.

Reagent	Oestriol	D_{604}	D_{601}	D_{606}	% Recovery $D_{corr.}$
1	Extract	0.179	0.053	0.018	89
	"	0.173	0.061	0.019	84
	Standard	0.180	0.032	0.004	
	"	0.186	0.033	0.005	
	Extract	0.165	0.052	0.013	85
	"	0.174	0.068	0.019	85
	Standard	0.179	0.031	0.004	
	"	0.175	0.025	0	
2	Extract	0.128	0.040	0.009	65
	"	0.143	0.047	0.006	74
	Standard	0.179	0.035	0	
3	Extract	0.140	0.078	0.029	55
	"	0.150	0.089	0.031	58
	"	0.157	0.088	0.033	60
		0.151	0.088	0.032	59
	Standard	0.192	0.048	0	
		0.190	0.048	0	

Reagent 1 - 2 g. of hydroquinone finely powdered dissolved in 76% (v/v) H_2SO_4 by shaking at room temperature; used within 12 hr.

Reagent 2 - as for reagent 1, but heated at 40-50° C. to aid solution.

Reagent 3 - as for reagent 1, but 3 weeks old.

'Standard' - pure oestriol.

'Extract' - oestriol put through extraction procedure facing p.25.

Hydroquinone monosulphonate is a weaker reducing agent than hydroquinone (Pinnow, 1915), so it is considered likely that reagents prepared and used as suggested by Brown (1952b) permit Type I inhibition because of lessened reducing power. The change of this type of reagent has been noted before (Chamot and Pratt, 1909, 1910). Dr Clayton in this laboratory in 1949 noted that the best results in the determination of oestriol in the presence of solvent residues were obtained with a reagent made up as suggested by Venning et al. (1937) if the reagent was not more than three days old. Marlow (1950) also used a freshly prepared reagent. The results of experiments designed to show improvement in the inhibition of colour development by ether residues are shown in Table 7 on the facing page.

It is now possible to explain the action of m-cresol in stabilizing the hydroquinone reagent on the basis that it is a stronger reducing agent than hydroquinone monosulphonate. An attempt was made to obtain a reagent which would be stable on ageing by substitution of duroquinol for hydroquinone, but it was found to be too strong a reducing agent, oxidation to intensely coloured products occurring on heating in 76% (v/v) sulphuric acid. It was decided, therefore, to retain hydroquinone as the reducing agent as suggested by Brown (1952b), but with the essential/

essential requirement that hydroquinone must be freshly added to the reagent immediately before the colour reaction was carried out.

In the case of oestrone and oestradiol it has been found that since there is no necessity for free hydroquinone in the reagent ageing is of no importance. The recoveries of oestrone and oestradiol from aqueous acid by extraction with ether are satisfactory whether freshly prepared or one month old reagents are used. This is shown in Table II, Appendix IV, p. 18-19.

3. Type II Inhibition

The second stage of the Kober reaction in its various modifications consists of reheating the yellow fluorescent product of the first stage in more dilute H_2SO_4 during which there is a conversion of yellow to pink. This change occurs only at concentrations of H_2SO_4 below 50% (cf. Cohen and Bates, 1947; Brown 1952b). Brown (1952a) showed that this change could be facilitated by the addition of ferric ion or hydrogen peroxide with the diluting water. This observation has been confirmed and cupric ion also found to be effective. These facts suggest that this second stage is oxidative in character. Haenni (1950) found that the addition of traces of Fe^{+++} to a phenolsulphonic acid reagent aided the complete conversion to pink. This second/

Table 8. Variations in optical density obtained on development of colour with pure oestrogen residues using hydroquinone reagents.

Expt.	Oestrogen used	D_{604}
1	Oestriol (25μg.)	0.125, 0.125
2		0.176, 0.180
3		0.156, 0.160
4		0.153, 0.153
5		0.139
1	Oestradiol-17β (25μg.)	0.196, 0.193
2		0.223
3		0.186, 0.175
4		0.186, 0.186
5		0.194, 0.196
1	Oestrone (25μg.)	0.237, 0.240
2		0.268, 0.268
3		0.235, 0.235
4		0.242, 0.242
5		0.224, 0.224

This second stage is followed in most modifications by a subsequent dilution to lower sulphuric acid concentrations for ease of colorimetry. In at least two methods (Bachman, 1939; Brown, 1952b), this step is in fact an active process, in that further conversion of yellow to pink occurs during standing at room temperature. It was felt that this uncontrolled, almost fortuitous completion of the reaction was undesirable in that contaminants from solvent or urine residues might interfere. Moreover, with the hydroquinone reagents (Brown, 1952b), appreciable day-to-day variations were encountered in the final colour produced by standard solutions of the three oestrogens as is shown in Table 8 on the facing page. These results were obtained in the period December 4-12th, 1951. The reagents used were made from B.D.H.(A.R.) sulphuric acid exactly as recommended by Brown (1952b); the colour reaction was carried out as detailed in Appendix II (p. 7), with the exception that in the case of oestriol only, 0.2 ml. of ethanol was added to the dry residue in the reaction tubes (including the blank) immediately before carrying out the procedure. It might be argued that this variation is unimportant in that it could be corrected merely by always including standards with the unknown solutions during the colour reactions. In this period, however, variations

as/

as great as 15-20% were encountered in the recoveries obtained with the complete method. These variations were not obtained in the quadruplicates done, but between identical experiments carried out on different days. For example, one set of recoveries for oestradiol by the colour correction formula gave values of 68%, 80%, 80%, 80%, and on repeating the experiment two days later recoveries of 89%, 92%, 93% and 95% were obtained. There appeared to be some uncontrolled variable which was influencing the colour developed by the oestrogens in the hydroquinone reaction. The possibility of traces of oxidizing agents in the glassware was ruled out in view of the rigidly controlled washing procedure which was introduced very early in the investigation (see Appendix II, p. 10).

Definite proof of the unsatisfactory nature of the colour reaction was found in a group of experiments, examples of which are given in Table 9. The oestrogen used in these experiments was oestriol, and the colour reaction in one case was carried out exactly as recommended by Brown (1952b) - Reaction 2 - and in the other - Reaction 1 - stabilized by the addition of 0.1 ml. of m-cresol to the dry residue of oestrogen in the reaction tube. A comparison is shown between colours obtained from oestrogen extracted from aqueous acid in the method described on p. 25 , and those obtained from oestrogen to which the solvent residues resulting from

a/

a blank extraction were added:

Table 9. Depression of colour produced by hydroquinone reagent with solvent residues.

Reaction	Oestriol	D_{604}	D_{601}	D_{606}	$D_{corr.}$ Recovery
1[x]	Standard	0.180	0.028	0	
	Extract	0.152	0.038	0.003	81%
	Blank extract + standard	0.165	0.052	0.009	84%
	Blank extract	0.014	0.017	0.004	
2	Standard	0.162	0.026	0	
	Extract	0.150	0.078	0.033	70%
	Blank extract + standard	0.150	0.061	0.022	78%
	Blank extract	0.028	0.043	0.015	

x Stabilized with m-cresol (see text)

Evidently the apparent loss of oestriol by extraction was mostly due to inadequate development of colour in the presence of solvent residues. Attention was next directed to the precise stage of the colour reaction which was causing the variability of results. In Brown's publication (1952b) no suggestion was made that prolonged standing at room temperature of the finally diluted solution was necessary for full development of the colour. In his Fig.5(loc.cit.), however, it can be seen that dilution to 40% without further heating increased the optical density almost 15% over that obtained at 60%. It was felt that this increment in colour might be the cause of Type II inhibition. The results shown in Table 10 indicated that this increase in the colour of the finally diluted solution did in fact/

fact take considerable time.

Table 10. Increase in colour in final stage of hydroquinone reaction on standing at room temperature (oestriol).

Extracted from ether	D_{504}	D_{501}	D_{505}
Read in 10 min.	0.169	0.083	0.013
2 hr.	0.173	0.065	0.012
12 hr.	0.178	0.058	0.012
Pure oestriol			
Read in 10 min.	0.163	0.042	0
2 hr.	0.168	0.035	0
12 hr.	0.178	0.028	0

In this experiment the colour development proceeded at approximately equal rates in both cases. Brown (1953) has confirmed this result and extended it to show that in the presence of urine residues the development of the final colour at room temperature was delayed.

This defect in the colour reaction once demonstrated, the correction seemed to be obvious. Other workers (cf. Venning et al., 1937; Cohen and Bates, 1947; Brown, 1952b) had shown that decreasing the sulphuric acid concentration of the second stage aided the conversion of yellow to pink. Accordingly a re-investigation of the amount of water added and of the heating time of the second stage was carried out for oestriol.

25 μg. of oestriol in a test tube (18 mm. x 150 mm.) were dissolved in 0.1 ml. of m-cresol and 4 ml. of 2% hydroquinone (w/v) in 76% (v/v) A.R. H_2SO_4 added. The solutions were heated in a boiling water bath for 20 minutes/

Table 12. Effect of time of heating in second stage on modified oestriol colour reaction.

Time of heating min.	D_{s04}	D_{s01}
0	0.126	0.046
1	0.132	0.033
2	0.147	0.029
3	0.155	0.025
4	0.159	0.025
5	0.161	0.025
6	0.161	0.025
7	0.168	0.025
8	0.166	0.023
9	0.164	0.024

N.B. Solutions were diluted to 15 ml. with 33% (v + v) A.R. sulphuric acid to give a final concentration of approximately 40%.

minutes, shaken thoroughly at 2 and 5 minutes. After cooling, distilled water in the amounts shown below was added, thoroughly mixed by shaking, and the solutions reheated at 100°C. for 8 minutes. After cooling the solutions were diluted to 15 ml. to give a final H_2SO_4 concentration of 40% (39 and 38% in the case of the 2.5 and 3.0 ml. additions), and read after 10 minutes with a blank to which 2 ml. of water had been added. The results are shown in Table 11.

Table 11. Effect of amount of water added in second stage on colour developed by oestriol (25 µg.)

Amount of water added ml.	D_{504}	D_{501}
0.5	0.128	0.058
1.0	0.139	0.049
1.5	0.150	0.045
2.0	0.155	0.031
2.5	0.155	0.020
3.0	0.140	0.018

It will be seen that 1 ml. of water was insufficient to cause maximal formation of pink colour.

In order to determine the optimal heating times a similar experiment was carried out adding 2 ml. of water to all the tubes prior to the second stage, and varying the length of heating time. The results are shown in Table 12 on facing page.

This modified reaction thus appeared to have overcome the Type II inhibition previously noted. The discrepancy with Brown's results (1952b) remained unexplained, and use of this reaction still gave considerable/

considerable day-to-day variation in the colours obtained with standard solutions. It was suspected that other factors as yet undiscovered were involved, and these suspicions were confirmed as will be seen in the following section.

4. Fading.

It has long been recognized that the pink colour obtained may in turn be converted to a colourless compound. Kober (1931) demonstrated fading by neutralization or oxidation. The importance of this type of interference in explaining depression of colours obtained by urine residues was first recognized by Cohen and Marrian (1934), who prevented the fading by increasing the sulphuric acid concentration of the second stage. Brown (1952c), by the same means, obtained more consistent results with the method of Venning et al. (1937). In the present investigation it was observed that sunlight, to the extent found within a laboratory, plays a major role in Type II inhibition and fading. Typical results for oestriol are shown in Table 13.

The reagent used was 2% hydroquinone in 76% (v/v) A.R. sulphuric acid. In the reaction 4 ml. of the reagent added to the dry residue were heated in a boiling water bath for 20 minutes, shaken vigorously at 2 and 5 minutes. After cooling, an amount of 1 or 2 ml. of water was added as shown and after thorough mixing/

mixing the solution heated for 5 minutes. The tube was cooled and the contents diluted to 15 ml. by the addition of either 30% (v+v) or 33% (v+v) sulphuric acid to give a final concentration of approximately 40% sulphuric acid. The colour intensity was read at 5 minutes and subsequently as shown.

Table 13. Influence of sunlight on development of oestriol colour.

a. In sunlight in all stages

Min.	1 ml. H_2O D_{604}	1 ml. H_2O D_{601}	2 ml. H_2O D_{604}	2 ml. H_2O D_{601}
5	0.170	0.044	0.151	0.048
15	0.160	0.042	0.148	0.036
90	0.155	0.043	0.140	0.025
120	0.153	0.041	0.132	0.032

b. In laboratory at night

	1 ml. H_2O D_{604}	1 ml. H_2O D_{601}	2 ml. H_2O D_{604}	2 ml. H_2O D_{601}
5	0.178	0.079	0.198	0.059
15	0.189	0.072	0.200	0.053
90	0.194	0.065	0.199	0.049
120	0.198	0.057	0.199	0.048

These results are obviously most complex and show the interplay of two separate variables, viz. illumination and H_2SO_4 concentration. The following facts seem to be established:

i. Lowering sulphuric acid concentration has aided the conversion of yellow to pink as shown by lower D_{601} readings in both cases with the 2 ml. diluent.

ii. A similar facilitation is shown by the amount of illumination present in the laboratory on this particular day.

iii. /

iii. In the absence of sunlight, 1 ml. H_2O diluent is insufficient to bring about full conversion of yellow to pink during the colour reaction; this requires prolonged standing of the finally diluted solution. This is indicative of the Type II inhibition to which reference has already been made.

iv. In the presence of sunlight even with 1 ml. of water as diluent, the low initial optical density suggests that fading has occurred during the second stage or immediately on dilution. This effect is markedly increased at the lower concentration of sulphuric acid for the second stage with which even on immediate colorimetry a 25% depression of colour intensity has occurred.

v. Only in one case was a satisfactory stable coloured product obtained. This was done by carrying out the reaction away from bright light at all stages and avoiding Type II inhibition by the use of a second stage of approximately 50% rather than the recommended (Brown, 1952b) 60%.

These results offered a ready explanation for the day-to-day variations in the intensity of colour produced, and for the discrepancies between my results (Table 11, p. 36) and Brown's (1952b) in the optimal amount of water added in the second stage. The kinetics of the photochemical reaction involved were not investigated. This experiment was conducted under conditions/

conditions of illumination similar to those under which the original work had been carried on by Dr Brown (i.e. western exposure). The heating procedures were carried out in a metal bath, but in the diluting steps the tubes were exposed in an open rack. This finding offered a logical explanation of the discrepancies obtained in the investigation of the hydroquinone colour reaction: since the laboratory never received any direct sunlight. Under the usual operating conditions, therefore, it was to be expected that with the recommended 60% sulphuric acid concentration for the second heating period difficulties would be encountered in full conversion of the yellow to the pink, Type II inhibition being present unless there was a long interval between final dilution and colorimetry. This effect was of such practical importance that it was reinvestigated under differing conditions of light. The results of these experiments and the results obtained with oestrone and oestradiol-17β are shown in Appendix IV, p. 21-3. It is of interest that the sulphuric acid colour reaction for cholesterol is known to be photosensitive (cf. Kenny, 1952), and that Szego and Samuels (1943) kept their solutions in the dark at one stage of the reaction. Kober (1938), too, noted the effect of sunlight on his β-naphthol reagent.

D. /

D. Development of a Modified Hydroquinone Sulphuric Acid Reaction.

1. General Discussion

It was apparent from the results already presented that the use of the modified Kober reaction developed by Brown (1952b) was an unsatisfactory method for quantitative estimation of small amounts of oestrogen in presence of relatively large amounts of solvent or urine residues. Three possible forms of interference with the colour development have been demonstrated. In the first of these, the Type I inhibition, it appears that in the case of oestriol the absence of an effective reducing agent during the first heating stage of the reaction allows a yellow coloured complex to be produced which fails to undergo conversion to pink on reheating with diluted sulphuric acid. In practice this condition is found when an aged reagent (in which all the hydroquinone is sulphonated) is used for the determination of oestriol in the presence of ether or urine residues. This fault can be eliminated by the addition of ethanol or m-cresol to the reagent immediately before production of the colour. This, however, frequently results in the appearance of turbidity in the coloured solutions. A more satisfactory method is to use only freshly prepared reagent or to add hydroquinone/

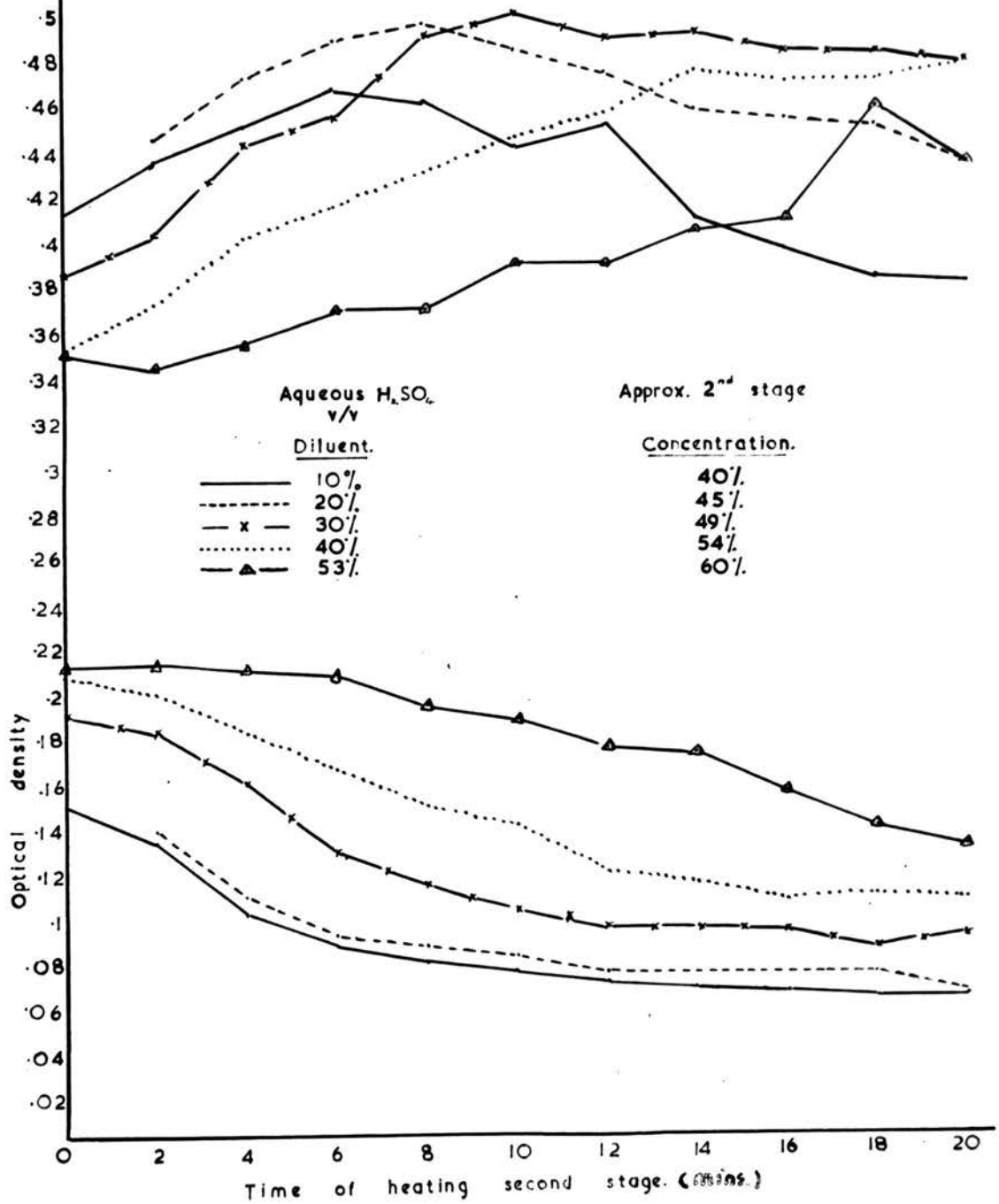
immediately before use.

The other two types of colour interference are found with all three oestrogens and are due to the fact that the colour change from yellow to pink is a graded oxidation which, if excessive, results in disappearance of the colour. There are thus two additional forms of interference with colour development. One of these, the Type II inhibition, the failure to convert the yellow complex to the pink derivative, is overcome by the presence of sunlight or the use of a more dilute sulphuric acid concentration for the second stage. The other, fading of the pink colour, results when the second heating stage is carried out at a sulphuric acid concentration of 50% in a brightly illuminated laboratory.

2. Modification of Procedure

Brown had shown that the colour produced by using a 60% sulphuric acid concentration was stable even on heating for 2 hours. This suggested that elimination of the final dilution to 40% would remove any tendency to fading. Moreover, since only 7.5 ml. were required for colorimetry, it was decided to substitute this final volume for the 15 ml. recommended and thus double the intensity of the colour produced. In order to provide a convenient means of varying the sulphuric acid concentration of the second (and in this case the final/

Fig. 1. Effect of Variation in H_2SO_4 concentration of second stage (oestrone)



final) stage, dilution was effected with aqueous sulphuric acid in fixed volume rather than pure water. For convenience the procedure was adopted of adding 4 ml. of the reagent, prepared as in Appendix II, p. 7 and heating for 20 minutes. After cooling, 3.5 ml. of aqueous sulphuric acid were added, and the effect of heating times investigated.

The results obtained with oestrone are shown in Fig. 1 opposite. It will be seen that adding 3.5 ml. of 53% (v/v) H_2SO_4 to a series of 11 tubes each containing 25μg. of oestrone, and withdrawing samples at 2 minute intervals from 0-20 minutes gave values of optical density increasing in direct proportion to the heating time with the green 604 filter (upper graph) and steadily diminishing values with the violet 601 filter (lower graph). Even with 20 minutes heating maximum formation of pink was not achieved. This then is indicative of Type II inhibition. On the other hand, when 3.5 ml. of 10% (v/v) H_2SO_4 were used, giving a second stage concentration of approximately 40%, the maximum intensity of the pink colour was found after only 6 minutes of heating. (The samples which were heated longer showed lower values of $O.D._{604}$, indicating fading). The residual yellow colour as shown by $O.D._{601}$ was lowest under these conditions. A similar but less marked effect was found in the time heating curve obtained with 3.5 ml. of 20% H_2SO_4, giving/

giving a second stage concentration of approximately 45%. The graph also shows that with 3.5 ml. of either 30 or 40% (v/v) H_2SO_4 and 15-20 minutes heating full conversion of yellow to pink was achieved (i.e. Type II inhibition overcome) with no tendency to fading.

In these experiments Pyrex test tubes (18 mm. x 150 mm.) were used. In order to minimize changes in final volume due to the loss of water from the solutions or condensation of steam inside the tubes during the heating periods, tubes of larger bore with standard taper glass socket joints fitted with tops (see Appendix III, p. 13) were introduced. It was found that this increased both $O.D._{.504}$ and $O.D._{.501}$, the increase in the former being proportionately greater. It was felt that this enhancement was due to increased exposure to atmospheric oxygen. This was confirmed by showing that continual stirring (breaking the surface of the solution) in the second stage increased the colour obtained in the narrow-bore tubes to that obtained in the others. The larger bore, however, allowed optimal aeration since continual stirring produced no increase of colour over that obtained in the standard procedure of shaking the tube 5-10 times after dilution and prior to heating. Thus in the small-bore tubes $O.D._{.504}$ for oestrone obtained with

3.5/

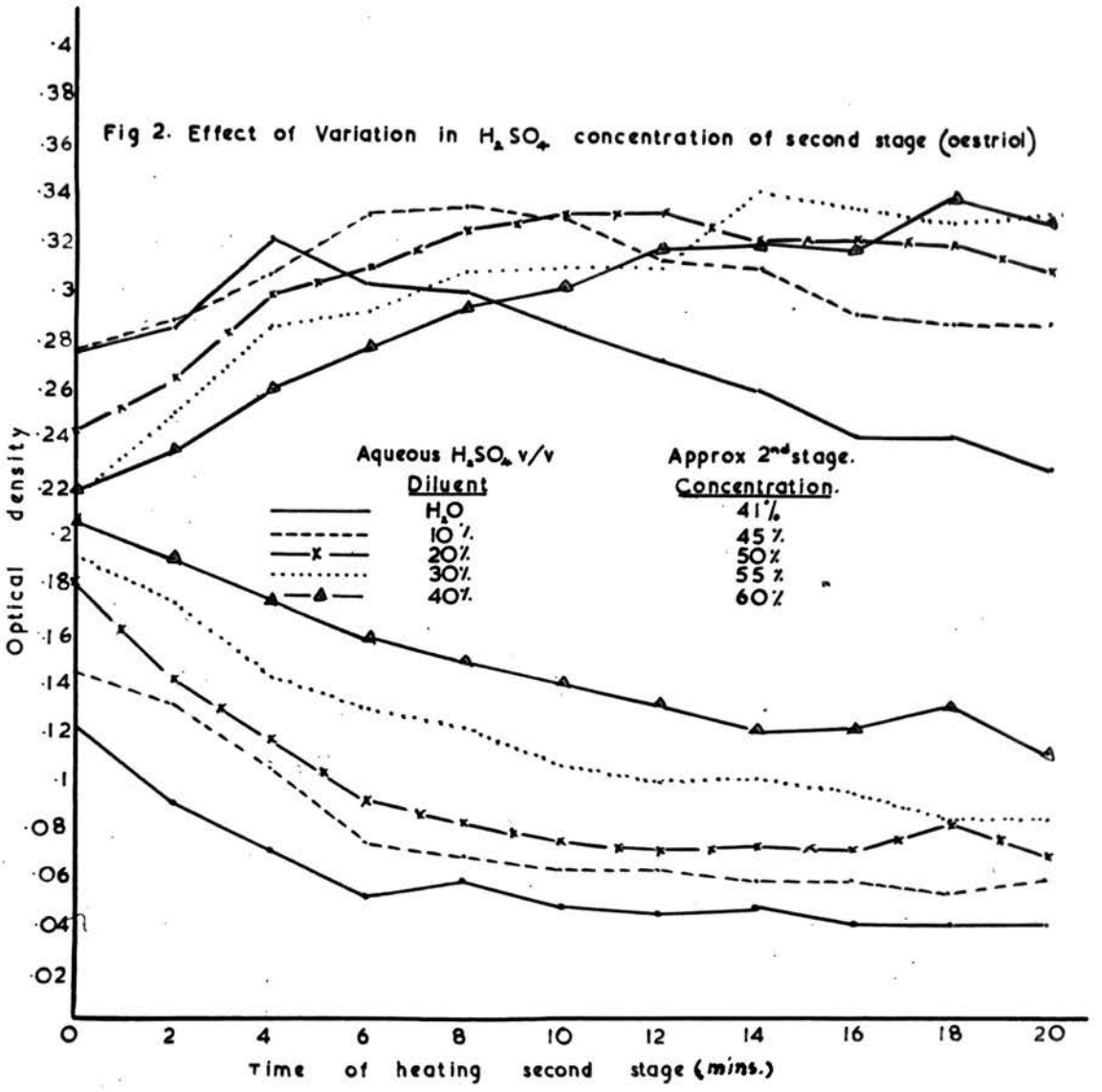


Fig 2. Effect of Variation in H_2SO_4 concentration of second stage (oestriol)
Optical density
·4
·38
·36
·34
·32
·3
·28
·26
·24
·22
·2
·18
·16
·14
·12
·1
·08
·06
·04
·02
Aqueous H_2SO_4 v/v Diluent
H_2O
10%
20%
30%
40%
Approx 2nd stage. Concentration.
41%
45%
50%
55%
60%
0
2
4
6
8
10
12
14
16
18
20
Time of heating second stage (mins.)

3.5 ml. of 30% (v/v) H_2SO_4 and a second heating period of 15 minutes was 0.465, 0.459 with mixing only at start of second stage, and 0.483, 0.479 with continuous stirring during the second heating. With the larger-bore (hereafter called Kober) tubes values of 0.479, 0.483 were obtained with mixing only, and 0.480, 0.483 with continuous stirring. Subsequently the Kober tubes were used and the contents mixed vigorously shaking 5-10 times before the second heating. The final colour obtained in all cases remained stable for hours. Thus if the second stage conditions are unsatisfactory for full conversion of yellow to pink, the resulting Type II inhibition cannot be overcome by further reaction on standing at room temperature. Similarly if the conditions of the second stage are such as to cause fading, this will occur during the heating period and will not be increased on standing at room temperature.

A similar experiment showing the relation between time of heating and H_2SO_4 concentration of the second stage was carried out using 25μg. quantities of oestriol. The results are shown in Fig. 2 opposite. Again Type II inhibition and fading were demonstrated at second stage concentrations of 40% and 60% respectively. The graphs obtained with 30% or 20% (v/v) /

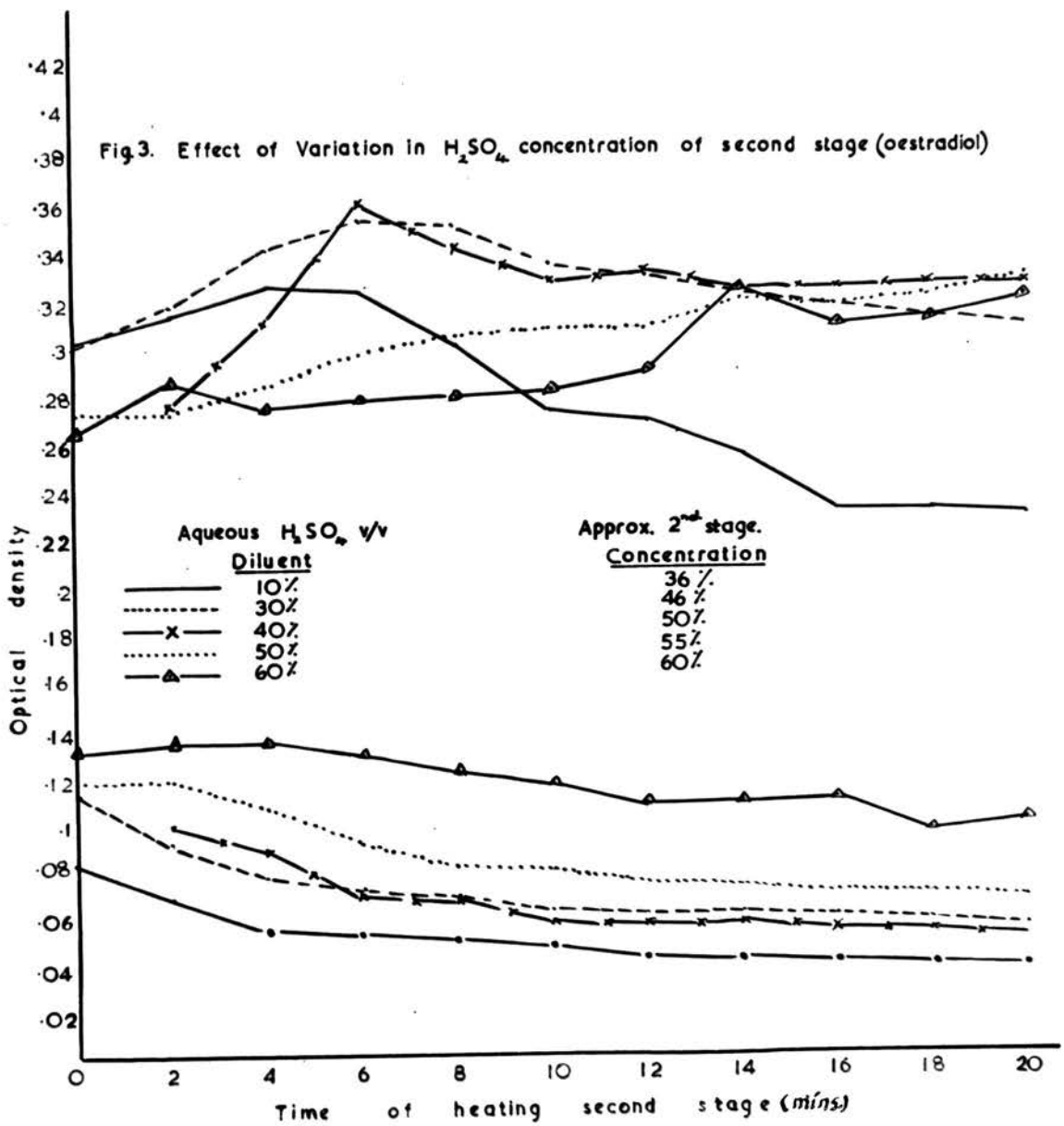


Fig.3. Effect of Variation in H_2SO_4 concentration of second stage (oestradiol)
Optical density
·42
·4
·38
·36
·34
·32
·3
·28
·26
·24
·22
·2
·18
·16
·14
·12
·1
·08
·06
·04
·02
Aqueous H_2SO_4 v/v
Diluent
10%
30%
40%
50%
60%
Approx. 2nd stage.
Concentration
36 %.
46 %
50%
55%
60%
0
2
4
6
8
10
12
14
16
18
20
Time of heating second stage (mins.)

(v/v) H_2SO_4 indicate freedom from both Type II inhibition and fading with heating times of 15-20 minutes. These results were obtained with a reagent prepared the same day by shaking finely powdered hydroquinone (2% w/v) in 76% (v/v) H_2SO_4 at room temperature. This reagent, since it contains free hydroquinone, would be capable of preventing Type I inhibition from solvent residues. Similar results were obtained with an aged (3 weeks) oestriol reagent, the same relationship existing between heating times and H_2SO_4 concentration.

Fig. 3 on the facing page shows the results of a similar investigation of the most suitable diluent and heating time for oestradiol-17β. Type II inhibition is demonstrated with a diluent of 60% H_2SO_4 (second stage concentration of 60%), and fading is well shown with 10% (v/v) H_2SO_4 as a diluent. In general these two effects were somewhat less marked than with the other oestrogens, but the greater irregularity of the graph suggests some instability of the reaction even at second stage concentrations of 50 and 55% H_2SO_4.

In this investigation of optimal conditions for the second stage reaction, it will be noted that for each of the oestrogens the H_2SO_4 concentration of the first stage was exactly as Brown (1952b) recommended. His experiments were repeated and confirmed in full. The use of 76%, 66% and 60% (v/v) H_2SO_4 for the preparation of/

of the reagents gave optimal formation of the yellow coloured complex in the first stage. Brown (1952b) originally suggested a 60% (v/v) reagent for both oestrone and oestradiol-17β, but subsequently (1952c) used separate reagents as above.

At this stage of the investigation it was thought that the difficulties of the hydroquinone colour reaction had been eliminated and that these modifications of procedure had removed the three possible forms of interference with colour development. With the removal of the unnecessary final dilution no effect of sunlight could be demonstrated. The modified procedures based on experiments such as have been shown in Figs. 1-3 were as follows:-

The reagents were prepared as described in Appendix II, p. 7 , with the exception that the solution of hydroquinone in the oestriol reagent was effected at room temperature and used within 24 hours. The appropriate reagent (4 ml.) was added to the dry residue of oestrogen in a Kober tube, a top fitted, and the solution heated for 20 minutes in a bath of boiling water, the tube being shaken at 2 minutes and 5 minutes, and then cooled in running tap water; 3.5 ml. of 30, 40, or 50% (v/v) H_2SO_4 were added to oestriol, oestrone and oestradiol-17β respectively, the tubes shaken vigorously 5-10 times, and reheated with/

with tops in place for 15 minutes. After cooling in running tap water the optical densities with the green 604, violet 601 and yellow 606 Ilford filters were read in a Spekker colorimeter against the appropriate reagent blanks.

3. Modification of the Reagent.

The application of this modified colour reaction to recovery experiments of the three oestrogens gave entirely satisfactory results. It was, however, noted that the solutions did not completely obey Beer's law, some deviation at the higher concentrations being found. More will be said of this later. At this time Cox (1952) working in this Department was finding a marked difference in behaviour of A.R. grades of sulphuric acid in the Barker and Summerson colour reaction which he had adopted for determination of acetaldehyde. It seemed possible, therefore, that different batches of 'pure' sulphuric acid might behave differently in the time heating curves for the second stage of the reaction. Venning (1952) noted the dependence of the conditions developed for the phenol-sulphonic acid reaction (Venning *et al.*, 1937) on the batch of sulphuric acid used. In the determination of oestrogens by their fluorescence in sulphuric acid, variation with the type of sulphuric acid has been noted (cf. Engel *et al.*, 1950). Before finalizing the

present/

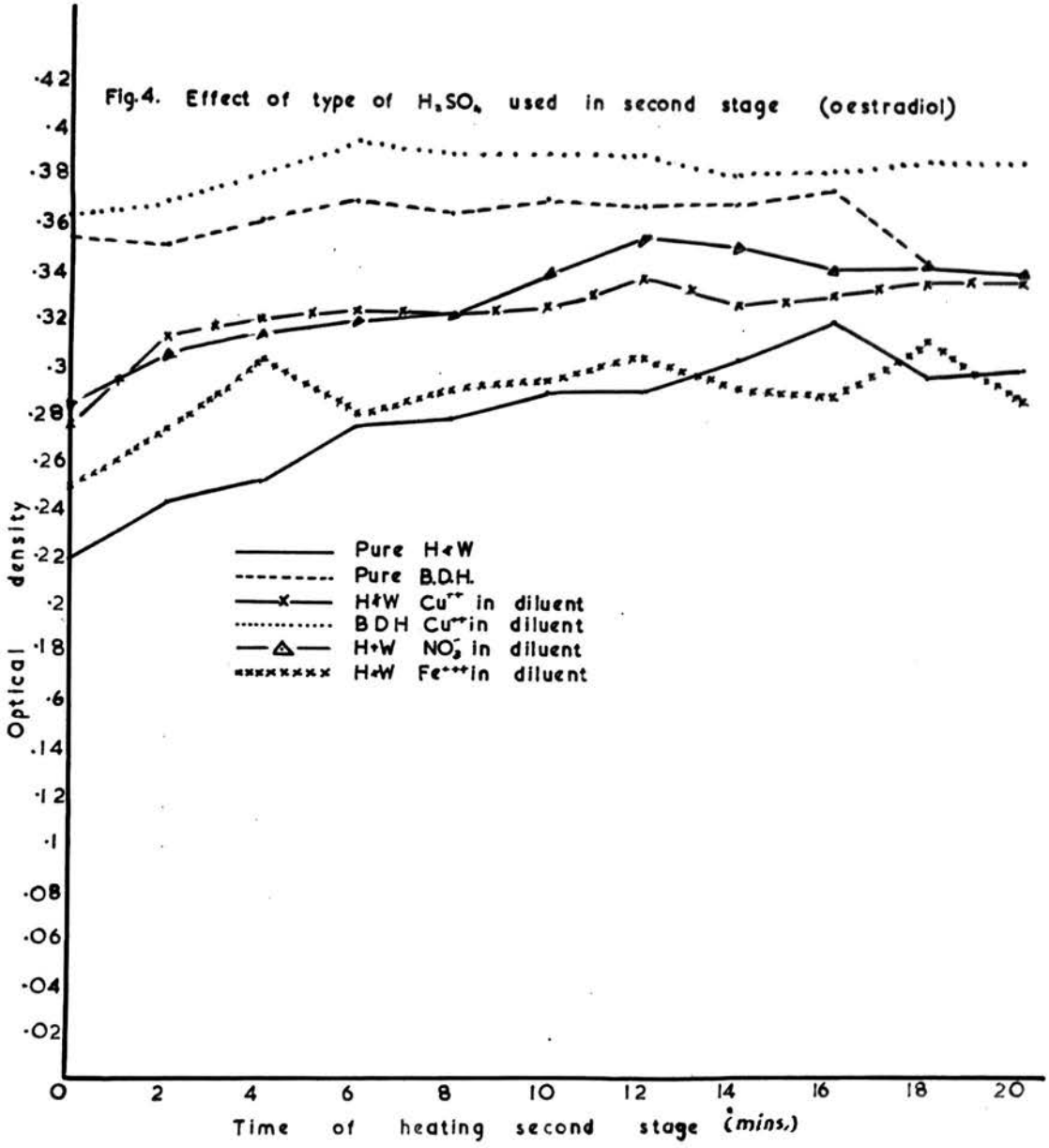


Fig.4. Effect of type of H_2SO_4 used in second stage (oestradiol)
Optical density
·42
·4
·38
·36
·34
·32
·3
·28
·26
·24
·22
·2
·18
·6
·14
·12
·1
·08
·06
·04
·02
0
2
4
6
8
10
12
14
16
18
20
Time of heating second stage (mins.)
Pure H+W
Pure B.D.H.
H+W Cu^{++} in diluent
B D H Cu^{++} in diluent
H+W NO_3^- in diluent
H+W Fe^{+++} in diluent

present colour method the effect of A.R. H_2SO_4 from two manufacturers (British Drug Houses and Hopkins & Williams) was tried. The results with oestradiol-17β are shown in Fig. 4 on the facing page. The marked difference between $O.D._{804}$ obtained with reagents made from B.D.H. sulphuric acid and those obtained from Hopkin & Williams reagents, as well as the markedly different slopes of the graphs, is apparent. The effect of inclusion of 30 mg.% (w/v) of $CuSO_4.5H_2O$ in the 60% H_2SO_4 used for dilution is also shown. Qualitatively similar effects were found by the addition of 0.3 mg.% $NaNO_3$, 30 mg.% $FeNH_4(SO_4)_2.12H_2O$.

These results were considered highly significant and it was felt that they rendered all conclusions so far reached virtually valueless. In the first place, it was apparent that the type of sulphuric acid used could cause differences in intensity of colour as wide as 15%, and although this could be corrected by the inclusion of standard solutions in each oestrogen assay, the possibility existed that certain batches of sulphuric acid would give so little colour as to diminish seriously the sensitivity of the method. Secondly, the investigation was clearly not being conducted under conditions optimal for the production of the colour, so that trace contamination introduced during the course of the reaction might cause erroneous results. Finally the alteration in the shape of the

time/

time heating curves suggested that conditions chosen as most suitable for the exclusion of Type II inhibition without fading might vary from one reagent to another. This was the most serious defect, and although not apparent from Fig. 4 is a real danger, as will be shown later.

It was felt at this time that these effects seen with different batches of sulphuric acid were related to the deviation from Beer's law found at the higher concentrations. It seemed possible that these sub-optimal conditions resulted from a deficiency of some particular oxidant in the sulphuric acid or reagent, and that this defect would become more pronounced at a higher concentration of oestrogens. As will be seen, this supposition was later proven to be at least partly false, but the idea was responsible for the use of 50μg. quantities of oestrogen in the subsequent investigations.

The initial approach to the problem was entirely empirical. It was felt that an ideal oxidizing agent had to be found similar to that presumably present in B.D.H. H_2SO_4. On this basis literally scores of experiments were carried out using possible contaminants of sulphuric acid such as Pb, NO_3^-, NO_2^- and Fe^{+++}. The results were inconclusive, demonstrating only the instability of the reagents and the enhancing effects/

effects of oxidizing agents in general. The inclusion of the oxidant in the first stage gave more intense colours than if it was merely added with the second stage diluent; moreover the intensity varied with the age of the reagent. A most informative experiment is shown in Table 14.

Table 14. Effect of time of addition and age of reagent on enhancement of colour produced by hydrogen peroxide (oestradiol-17β, 50μg.)

Addition of 50μg. of H_2O_2 in first stage.

	D_{604}	D_{601}
5 weeks old reagent	0.682	0.161
	0.697	0.192
Fresh reagent	0.770	0.165
	0.762	0.166

Addition of 50μg. of H_2O_2 in second stage.

	D_{604}	D_{601}
3 weeks old reagent	0.613	0.190
	0.698	0.176
Fresh reagent	0.666	0.169
	0.687	0.170

N.B. 3.5 ml. of 60% (v/v) Hopkins & Williams H_2SO_4 added in second stage, heated 15 minutes.

One notices that greater intensities were produced by the addition of the oxidizing agent in the first stage of the reaction. Moreover the deeper colour was found with the freshly prepared reagent. This suggested that the degree of sulphonation of the hydroquinone/

hydroquinone in the reagent affected the enhancement of colour by the added oxidizing agent. Since hydroquinone monosulphonate is more stable to oxidation than free hydroquinone (Pinnow, 1917) it was possible that the oxidants were exerting their effect on the reaction of the oestrogens indirectly, by oxidation of the hydroquinone itself. When Professor Marrian's suggestion that quinone be added to the reagent was investigated, it was demonstrated that a large excess of hydroquinone was required in the reagent in addition to the quinone. Moreover, the order in which the quinone and hydroquinone were added made a significant difference. The results of a typical experiment using 60% H_2SO_4 concentration for the second stage are shown in Table 15.

Table 15. Effect of mode of addition of quinone and hydroquinone in preparation of the reagent.

Reagent	D_{604}	D_{601}
1	0.775	0.159
	0.761	0.162
2	0.685	0.183
	0.674	0.192
3	0.663	0.186
	0.654	0.196

Solvent throughout was 60% (v/v) Hopkins & Williams H_2SO_4.

Reagent 1 - 2 mg.% quinone in solution, then made 2% hydroquinone by addition of solid (opalescent)

Reagent 2 - 2% hydroquinone in solution, 2 mg.% quinone added.

Reagent 3 - 4% hydroquinone in solution added to equal volume of 4 mg.% quinone in solution.

One/

Table 16. Effect of varying mixtures of quinone/hydroquinone in the reagent.

Reagent	D_{604}	D_{601}
1. 2 mg.% quinhydrone in 60% H_2SO_4	0.553 0.553	0.243 0.243
2. Reagent 1 + 0.5% hydroquinone	0.638 0.658	0.174 0.179
3. Reagent 1 + 2% hydroquinone	0.694 0.684	0.170 0.170
4. 100 mg. hydroquinone in 60% H_2SO_4 containing 1 mg.% $NaNO_3$	0.569 0.550	0.158 0.150
5. 2 g. hydroquinone in 60% H_2SO_4 containing 1 mg.% $NaNO_3$	0.690 0.676	0.167 0.167

One sees that the most effective reagent was prepared by adding solid hydroquinone to a solution of quinone in 60% (v/v) H_2SO_4 and that the least effective resulted from mixture of the two in solution. This finding was confirmed repeatedly. The former reagent was opalescent but removal of this opalescence by filtration through porosity 4 sinter glass did not lessen its effectiveness. It seemed desirable before proceeding further to obtain some idea of the mechanism of these peculiar effects.

Brown (1952c) suggested that the hydroquinone reagent on ageing in fact represented an oxidation-reduction system of the hydroquinone - quinone pair. The peculiar results just reported suggested that something more than this was in fact required, and experiments were carried out to test the hypothesis. The findings, shown in Table 16 opposite, suggest that a poised system of hydroquinone/quinone is not desirable but that a large excess of hydroquinone is required, since reagents 2 and 3 are better than reagent 1, and reagent 3 than reagent 2. The results obtained on addition of hydroquinone to 60% sulphuric acid containing an oxidizing agent were highly significant. Reagent 4 was a pure lemon yellow much more intense than reagent 5. Assuming this to be due to the greater content of quinone in the former reagent/

reagent, one might suspect that in the preparation of reagent 4 there was enough oxidizing agent to convert the hydroquinone through the semiquinone to the quinone, whereas in reagent 5, with its much larger content of hydroquinone, more of the available oxidizing agent was utilized in the conversion of hydroquinone to the intermediate stage. At any rate, no other explanation of this effect suggests itself. It is true that semiquinones do not exist in dilute acid solution due to the availability of hydrogen ions (Michaelis, 1935), but it is conceivable that this situation does not apply in strong H_2SO_4 solutions.

It was demonstrated that hydroquinone was much more soluble in 60% H_2SO_4 than quinone, and quinone more soluble than quinhydrone. Dissolving quinone in 60% H_2SO_4 gave a colourless solution which on heating turned green and became cloudy. This occurs in all types of sulphuric acid and is not prevented by oxidizing agents. Addition of solid hydroquinone causes a disappearance of the green colour, the solution becoming pale yellow and cloudy. The turbidity may be due to the formation of the semiquinone radical, but regardless of the mechanism, for each individual type of sulphuric acid, this method of preparing the reagent gives a cloudy solution which after filtration causes maximum pink colour formation with oestrogen on

applying/

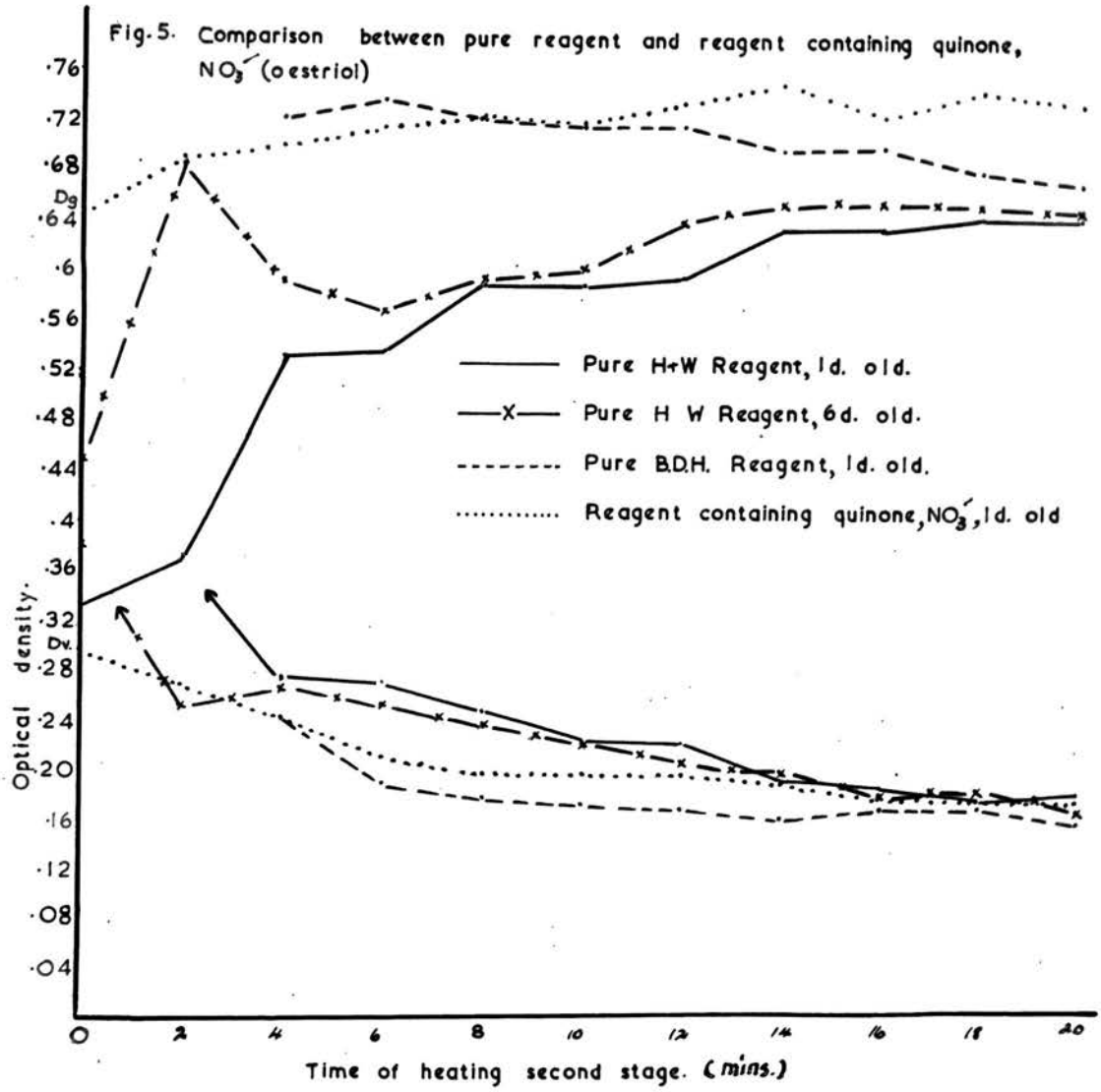


Fig. 5. Comparison between pure reagent and reagent containing quinone, NO_3^- (oestriol)
Optical density.
Dg
Dv.
·76
·72
·68
·64
·6
·56
·52
·48
·44
·4
·36
·32
·28
·24
·20
·16
·12
·08
·04
0
2
4
6
8
10
12
14
16
18
20
Time of heating second stage. (mins.)
Pure H+W Reagent, 1d. old.
Pure H W Reagent, 6d. old.
Pure B.D.H. Reagent, 1d. old.
Reagent containing quinone, NO_3^-, 1d. old

applying the modified hydroquinone reaction. A slight difference (about 5%) can still be found between H_2SO_4 from different suppliers. This difference can be eliminated by the addition of 1 mg.% $NaNO_3$ to the diluted H_2SO_4 before the quinone. Since the type of sulphuric acid used in the diluent was found to influence the reaction slightly, this possible variation was eliminated by returning to the former practice of adding distilled water in the second stage in order to obtain the required sulphuric acid concentration.

4. Modified Procedure and Reagent

A comparison of the two reagents in the modified procedure with 50μg. of oestriol is shown in Fig. 5 on the facing page and Appendix IV, p. 23 . Preliminary experiments showed that a second stage concentration of approximately 55% was required in order to avoid Type II inhibition with the Hopkins & Williams reagent, and this was obtained by heating 5.5 ml. of the reagent and 100 mg. solid hydroquinone for 20 minutes and diluting with 2 ml. of water. The figure shows that low values of $O.D._{.604}$ are obtained, that the ratio of $O.D._{.604}/O.D._{.601}$ gradually rises with increasing heating time, and that occasional high values result, due probably to chance contamination. Ageing of the reagent aided the conversion somewhat but did not increase the intensity significantly or lessen the irregularity/

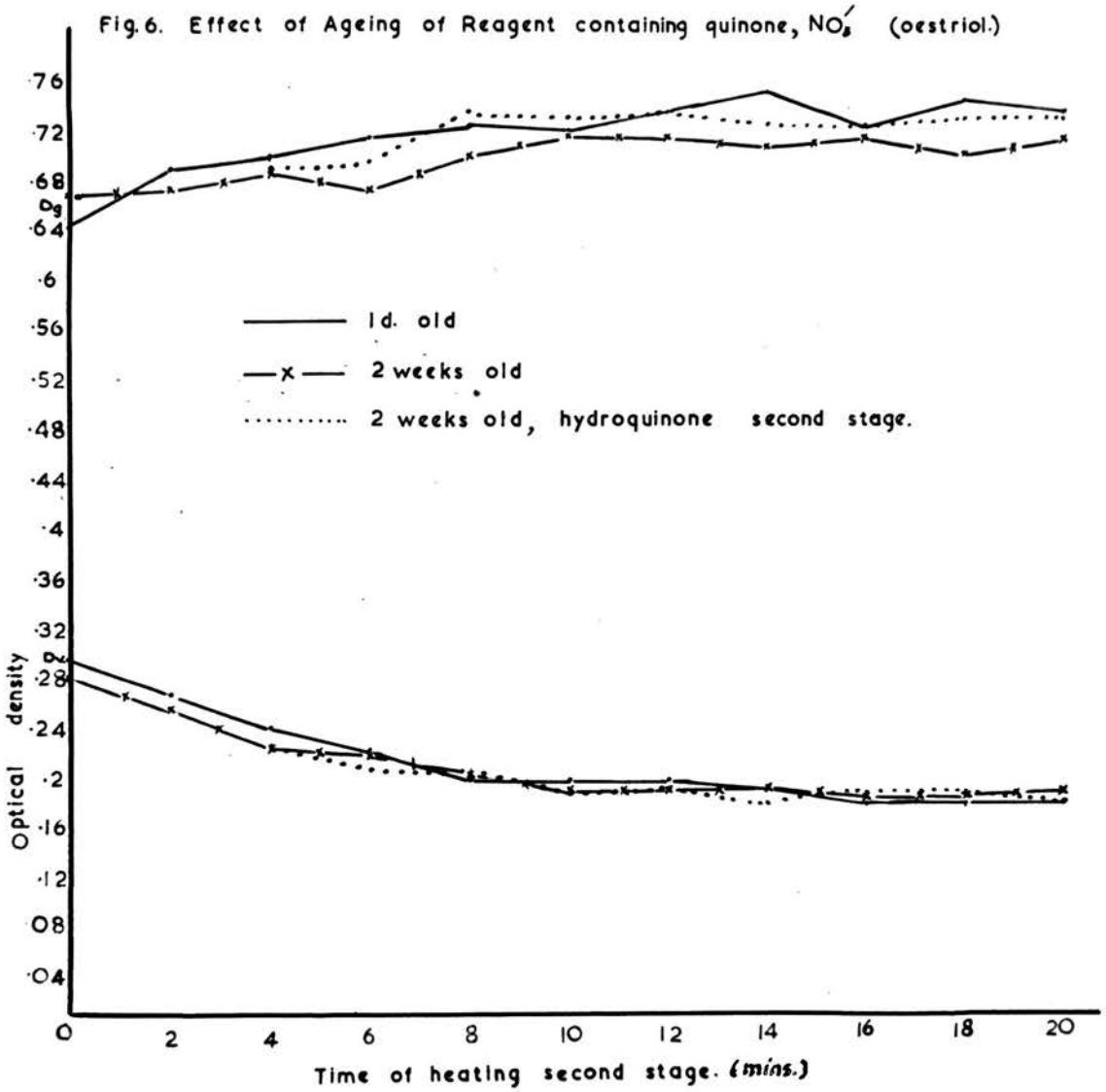


Fig. 6. Effect of Ageing of Reagent containing quinone, NO3' (oestriol.)
·76
·72
·68
·64
·6
·56
·52
·48
·44
·4
·36
·32
·28
·24
·2
·16
·12
·08
·04
Optical density
1d. old
2 weeks old
2 weeks old, hydroquinone second stage.
0
2
4
6
8
10
12
14
16
18
20
Time of heating second stage. (mins.)

irregularity. It is apparent that the conditions optimal for avoidance of Type II inhibition with the Hopkins & Williams reagent in fact result in a tendency to fading when the B.D.H. reagent is used. The use of freshly prepared modified reagent containing NO_3^- and quinone and a second stage concentration of 60%, clearly facilitated the conversion of yellow to pink and produced maximum intensity of colour.

Fig. 6 of the facing page shows the effect of ageing on the modified reagent. There is a slight decrease in $O.D._{504}$ attended by a diminution in $O.D._{504}/O.D._{501}$. The latter effect is shown more clearly in Appendix IV, p. 25 . This was felt to be undesirable when a colour correction formula is being used, and it is seen that the effect can be reversed by adding another 100 mg. hydroquinone at the start of the second stage. A similar addition to the pure reagent also corrected this tendency but did not remove the other defects already noted. Since the precise amount added is not critical, for convenience a spoon spatula was cut down so as to hold when filled the required amount of powdered hydroquinone. This readily dissolves within 4 minutes. On the basis of these results this reagent and a second stage heating time of 15 minutes were adopted. The full details of /

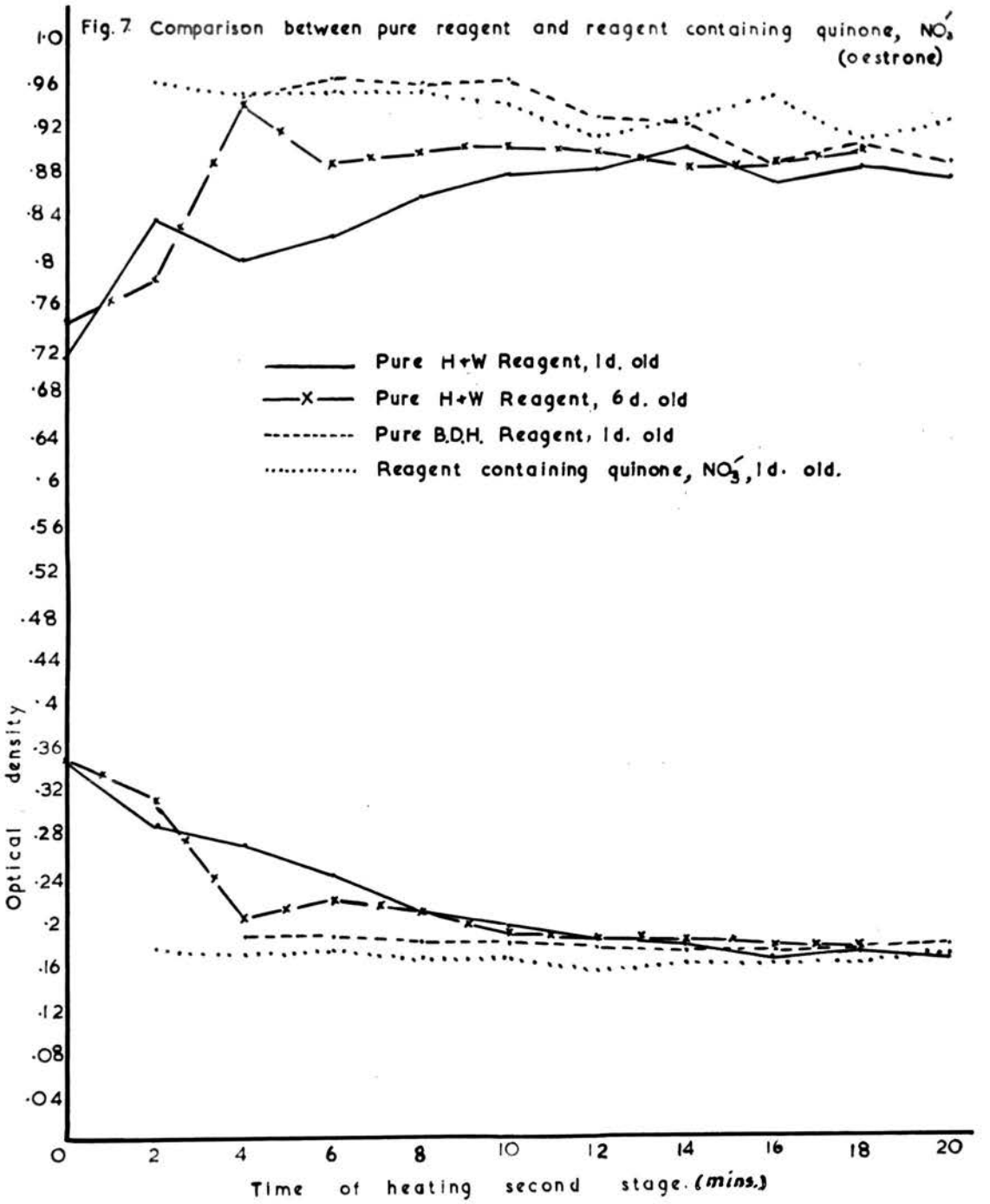


Fig. 7 Comparison between pure reagent and reagent containing quinone, NO_3^- (oestrone)
Pure H+W Reagent, 1d. old
Pure H+W Reagent, 6d. old
Pure B.D.H. Reagent, 1d. old
Reagent containing quinone, NO_3^-, 1d. old.
Optical density
1·0
·96
·92
·88
·84
·8
·76
·72
·68
·64
·6
·56
·52
·48
·44
·4
·36
·32
·28
·24
·2
·16
·12
·08
·04
0
2
4
6
8
10
12
14
16
18
20
Time of heating second stage. (mins.)

of the procedure are given in Appendix II, p. 8.

A comparison between the pure and modified reagents under optimal conditions for each is shown in Fig. 7 on the facing page and Table VII, Appendix IV, p. 26 . Preliminary experiments showed with all reagents that there is an increase in D_{601} with ageing and that this could be largely prevented by the addition of 100 mg. of solid hydroquinone at the start of the reaction. This is similar to the procedure for oestriol, in which in any case fresh hydroquinone is required in order to prevent Type I inhibition. The addition was therefore incorporated into all the reactions. The results shown are quantitatively similar to those found with oestriol. Using 2% hydroquinone in 66% (v/v) H_2SO_4 (Hopkins & Williams), i.e. 'pure reagent', one finds a certain amount of Type II inhibition, but with 15 minutes heating at a second stage concentration of 53% H_2SO_4, this is avoided without fading, though an occasional erratic result is seen. Ageing of the reagent under these same conditions exhibits fading and greater intensity of colour. With the modified reagent maximum intensity is obtained together with stability, freedom from fading and the highest $O.D._{604}/O.D._{601}$. The actual difference in intensity between the two reagents is less than in the case of oestriol but still significant.

The/

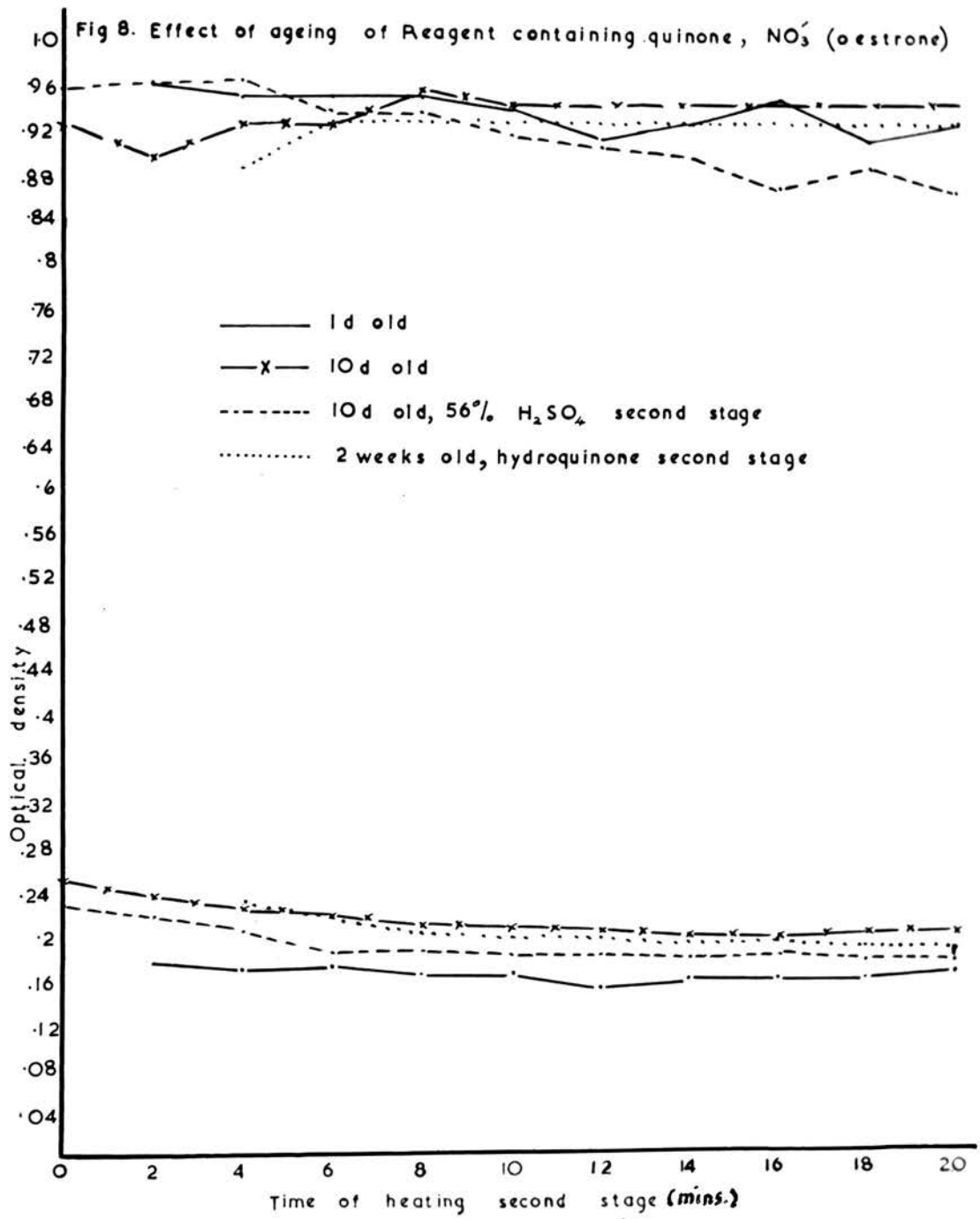


Fig 8. Effect of ageing of Reagent containing quinone, NO_3^- (oestrone)
1·0
·96
·92
·88
·84
·8
·76
·72
·68
·64
·6
·56
·52
·48
·44
·4
·36
·32
·28
·24
·2
·16
·12
·08
·04
Optical density
1 d old
10 d old
10 d old, 56% H_2SO_4 second stage
2 weeks old, hydroquinone second stage
0
2
4
6
8
10
12
14
16
18
20
Time of heating second stage (mins.)

The effect of ageing of the modified oestrone reagent is shown in Fig. 8 on the facing page. The ageing removes the slight irregularities seen with the fresh reagent and gives a perfectly stable reaction with no tendency to Type II inhibition or fading. It has caused a diminution in the $O.D._{SO_4}/O.D._{SO_1}$ which in this case is slightly improved by the addition of hydroquinone. Here too, for uniformity of procedure it was decided to add 100 mg. of hydroquinone in the second stage as well. Also shown are the results obtained with this modified reagent at a second stage concentration of 56%. These show a slight tendency to fading. This result is included because, as will be seen, it was one of the methods used in an endeavour to make the reaction conform completely with Beer's law.

The procedure adopted therefore was to use the modified reagent at a second stage concentration of 60% H_2SO_4, adding 100 mg. of solid hydroquinone at the start of the first and second stages. The precise details of the procedure are given in Appendix II, p. 8 . The stability of this reaction was seen in experiments in which trace amounts of $SnCl_2$, $CuSO_4.5H_2O$, As_2O_3, $NaNO_3$, added with the diluting water in the second stage, produced virtually no change in the intensity of the coloured product.

Fig. /

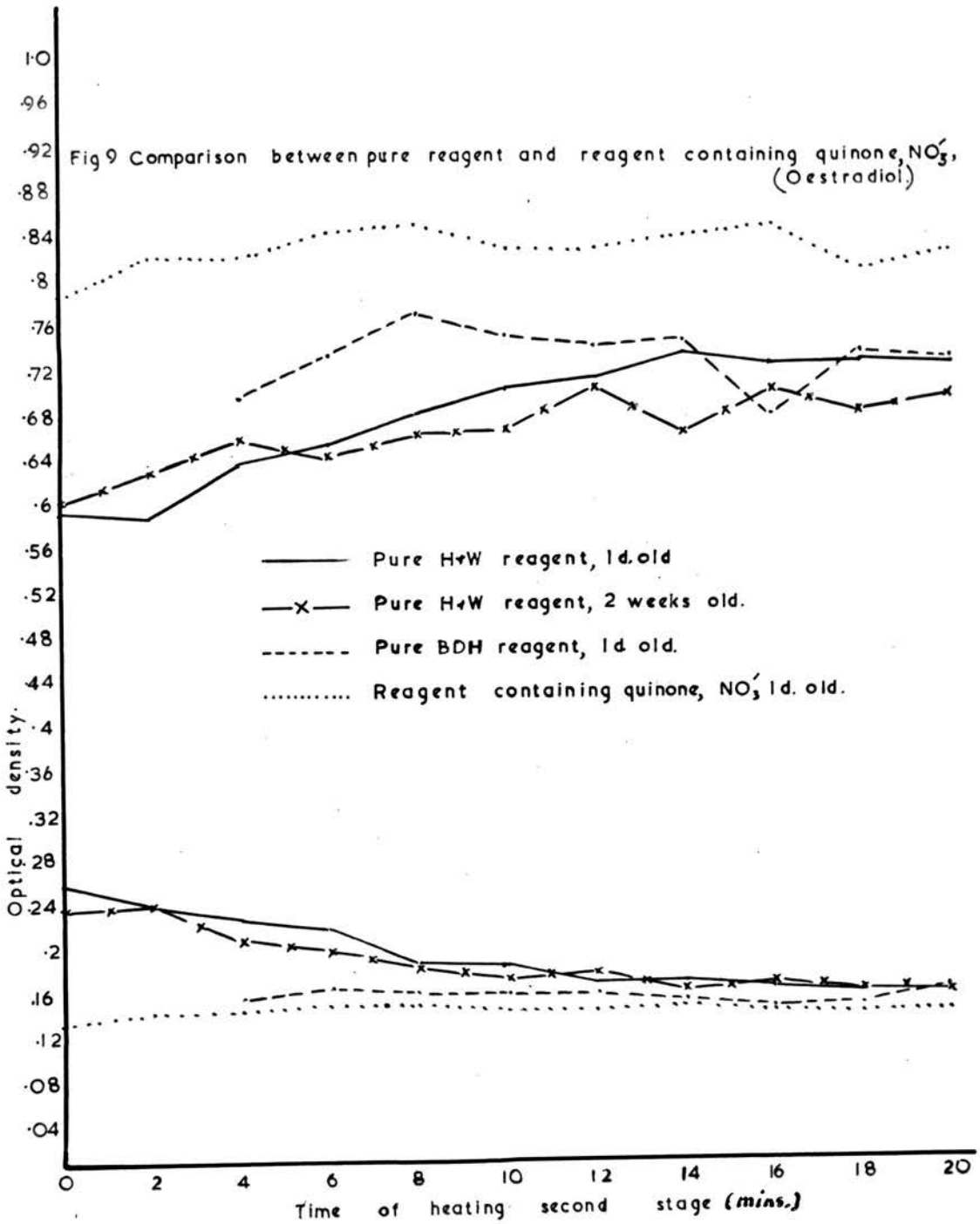


Fig 9 Comparison between pure reagent and reagent containing quinone, NO3', (Oestradiol.)
Pure H+W reagent, 1d. old
Pure H+W reagent, 2 weeks old.
Pure BDH reagent, 1d. old.
Reagent containing quinone, NO3' 1d. old.
Optical density.
1·0
·96
·92
·88
·84
·8
·76
·72
·68
·64
·6
·56
·52
·48
·44
·4
·36
·32
·28
·24
·2
·16
·12
·08
·04
0
2
4
6
8
10
12
14
16
18
20
Time of heating second stage (mins.)

Fig. 9 on the facing page and Appendix IV, p. 29 show the result of a comparison for 50μg. of oestradiol-17β between pure and modified reagents under optimal conditions for both. Hydroquinone (100 mg.) is added in the first stage to prevent the slight decrease in intensity otherwise seen with all the reagents. The pure reagent with Hopkins & Williams H_2SO_4 requires a 56% second stage concentration to avoid Type II inhibition without fading. Ageing causes a slight fall off in intensity and diminution of $O.D._{604}/O.D._{501}$. Under the same conditions a pure reagent prepared from B.D.H. H_2SO_4 shows a very slight tendency to fading. Maximal intensity is obtained with the modified reagent at a second stage concentration of 60%. An effect specific to oestradiol-17β is seen in these charts: with all reagents there is a variability which is unrelated to the time of heating. A similar effect can be found by carrying through quadruplicate determinations. The variation is unrelated to type of reagent used or the H_2SO_4 concentrations of the first or second stages and seems to be specific for oestradiol-17β. This may be due to a difference in the colour complex obtained (see Fig. 11, p. 61) for difference in absorption spectra . The addition of various oxidizing and reducing agents in the second stage was without effect but when the standardized procedure was used with the modified/

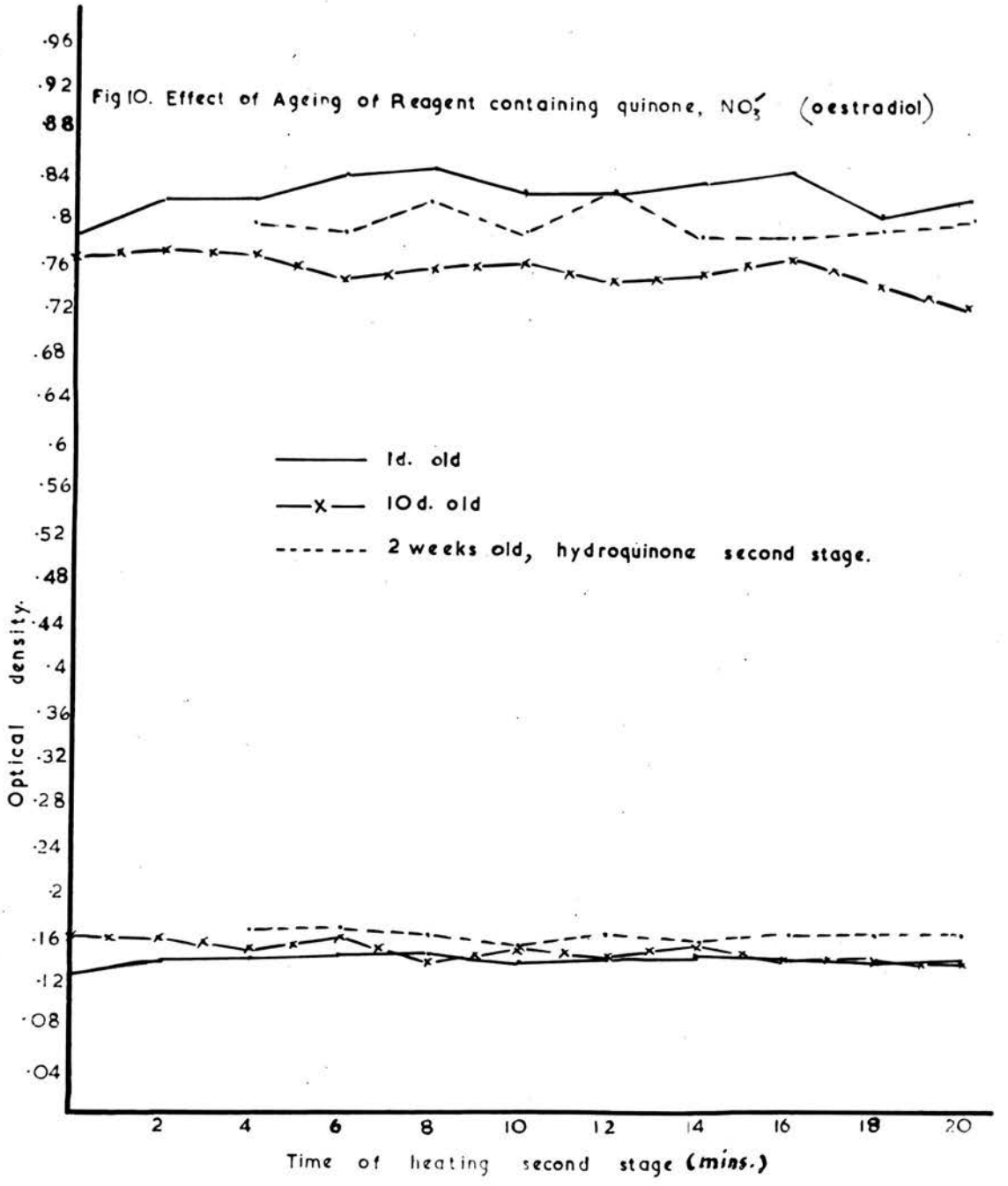


Fig 10. Effect of Ageing of Reagent containing quinone, NO_3^- (oestradiol)
1d. old
10d. old
2 weeks old, hydroquinone second stage.
Optical density.
·96
·92
·88
·84
·8
·76
·72
·68
·64
·6
·56
·52
·48
·44
·4
·36
·32
·28
·24
·2
·16
·12
·08
·04
2
4
6
8
10
12
14
16
18
20
Time of heating second stage (mins.)

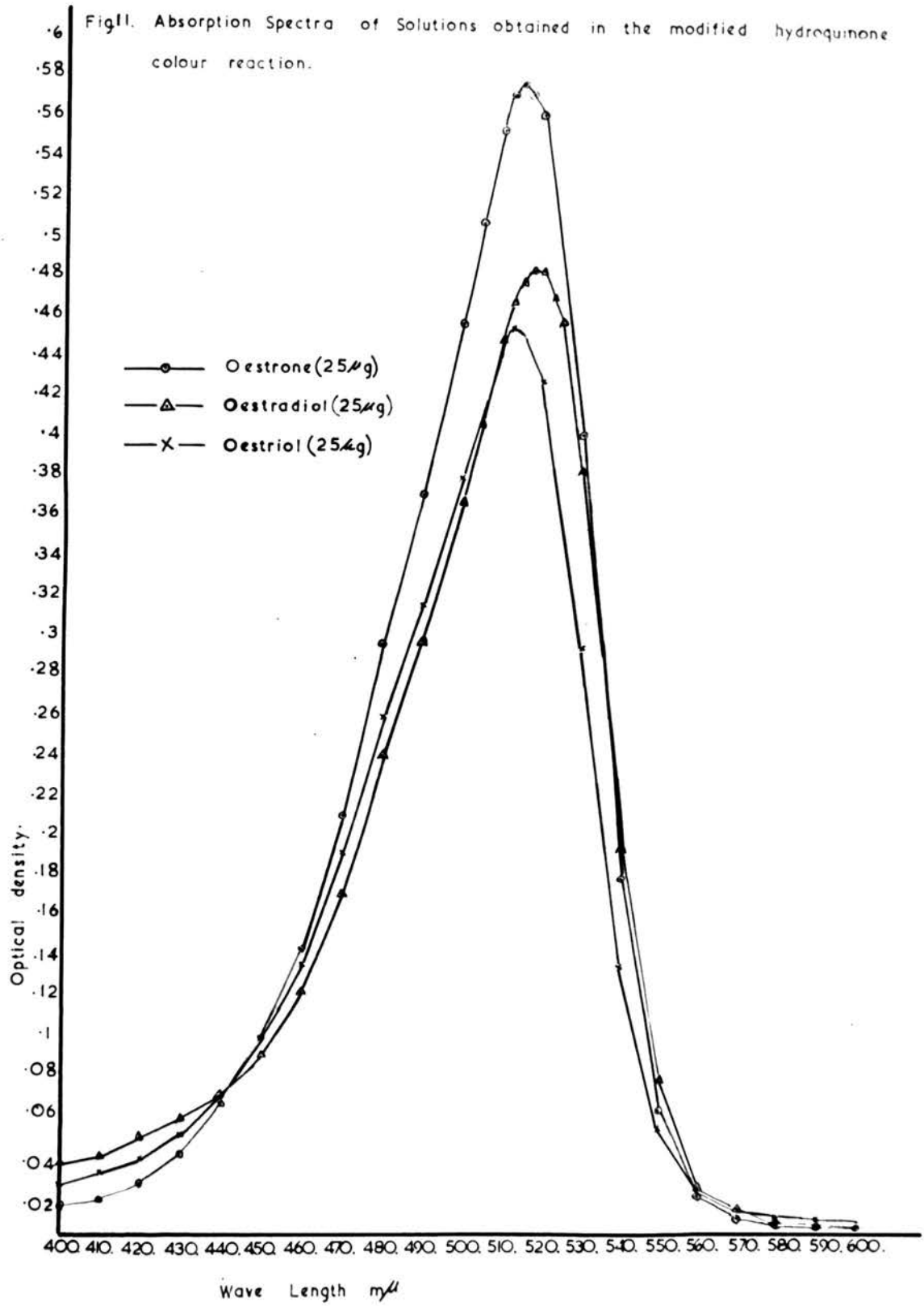
modified reagent variation in quadruplicates dropped from 9% with a fresh reagent to 4% after 5 days ageing, with no scatter at all after two weeks.

Fig. 10 completes the series in showing the effect of ageing the modified reagent. Here again the addition of solid hydroquinone in the second stage appears to be advantageous in preventing the diminution in intensity. It has therefore been incorporated in the finalized procedure, again to achieve uniformity. The details of the modified colour reaction and reagent are given in Appendix II, p. 8.

The results reported in this section show the necessity of modifying the hydroquinone reagent in order to secure satisfactory colour development. There is such a large variation in different types of A.R. sulphuric acid that conditions cannot be made suitable for all brands. Thus a Hopkins & Williams reagent requires a second stage of 50-55% H_2SO_4 in order to overcome Type II inhibition; at these concentrations reagents made from B.D.H. acid show fading. This precludes the use of an unmodified reagent and can only be avoided by pooling large amounts of acid and developing the operating conditions at the start of each series. Moreover it is possible that certain brands of untreated sulphuric acid inhibit the formation of the colour completely. It is of interest that Miller and Muntz (1938) were unable to

obtain/

Fig 11. Absorption Spectra of Solutions obtained in the modified hydroquinone colour reaction.



obtain any colour with acetaldehyde using p-hydroxy-diphenyl in sulphuric acid purified by distillation. If sulphuric acid is treated after dilution with 1 mg.% of sodium nitrate uniformity is achieved in the oxidation reduction potential of the system. The addition of hydroquinone to 2 mg.% quinone in acid so treated yields a reagent which does not require ageing to reach maximum values in colour development and which is stable. Its sole disadvantage is that the blanks are slightly coloured, but this has not been found to cause any inaccuracies. Reagents prepared in this way from B.D.H. A.R. sulphuric acid have exactly the same characteristics in the second stage as Hopkins and Williams modified reagents in regard to intensity, pattern of development and stability.

The absorption characteristics in the visible range of the colours developed by oestrone, oestradiol-17β and oestriol with the modified reagents and procedure (Appendix II, p. 8) are shown in Fig. 11 on the facing page, the ratio of intensities being 1:0.84:0.79 respectively. Measurements were carried out in 1 cm. cell with Unicam spectrophotometer SP-500. There is a slight difference in the absorption at 400-430 mμ., and this also is apparent from Tables vi-xi of Appendix IV, p. 23 , where the $O.D._{604}/O.D._{601}$ varies slightly for the three oestrogens. This necessitated/

necessitated a slight modification in the divisor of the correction formula (Brown, 1952c) which up to this time had been:

$$D_{corr.} = \frac{D_{s04} - 3D_{s06} - D_{s01}}{3.8}$$

Table 17. Investigation of appropriate factor for colour correction equation.

	D_{s04}	D_{s01}	D_{s06}	Factor
12.4μg. of oestrone	0.258	0.058	0.005	3.72
	0.249	0.051	0	3.81
	0.252	0.049	0	3.81
	0.260	0.052	0.002	3.78
24.8μg. of oestrone	0.480	0.100	0.002	3.79
	0.490	0.100	0.004	3.78
	0.495	0.107	0.006	3.75
	0.502	0.111	0.006	3.74
49.6μg. of oestrone	0.930	0.190	0.006	3.78
	0.930	0.192	0.008	3.77
	0.940	0.195	0.010	3.76
	0.930	0.196	0.008	3.77

The results of a recheck into the appropriate factor for the new procedure and reagent are shown in Table 17 for oestrone. The factor is obtained in each case by calculation in the above equation making $D_{s04} = D_{corr.}$. The mean of all the determinations is 3.77 so that this was selected as the divisor for oestrone. Similar experiments established factors of 3.72 for oestradiol-17β, and 3.69 for oestriol.

5. Deviation from Beer's Law

Clayton (1949) had noted the failure of the phenol-sulphonic acid reaction to conform to Beer's law. Throughout this investigation with all the reagents used the same tendency was found. On the other hand

most/

Table 18. Deviation from Beer's law due to Type II inhibition.

	Reaction 1				Reaction 2			
µg. oestrone	D_{604}	D_{601}	$\frac{D_{604}}{D_{601}}$	$\frac{D}{D_{12.5}}$	D_{604}	D_{601}	$\frac{D_{604}}{D_{601}}$	$\frac{D}{D_{12}}$
12.5	0.223	0.084	2.7		0.242	0.044	5.5	
	0.222	0.084	2.6		0.232	0.054	4.3	
25.0	0.400	0.148	2.0	1.8	0.473	0.094	5.0	2.0
	0.400	0.150	2.9		0.473	0.094	5.0	
37.5	0.607	0.208	2.9	2.7	0.690	0.138	5.0	2.9
	0.592	0.203	2.9		0.680	0.138	4.9	
50.0	0.780	0.279	2.8	3.51	0.868	0.182	4.8	3.7
	0.775	0.277	2.8		0.892	0.195	4.6	

most of the published methods (cf. Venning et al., 1937; Brown, 1952b) claim a linear relationship between optical density and concentration. Szego and Samuels (1943) on the other hand found deviations from linearity at higher concentration. In the present investigation a very large number of experiments has been done in an attempt to explain this discrepancy. It appears that three separate and distinct factors are involved and these will be discussed in turn.

a. Deviations from Beer's law due to Type II inhibition

On theoretical grounds it seemed likely that when the colour reaction was carried out under conditions in which the conversion of yellow to pink was difficult greater relative intensities could be produced at the lower concentrations. An example of this effect is shown in Table 18 shown on the facing page.

In the first reaction 6.8 ml. of a three month old 2% hydroquinone in pure Hopkins& Williams 66% (v/v) H_2SO_4 were added to the dry residues in Kober tubes and heated for 20 minutes. After cooling 0.7 ml. H_2O was added, and reheated for 15 minutes after thorough mixing. For the second reaction the same reagent and heating times were used, but 6 ml. only used in the first stage and 1.5 ml. H_2O added as the diluent. It will be seen that $D/D_{12.5}$ deviates more from the theoretical values of 2, 3 and 4 in the former/

former case. The low D_{e04}/D_{e01} seems to indicate Type II inhibition and is indeed a possible cause of deviation from Beer's law. In the development of the modified procedure, however, 50μg. of oestrogen were used and second stage concentrations were adjusted to avoid Type II inhibition. Under these optimal conditions for ease of conversion of yellow to pink deviations from Beer's law cannot be due to Type II inhibition.

b. Conformity with Beer's law due to fading.

By the same reasoning it should be possible to use conditions for the second stage which would allow fading to occur, thus affecting the lower concentrations more. An example of this type of effect is shown in Table 19.

Table 19. Effect of fading on relation between optical density and concentration

μg. oestriol	D_{e04}	D_{e01}	$\frac{D_{e04}}{D_{e01}}$	$\frac{D}{D_{12.5}}$
12.5	0.171	0.030	5.7	
	0.171	0.031	5.5	
25.0	0.334	0.061	5.5	1.99
	0.346	0.068	5.0	
37.5	0.504	0.104	4.8	2.93
	0.510	0.110	4.6	
50.0	0.685	0.150	4.6	4.02
	0.690	0.155	4.4	

These results were obtained by using the modified oestriol reagent containing quinone and nitrate, but the/

the reaction was altered from the standard one by using 5.5 ml. of reagent in the first stage and diluting with 2 ml. of H_2O. These conditions were shown to cause fading on heating during the second stage. It will be seen that linearity has been achieved but that D_{604}/D_{601} falls as the concentration rises. In this example the fading has been just enough to counteract the tendency to deviation from Beer's law which seems to be inherent in the reaction, and thus achieves linearity of the relationship between optical density and concentration. Obviously on occasion the two opposing effects may be unequal, so that relatively more intense colours could apparently be developed at the higher concentrations. It is significant that Clayton (1949) also noted this effect.

c. Inherent lack of conformity with Beer's law.

In the procedures which have been developed in this investigation Type II inhibition and fading have been effectively eliminated from the colour reaction. Nevertheless strict conformity with Beer's law has never been obtained even under optimal conditions and regardless of the reagent used. This has already been shown in Table 18, p. 63 for a pure Hopkins & Williams reagent. It is true also of B.D.H. pure reagent and the modified reagent recommended in this investigation. This fact (with the probable explanation/

explanation) is shown in Table 20.

Table 20. Effect of instrument on deviation from Beer's law.

µg. oestriol	Spekker OD_{604}	D $\overline{D}_{12.5}$	Junior Unicam $OD_{512.5}$	D $\overline{D}_{12.5}$	Unicam SP_{500} $OD_{512.5}$	D $\overline{D}_{12.5}$
12.5	0.189 0.191		0.174 0.174		0.237 0.231	
25.0	0.368 0.362	1.93	0.313 0.323	1.8	0.454 0.450	1.93
37.5	0.560 0.555	2.94	0.455 0.457	2.62	0.684 0.686	2.93
50.0	0.726 0.715	3.8	0.586 0.585	3.36	0.920 0.896	3.88

These results suggest that the slight apparent deviation from Beer's law is inherent in the colour reaction and the degree varies with the particular instrument used. Strict linearity of the relationship between concentration and optical density is only obtained with monochromatic light. Where heterochromatic incident light is used solutions with sharp absorption maxima such as we have here tend to show deviations from Beer's law (West, 1946).

The relation between optical density and concentration for the other two oestrogens in the standardized procedure - Appendix II, p. 8 , is shown in Table 21, the Spekker colorimeter being used.

Table/

Table 21. Relation between optical density and concentration for oestrone and oestradiol-17β.

	Oestrone		Oestradiol-17β	
µg. oestrogen	OD_{604}	$\frac{D}{D_{12.5}}$	OD_{604}	$\frac{D}{D_{12.5}}$
12.5	0.266 0.266		0.218 0.228	
25.0	0.516 0.520	1.95	0.441 0.435	1.97
37.5	0.732 0.735	2.76	0.650 0.640	2.9
50.0	0.944 0.944	3.56	0.812 0.824	3.68

This slight deviation could really be disregarded and the readings taken from a standard curve, but for increased accuracy it is preferable to include known amounts of oestrogen in the range expected to be found in the unknown solutions.

6. Mechanisms of Kober Reaction.

It is considered desirable to record briefly some of the concepts of the mechanism of the Kober reaction which developed in the course of this investigation. Brown's finding(1952b),that the use of aqueous sulphuric acid reagents for the first stage resulted in enhanced colour formation, led him to the belief that sulphonation was notimportant in the reaction. Pinnow (1915-1917) had demonstrated that sulphonation of hydroquinone to form the o- or p-monosulphonate occurred very readily with 15-20M-H_2SO_4, and that use of concentrated H_2SO_4 led to disulphonation and oxidation/

oxidation. A similar effect occurring with the oestrogens would offer an alternative explanation for the decrease in colour at the higher acid concentrations. Certainly the results quoted with m-cresol suggest sulphonation of this monohydric phenol and it is possible that mono-sulphonation of the oestrogens occurs in the first stage of the Kober reaction.

The evidence suggesting that the second stage is oxidative has already been summarized (p. 31). The relation between dilution with water and oxidation is not readily apparent. Raman spectra (Woodward and Horner 1934), and conductivity measurements (Hall and Voge 1933), indicate that dilution of sulphuric acid to 60% (v/v) should actually increase ionization and thus the acidity of the solution. Acidity function determination (Hammett 1940) shows the reverse to be true. Professor R.A. Morton suggested that the discrepancy was due to the formation of ion pairs only revealed by H_0. Brown's theory (1952b) that dilution decreases the acid concentration is therefore probably true. This, however, would have little effect on the oxidation potential of the system.

It is more possible that the main result of the dilution with water is to alter the degree of ionization of the sulphonated oestrogen complexes.

Some/

Some evidence of this was obtained by measurement of the absorption characteristics, in the ultra-violet region of the spectrum, of solutions of oestrone in 60%-100% H_2SO_4. This represents a possible method of further study of the problem, and it is to be hoped that the investigation will be continued. It is of interest that Lantz (1939) demonstrated definite changes in the ionization of aromatic sulphonic acids in the vicinity of 60% (v/v) sulphuric acid. Linford (1952) correlated the change in peaks of absorption in sulphuric acid solutions of oestradiol-17β with the hydroxyl groups in the molecule.

Since oestriol requires a significantly higher concentration of sulphuric acid and longer heating time in the first stage, it is probable that an additional reaction is involved. The similarity of the absorption spectra of the coloured complexes formed with oestrone and oestriol suggests that this may be dehydration of the 16, 17-dihydroxy grouping to oestrone. It is interesting that <u>iso</u>oestriol, in which the hydroxyl groups have a cis relationship to one another, is more sensitive than oestriol in the Kober reaction (Marlow, 1950). Marrian (1938) showed that oestriol but not oestrone could be readily oxidized to a product which turned pink in dilute sulphuric acid, and suggested that the oxidative product was the 16, 17-diketo/

diketo derivative which formed a pink oxonium salt in sulphuric acid. It is the author's belief that in the absence of an effective reducing agent oestriol is oxidized rather than dehydrated to oestrone. Marlow (1950) showed that the 16, 17-diketo derivative did not form a pink colour in the Kober reaction. It is conceivable that the formation of this compound by oxidation is the cause of Type I inhibition, and that reducing agents prevent this formation and allow dehydration to occur.

III. DEVELOPMENT OF A METHOD FOR THE PURIFICATION AND SEPARATION OF OESTRONE, OESTRADIOL-17β AND OESTRIOL IN URINE.

A. Historical Introduction.

The various procedures about to be described for the purification and separation of urinary oestrogens are based directly on earlier work in this field. The modifications are extensions rather than new departures except for the use of partition chromatography, which has been developed independently of the simultaneous work of others in the field. It is therefore considered that a survey of previous investigations is required as an introduction to the results presented below. For simplicity, the discussion will be confined to methods utilizing the Kober reaction or its modifications.

The earliest attempt to place urinary oestrogen determination on a quantitative basis was made in 1934 by Cohen and Marrian. The method was developed as a guide to isolation studies and these workers did not claim strictly quantitative recoveries. However, since their procedure has formed the foundation of all future studies it will be considered at some length. Firstly, they made a significant contribution to the problem of releasing the oestrogens from their water-soluble conjugates/

conjugates found in urine. During the course of the present investigation a review was prepared of the various hydrolytic procedures that have been applied in the initial step of urinary oestrogen determinations. A reprint is appended to this thesis and no further comments will be made on this subject. Cohen and Marrian also fully appreciated the other problems involved and made significant contributions towards their solution.

They selected ether as the solvent for the initial extraction and stressed that it must be peroxide-free. This selection has been fully justified by all subsequent work. Initial purification of the ether extract was achieved by washing with aqueous sodium carbonate. A method of separation of oestrone and oestriol was developed based on differences in their pK values. Thus they demonstrated that oestriol could be removed from ether by washing with N/10 sodium hydroxide without contamination by oestrone. Moreover, they showed that in order to separate oestrone from non-phenolic 'neutral' components in the extract the ether had to be removed and extraction done from a less polar solvent (toluene) with N-sodium hydroxide. In view of these findings they introduced the terms strong and weak phenols for the oestriol and oestrone fractions respectively, retaining the terms acids/

acids and neutrals for the bicarbonate washings and toluene residue respectively. Cohen and Marrian did not claim that their partition was quantitative. They pointed out that traces of oestriol were not in fact removed from the ether by N/10 sodium hydroxide. Moreover, they reported no results for oestradiol-17β.

With the problem inherent in hydrolysis, extraction and partial separation demonstrated and effectively solved for their particular requirements, they turned their attention to the fourth and final problem. This was the removal of non-oestrogenic components which when present in the Kober reaction gave not the typical pink colour (since the reaction is specific for oestrogens), but a brown background colour capable of significant contribution to the absorption in the pink range of the spectrum. Their method of separating acidic and neutral fractions was of considerable benefit. Their extracts, however, still showed the presence of these non-specific chromogens, which were only partially compensated for by addition of hydrogen peroxide to the final coloured product. This caused fading of the pink due to the oestrogens, and had little effect on the non-specific brown colour.

Cartland and his co-workers (1935) used butanol as the initial extractant, removed the acids with sodium carbonate, and introduced the use of partition between/

between aqueous alcohols and hydrocarbons for partial purification and separation. Venning and others (1937) by use of the newly developed photoelectric colorimeter approached the problem from a different standpoint. They found that hydrolysis of the oestrogenic conjugates obtained by butanol extraction of urine caused a significant decrease in non-specific contaminants. In order to avoid losses they did not attempt separation or purification but carried out the phenol-sulphonic acid reaction directly on the residue from ether extraction of the aqueous phase after hydrolysis. The presence of chromogenic impurities was allowed for by a formula based on differences in absorption maxima of the products formed in the Kober reaction by oestrogens and their contaminants.

Smith and her co-workers (1939) reported quantitative separation of oestriol, oestrone and oestradiol-17β by the Cohen-Marrian (1934) procedure, stating that the diol was retained in the weak phenolic fraction. Thus with its isolation from urine (Huffman et al., 1940), they had no hesitation in applying the partition in a study of these two distinct fractions in the urine of women. Since they used bioassay, any loss of oestradiol-17β into the oestriol fraction would have seriously altered their findings. That such loss undoubtedly occurred has been demonstrated by subsequent work (cf. Bachman and Pettit, 1941/

1941). Thus began a series of difficulties which has continued to the present (cf. Smith and Smith, 1952).

An important advance came with the publication of partition coefficients of oestrogens in many of the common solvents (Bachman and Pettit, 1941; Mather, 1942), as the pure oestrogens became available for such studies. From these it became evident that sodium carbonate would remove some oestriol from ether along with the acids. On the basis of the distributions established, a reasonably quantitative method was developed by Bachman and Pettit (1941) for the chemical determination of urinary oestrogens. Ether, or benzene (4 x 1 volumes, urine saturated with salt), was used for the initial extraction, somewhat purer residues being found with benzene. Oestriol was separated from oestrone, oestradiol-17β by extraction from benzene with sodium carbonate solutions. The efficiency of this method was apparent from the partition coefficients and was established independently by Mather (1940). Bachman and Pettit showed also that water was equally effective except in the presence of large amounts of urinary residues. An additional purification of the oestrone, oestradiol-17β fraction was obtained by washing the benzene with strong sulphuric acid. This step caused considerable purification but (as appeared from the partition coefficients) a loss of about 10% of the oestradiol-17β /

17β. It is obvious that purification was still inadequate since the final colour was extracted with benzene to remove impurities.

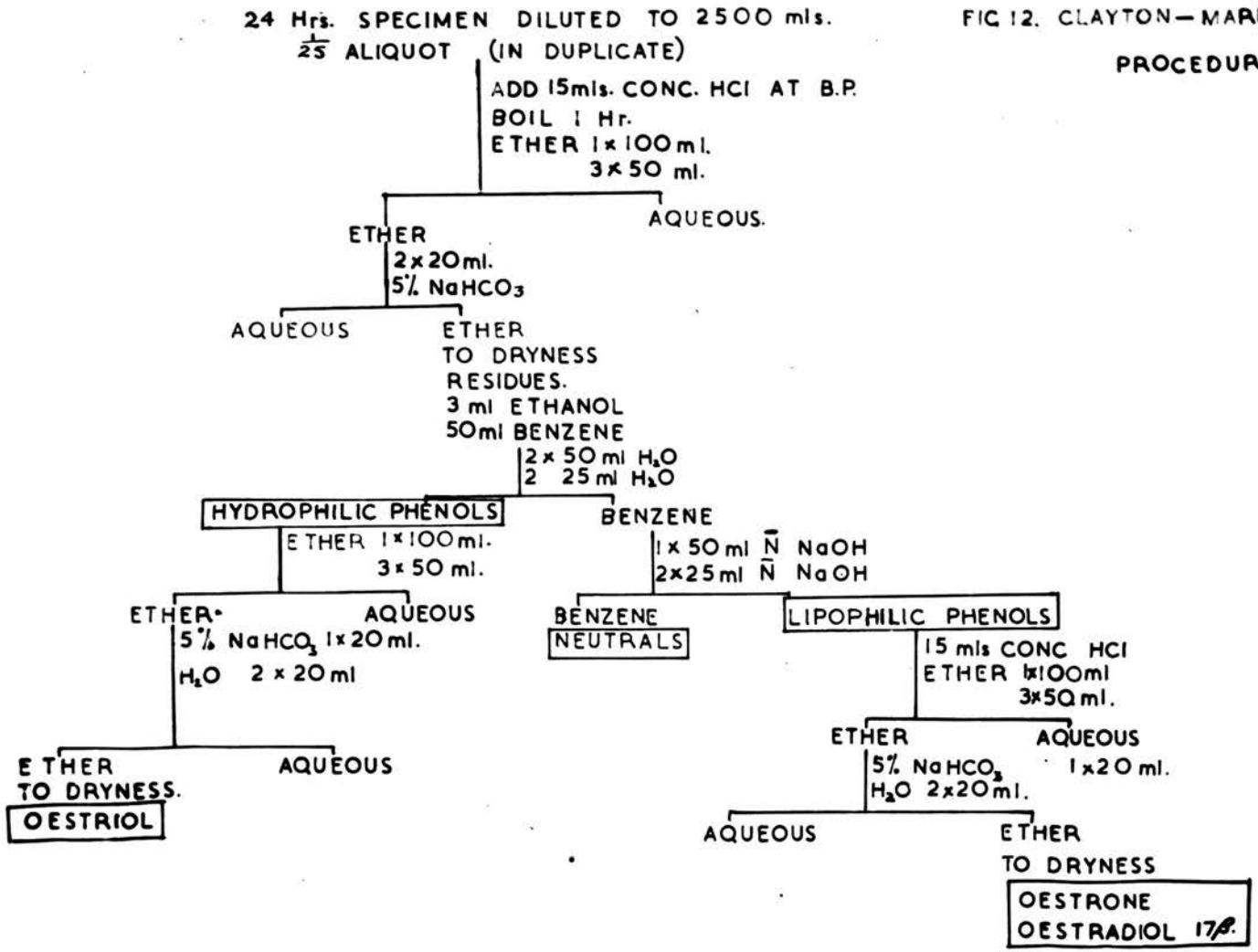
Bachman and Pettit (1941) also pointed out that separation of oestrone and oestradiol-17β was necessary for maximal accuracy in colorimetric assay. At this time techniques using Girard's reagent were applied to this aspect of the problem (Talbot et al., 1941, Pincus and Pearlman, 1941).

In the post-war period, a new approach was made to the problem by the use of adsorption chromatography on columns (Stimmel, 1946a). This technique had been widely used in oestrogen isolation studies (cf. Heard et al., 1941). Stimmel (1946b) reported complete separation of oestradiol-17β, oestrone and oestriol with considerable purification, although a correction was still necessary for the non-specific background colour. Other adaptations of the countercurrent principle followed. Engel and his co-workers applied the discontinuous countercurrent distribution method to the purification and separation of oestrone, oestradiol-17β and oestriol (1950). Haenni and his co-workers (see Banes et al., 1950, Carol et al., 1950) applied partition chromatography with 0.4 N-sodium hydroxide as stationary phase, benzene as mobile phase, to the separation/

separation of various closely related oestrogens. Friedgood and others (1950) using paper chromatography with a propanol-water system determined R_f values for various steroids. The similarity of the values obtained for oestrogens made it clear that this system was unsuited for their separation. Swyer and Braunsberg (1951) have utilized a stationary phase of 2.3 N-sodium hydroxide, mobile phase of benzene to separate and purify oestrone and oestradiol-17β. This has been extended (Stern, Braunsberg and Swyer, 1952) to the separation of oestriol. Nyc and others (1951) used reverse phase partition chromatography for the separation of pure oestrogens. Boscott (1951) has devised a variety of solvent systems for paper chromatography analysis. Mitchell (1952) has described a similar method.

Concurrently there have been occasional reports of chemical separation. Stimmel in 1950 advocated the partition of oestriol and oestradiol-17β as their hemi-phthalates, oestrone as the Girard derivative. It is clear from the variety of published methods that the problem is in a healthy state of activity, but that no entirely adequate method has yet been devised. That familiarity with the literature is essential is shown by a recent attempt (Finkelstein, 1952) to remove phenols from ether instead of benzene (cf./

FIG 12. CLAYTON—MARRIAN PROCEDURE.



(cf. Cohen and Marrian, 1934).

B. General Discussion.

The basic method for the present investigation is shown in Fig. 12 on the facing page. The procedure was developed in this Department (Stevenson and Marrian, 1947; Clayton and Marrian, 1949) and represented the best combination of the classical methods of extraction, purification and partial separation of the oestrogens at that time. Clayton (1949) modified the procedure by substituting ethyl acetate as the extractant at all stages. Quantitative extraction was achieved by the use of only two extractions, one with 100 ml., the second with 50 ml. A disadvantage was that the aqueous phase prior to extraction was neutralized in order to overcome the apparent losses on extraction from acid solutions, in the belief that these were due to hydrolysis of the solvent with release of ethanol. While it is true that mineral acids will catalyse the hydrolysis of ethyl acetate, even in their presence the reaction is a very slow process at room temperature (Lowry and Cavell, 1947). A more probable explanation is the appearance of a trace impurity which interferes with the development of colour. The phenol sulphonic acid reaction was found to be particularly unstable in regard to impurities arising from ethyl acetate (? acetaldehyde)

/see/

(see p. 19), and a return to the use of ether was made at an early stage of the present investigation.

The separation of oestriol from oestrone and oestradiol-17β represented a definite simplification of the older methods. It was shown (Clayton and Marrian, 1949) to be no less efficient than partition with disodium hydrogen phosphate (Friedgood et al., 1948). It is therefore preferable to discard the terms strong and weak phenols, substituting the terms hydrophilic and lipophilic phenols. The dissociation constants for oestrone and oestriol being 0.44×10^{-9} and 0.77×10^{-9} (see Marrian, 1938b), it seemed unlikely that separation could be achieved on this basis. The evidence suggesting separation by partition with water was presented by Bachman and Pettit (1941) and the significance of these findings noted by Clayton and Marrian (1949).

One theoretical objection to their method was the omission of the water washes subsequent to the sodium bicarbonate washing of the urine extract. It was felt that traces of bicarbonate entrained in the ether might be converted to carbonate during the distillation and cause destruction of the oestrogens at this point. From the beginning of this investigation two washings with 20 ml. of water were introduced.

There were two problems in connection with further development of the method: separation of oestrone/

oestrone and oestradiol-17β, and further purification of all fractions. The separation of the two lipophilic phenols, as pointed out by Bachman and Pettit (1941), is necessitated by quantitative differences in their behaviour in all modifications of the Kober reaction. Further purification was essential for quantitative estimation of small amounts of oestrogens in urine. The best method of overcoming the non-specific colour produced by urine residues in the Kober reaction was the fading technique of Stevenson and Marrian (1947), and these authors considered it to be of value only in urines containing more than 2 mg. oestrogen per 24 hour specimen. The various methods which have been applied to the solution of these two problems are considered in the remainder of this section.

C. Preliminary Attempts to Purify the Phenolic Extracts of Urine.

1. Selection of Solvent.

Obviously, the ideal solvent for the initial extraction is one which will extract all three oestrogens efficiently but will have an unfavourable partition coefficient for the impurities. In order to facilitate this and subsequent discussions it is proposed to differentiate between solutes that prefer polar and those that prefer non-polar solvents.

In/

In the absence of such terms it has become the practice to refer to a solute that will partition between polar and non-polar solvents in favour of the former as a polar solute. Thus polarity of a solute is inferred from the polarity of the member of the solvent pair which it prefers. In this discussion the term polaripetal will be used to denote a solute which prefers the more polar solvent of a pair, and polarifugal to denote the reverse.

The difficulty in selecting a suitable solvent for the extraction of oestrogens is due entirely to the fact that oestriol is much more polaripetal than oestrone and oestradiol-17β. Indeed this fact has been used in the present partition procedure. As has already been stated, two extractions 1 x 1, 1 x ½ volume with ethyl acetate quantitatively remove oestriol from an aqueous solution. Unfortunately, they also remove those urinary residues which form brown coloured products in the Kober reaction. Benzene on the other hand is inefficient in removal of non-specific chromogenic material but the partition coefficient for oestriol between benzene and water is only 0.28. This can be increased to 2 by the addition of sodium chloride to the aqueous phase (20% w/v)(Bachman and Pettit, 1941). The standard extraction formula is (Craig and Craig, 1950)

$$Y_n = \left(\frac{1}{Kr + 1}\right)^n$$

where Y_n = amount of solute remaining in aqueous phase after n extractions

K = partition coefficient between organic and aqueous phase

r = ratio of volumes of organic and aqueous phases

n = number of extractions.

Use of this equation shows that in order to extract oestriol from 20% saline, four extractions with equal volumes of benzene (yield 98.8%) or six extractions with half volumes of benzene (yield 98.5%) are required. It is obvious that such procedures are not suitable for a routine method in which 500 ml. portions of urine are to be analysed. Swyer and others (1952) recommended benzene as the initial extractant and suggested volumes less than the theoretical ones listed above. It is perhaps significant that at the same meeting they reported contamination of their oestriol with a more polarifugal substance whose intensity in their fluorescence reaction (Braunsberg, 1952) was much greater than that of oestriol.

Since the partition procedure requires the use of benzene in the second extraction, it is obviously desirable to use benzene as the initial extractant. Accordingly it became necessary to investigate the practicability of continuous extraction with benzene.

Two/

Two problems appeared: destruction of oestrogen and inadequate extraction. Grant (1949) found losses of oestriol on boiling in benzene. Table 22 shows the results of his and other experiments.

Table 22. Effect on oestriol of boiling in benzene

Experiment	Time of boiling hr.	% loss
J.K.Grant	12	42
	6	19, 47
	3	10, 8
Present investigator	6*	29,27,25
	6	4, 2

* Done with redistilled A.R.benzene, labelled thiophene-free.

At this time Professor Marrian was experiencing difficulties with thiophene in toluene, and recommended purification of the benzene by refluxing with concentrated sulphuric acid. (The details of the procedure are given in Appendix I). Boiled in A.R. benzene thus purified, the loss of oestriol was reduced to the insignificant levels shown in the author's second experiment.

A standard continuous extractor (see Appendix II) was used to extract urine saturated with salt. Much difficulty was of course encountered with emulsification, but this was completely prevented by the use of urine purified by the ion exchange procedures to be discussed later. Even under these conditions the yields/

yields obtained were low and erratic, due to the separation of solid particles of oestriol at the interface. This could be overcome only by the addition at frequent intervals of alcohol or acetone to the extractor, and these solvents interfered with the subsequent water partition procedure. The conclusion was reached that the benefit to be gained by the use of benzene as the initial extractant was far outweighed by the difficulties involved in its use.

2. Variations in Hydrolysis

It was known that hydrolysis of urine in the presence of zinc as recommended by Smith and Smith (1937) prevented the visible charring which takes place when urine is boiled with acid in the absence of a reducing agent. A study of the chromogens produced from male urine was undertaken in order to see if the formation of chromogenic material was mainly oxidative in character. The results are shown in Table 23.

Table 23. Effect of reducing agent present during hydrolysis on the formation of chromogens (1/5 aliquot, 24 hr. specimen)

	O.D.	
	15% HCl	5% Zinc 15% HCl
Hydrophilic phenols	0.133	0.123
	0.140	0.120
Lipophilic phenols	0.114	0.077
	0.113	0.072

It was considered that the decrease obtained in

this/

this strongly reducing medium was insufficient to justify further investigation with the weaker reducing agents which would have to be used routinely in order to prevent reduction of oestrone.

Stimmel (1946) stated that preliminary extraction of the urine with butanol and hydrolysis of an aqueous solution of the residues diminished the production of chromogenic impurities. This procedure is not particularly suitable for introduction into a routine procedure. On the other hand it was considered possible that the removal of ether-soluble material before hydrolysis (and the addition of it to extracts of the hydrolysed urine) might prevent formation of certain impurities. This was tried but found to be completely unsuccessful: the residue extracted before hydrolysis was itself brown in colour, and when it was combined with the ordinary extracts no purification was demonstrated. A variation of this procedure, in which the urine before hydrolysis was taken to pH 13-14 and extracted once with an equal volume of either ether or benzene (these being discarded), did effect a purification of 18% in the one urine tried. Such procedures, however, were cumbersome and in view of their relative inefficiency were not investigated further.

3. Purification by Adsorption

Boscott (1949) stated that filtration of the oestriol fraction through a column of cellulose (Fortisan/

(Fortisan, British Celanese) was effective in the removal of indigoid pigments. The present investigator could not confirm his findings.

Stimmel (1946) used adsorption chromatography with alumina for the separation of oestriol, oestrone and oestradiol-17β. In his hands the method was most effective in the separation, the only defect reported being a consistent loss of 15% of oestriol which he ascribed to tailing. Other workers (Clayton and Marrian, 1949; Swyer, 1952) were unable to confirm his findings. In view of these irregular results, Professor Marrian felt that adsorption chromatography was not suited to the quantitative estimation of oestrogens. Nor was he alone in this opinion: Engel (1950) strongly advised against its use because of the wide variability of different batches of alumina, the inherent tendency to trailing boundaries due to curved partition isotherms, and the influence of impurities on the retention volume of the oestrogens. Dasler (1948) showed that decomposition of certain labile steroids occurred on alumina, and Dobriner (1950) reported irregular behaviour of oestrogens.

In view of these objections to adsorption chromatography and the obvious convenience of application of adsorption, Professor Marrian recommended adoption of a batch-wise use of alumina. This method was investigated at some length and found to be most useful/

useful in the removal of impurities chromogenic in the Kober reaction.

The technique adopted consisted of stirring a solution of urine residues and oestrogens in 10 ml. of a suitable solvent with 3 g. of alumina of known activity in a one inch sinter glass funnel (Pyrex SF1A, Porosity 3). After adequate contact (2 minutes) had been achieved, the funnel was sucked dry and the process repeated. The alumina used was purified and standardized as shown in Appendix II, p. 11.

It was anticipated that the difficulty would be greatest with oestriol and attention was first directed to this. Recoveries of pure oestriol from acid-washed Grade IV (Brockmann) alumina are shown in Table 24.

Table 24. Retention of oestriol by acid-washed alumina

Exp.	Oestriol applied	Eluant	Eluate (ml.)	Oestriol in eluate	Recovery %
1	107.6 µg.	1:4 Methanol Benzene	0-10	41.0	
			10-20	33.5	70
			20-30	1.0	
			30-40	0	
			40-65	0	
		1:1 Methanol Benzene	65-115	6.0	
2	109 µg.	1:4 Methanol Benzene	0-10	36.5	
			10-20	38.5	70
			20-30	0	
			30-40	1.0	
		1:1 Methanol Benzene	40-50	0	
			50-60	1.0	
3	121 µg.	1:4 Methanol Benzene	0-10	38.0	
			10-20	55.5	77
			20-30	0	
			30-40	0	

These typical results demonstrated a retention of oestriol by the columns not characteristic of tailing alone. The third experiment was done with oestriol subjected to preliminary purification on a similar column procedure to eliminate the possibility of the retained material being an impurity in the oestriol itself. Elution with more polar solvents like acetone failed to remove the oestrogen completely. Tiselius (1947) showed that acid-washed alumina functions in part as an anion exchanger, and it was believed that this was the cause of the retention.

Accordingly, alumina purified without preliminary washing with mineral acid was used subsequently. The results of the first experiments are shown in Table 25.

Table 25. Elution of oestriol from alumina.

Exp.	Oestriol added	Eluant	Eluate (ml.)	Oestriol in eluate μg.	Recovery %
1	83.6μg.	1:4	0-10	14.2	
		Methanol	10-20	50.2	95
		Benzene	20-30	13.5	
			30-40	2.2	
		Methanol	40-50	2.0	
2	39μg.	1:4	0-10	14.8	
		Methanol	10-20	19.5	103
		Benzene	20-30	5.2	
			30-40	0	

In the first experiment 3 g. of alumina were used and the tendency towards delayed elution (tailing) noted. This was overcome as shown in the second experiment by the use of only 2 g. The activity

was/

was Grade I (Brockmann). Alumina of this activity is not suited for routine laboratory procedures as it alters on standing. A change was therefore made to Grade II (Brockmann), since this activity can readily be obtained from alumina once activated to this grade merely by heating in a boiling water bath under diminished pressure to the degree obtainable from an efficient water pump. The recoveries of oestriol obtained using 2 g. Grade II activity alumina by this stirring procedure are shown in Table 26.

Table 26. Elution of oestriol from 2 g. alumina in 1" sinter glass funnel.

Exp.	Oestriol added µg.	Eluant	Eluate (ml.)	Oestriol in eluate (µg.)	Recovery. %
1	50.0	1:4 Methanol Benzene	0-10 10-20 20-30 30-40	14.5 28.8 4.5 1.2	98
2	50.0	1:4 Methanol Benzene	0-10 10-20 20-30 30-40	16.0 24.0 6.0 2.0	96
3	48.8	1:4 Methanol Benzene	0-10 10-20 20-30 30-40	12.2 28.5 3.8 1.5	94
4	113.5	1:4 Methanol Benzene	0-10 10-20 20-30 30-40	58.5 29.0 17.0 9.0	100
5	50.0	1:4 Methanol Benzene	0-40	49.5	99
6	50.0	1:4 Methanol Benzene	0.40	50.8	102
7	50.0	1:4 Methanol Benzene	0.40	50.8	102

The recoveries of oestriol having been established as quantitative, the method was applied to male urine residues from the oestriol fractions of the extraction procedure. In the normal urines which were tried alumina, under the conditions standardized above, removed approximately 50% of the non-specific chromogenic material. Other adsorbents were tried and Celite 535 (Kies elguhr, Johns Manville) proved equally effective. With this weak adsorbent and 3% methanol benzene it was possible to elute all the oestriol but only 50% of male urine residues.

A similar type of procedure was developed for the purification of the oestrone, oestradiol-17β fraction. In this case it was possible to remove oestrone quantitatively using 10% methanol-benzene. Typical recovery experiments are shown in Table 27.

Table 27. Elution of oestrone from 2 g. of alumina in 1" sinter glass funnel.

Exp.	Oestrone added µg.	Eluant	Eluate (ml.)	Oestrone in eluate (µg.)	Recovery %
1	40.6	10% Methanol Benzene	0-10 10-20 20-30 30-40	16.2 23.5 2.0 0.7	104
2	52.4	10% Methanol Benzene	0-10 10-20 20-30 30-40	45.1 7.7 1.0 0	103
3	52.4	10% Methanol Benzene	0-40	55.0	103

The application of this procedure to the lipophilic phenol fraction of male urine caused considerable purification/

purification. For example, in three different urines the removal of chromogens was 81%, 74% (ethyl acetate extraction) and 65% (ether extraction). A similar procedure with 2 g. of dry Celite 535 and 3% methanol-benzene was also found to be effective in purifying urinary residues (45-60%), and there was no loss of oestrone. These experiments were conducted at an early stage of the investigation. Since they still left the residual chromogens at too high a level and did not of course separate oestrone and oestradiol-17β, it was necessary to find other purification procedures. The development and incorporation into the method of these more efficient processes have removed the need for the adsorption techniques and they are not included in the routine method. Their inclusion is undesirable since it is more than probable that different behaviour patterns will be found with different batches of adsorbent. However, if experience with pathological urines should indicate the necessity of further purification, these methods could be applied. In such an event the preferable procedure would be the use of Celite since this could be applied to the residue from the initial extraction before partition between benzene and water.

4. Ion Exchange Adsorption

A full investigation of the use of Amberlite IR-4B, a weak base anion exchange resin, in purification of the urinary phenolic fractions was carried out. It was thought that separation of the oestrogens as their conjugates prior to hydrolysis might be useful. In fact it was found that the time-consuming processes of quantitative adsorption and elution were not suitable for incorporation into a routine method.

5. Removal of Urinary Components Prior to Hydrolysis

The most widely known method in oestrogen estimations of removing a group of substances by precipitation prior to hydrolysis is the use of phosphotungstic acid by Jayle and his co-workers (1949). In the present investigation no significant degree of purification was obtained by his method. Urine freed of urochrome by precipitation with potassium bismuth iodide (Lawson et al., 1950) did not char on hydrolysis, but the material removable from ether by normal sodium hydroxide gave as intense a colour in the Kober reaction as an untreated control. Removal of purine derivatives from urine prior to hydrolysis, by the method of Kruger and Schmid (Hawk et al., 1949), gave a somewhat diminished residue in certain urines. However, since all chemical methods were considered possible sources of loss of oestrogens, no further investigation was done along these lines.

D./

D. Methods Adopted for the Purification of Oestrogens.

1. General Characteristics of the Chromogenic Impurities.

a. Relation between brown colour of urine residues and brown colour developed in the Kober reaction.

The most rational approach to the problem of elimination of the non-specific brown impurities of the Kober reaction, it seemed, was to investigate the general properties of this group of substances. In the first place it was necessary to establish the relation between the brown residues of the final extracts and the brown colour developed in the course of the Kober reaction. It was found that the oestriol fraction corresponding to 1/25 of a 24 hour specimen of male urine gave in the phenol-sulphonic acid colour reaction an optical density of 0.198, whereas a similar procedure substituting concentrated sulphuric acid for the reagent gave 0.239. Thus less brown colour was developed from urine residues by the phenol-sulphonic acid reagent than by pure sulphuric acid. It was further demonstrated that a 1/25 aliquot from another male urine gave an optical density of 0.160 in the phenol-sulphonic acid reaction, whereas a similar aliquot, with 60% sulphuric acid (v+v) substituted for the reagent, gave an optical density of 0.201. Thus the phenol prevented the development of the non-specific colour/

colour not just by dilution but in some more direct manner, possibly by its anti-oxidant powers. This is an important point in fluorescence reactions where no phenol is used.

b. Chromogenic impurities arising from molecular oxidation.

In any event the brown colours developed during extraction were considered to be a guide to at least some of the constituents of the chromogenic impurities and attention was directed to them. By this stage of the investigation characteristic behaviour patterns of this group had been observed. It will be seen in the extraction procedure (p. 78) that bicarbonate washings of the separated phenols were done in addition to a similar wash of the original ether extract. This latter step invariably resulted in a brown wash the first time, but a third wash at this stage was always colourless. The colour is undoubtedly due to acidic material with pK less than 6 either present as such in the original urine or formed during the course of hydrolysis. These will be referred to as the preformed acids.

It was noted that the lipophilic phenolic fraction invariably turned brown during its removal from benzene by sodium hydroxide, that the colour of the aqueous phase diminished somewhat on acidification with hydrochloric acid, but that the bicarbonate wash of/

of this fraction after reextraction from ether was always coloured brown. On the other hand the oestriol fraction which was never exposed to alkali was rarely coloured brown nor was its bicarbonate wash. On occasion, however, the water washings from benzene were dark. Moreover, not infrequently it was found that duplicate extractions from the same urine behaved differently, some being brown on removal from water and giving a brown bicarbonate wash, others being colourless at both these stages. Finally it was noted that frequently oestriol residues darkened on standing or during transfer with ethanol to Kober tubes.

These various observations led to the conclusion that there were in the phenolic residues of urine a group of substances susceptible to oxidation to brown coloured products. This oxidation occurred readily in alkali and was apparently reversed to some extent by acidification; the products were at least in part capable of being removed from ether with a bicarbonate solution. Without exposure to alkali the oxidation was more uncontrolled, sometimes occurring at one or other of the different stages but frequently not. Dihydric phenols, known to occur in urine (Dobriner et al., 1942), have these characteristics and as a guide to/

to purification it was considered that weakly acidic material arising by oxidative changes in this group of substances constituted a component part of the urinary chromogenic impurities.

In the light of these facts the relationship between the chromogen content of urinary extracts and pH was investigated. It being established at the outset that sodium hydroxide will remove more material from benzene than from ether, the decision was taken to use in these initial experiments the somewhat less complex mixture of 'total phenols' obtained by extracting the bicarbonate-washed ether extract of hydrolysed urine with 1 x 1/2, 2 x 1/4 volumes of normal sodium hydroxide. The 100 ml. quantities of total phenols obtained in this manner were either acidified with HCl as called for in the procedure, or brought to neutrality (universal indicator paper) by passing CO_2 into them. The solutions were then extracted with ether (1 x 1/2, 2 x 1/4), the acidified one washed with 8% sodium bicarbonate (3 x 20 ml.) and both washed with water (3 x 20 ml.). The residues were taken through the phenol-sulphonic acid reaction. The results of such comparisons are shown in Table 28.

Table 28. Comparison between reextraction of total phenols from acidified and neutralized solutions.

Urine	μg. as oestriol	
	Acidified	Neutralized
1	113	89
2	75	55
3	76	70
4	130	100

The/

The explanation of this improvement was not at once apparent. With unchanged partition coefficient, certainly the washing with bicarbonate should have been more efficient than reextraction from bicarbonate. The acidification recommended in the procedure (20 ml. of concentrated HCl for 100 ml. of N-NaOH) would increase the ionic content over that obtained by neutralization. It was shown that when the pH of the aqueous phase was 8.5 the addition of sodium chloride to saturation increased the partition of chromogens in favour of the ether by 10%. The impression was formed at this time that much of the improvement with the neutralization by CO_2 was connected with the decrease of colour (? reversibility of oxidation) seen with acidification, but experiments designed to prove this were inconclusive.

Engel and others (1950) by adjusting the pH of the aqueous phase to 9 ± 0.5 obtained purification of the total phenolic fraction by reextraction of the alkaline washings of toluene. Their published partition coefficients showed that this allowed quantitative extraction of the oestrogens. It was thought that part of the effect shown in Table 28 might have been due to incomplete neutralization with carbon dioxide. Accordingly experiments were conducted in which 'total phenols' obtained as described above were either acidified/

acidified or neutralized and then taken to different values of pH before extraction. The ether solutions were then washed with 8% sodium bicarbonate and water in the usual way and the ether residues taken through the phenol-sulphonic acid reaction. The results are shown in Table 29.

Table 29. Effect of pH of aqueous phase on reextraction of 'total phenols'.

Urine	Acidified	pH for extraction	µg. as oestriol
1	+	0.5	126
	+	9.0	81
2	+	0.5	130
	+	9.0	101
3	+	0.5	130
	+	1.2	120
	+	2.7	100
	-	7.8	100
	-	9.0	80
4	-	8.05	66
	-	8.6	62
	-	9.05	46
	-	9.5	42
5	-	8.0	78
	-	8.8	63
	-	9.35	60
	-	9.5	54

These results confirmed the findings of Engel and his co-workers that purification can be achieved by adjusting the aqueous phase to pH 9 ± 0.5.

It was reasoned that if the chromogenic impurities resulted from molecular oxidation of phenolic material, it should be possible to forestall their appearance by/

by introducing mild reducing agents during contact of the urinary residues with alkali. In order to check this, 'total phenols' were extracted from ether in the usual way by N-sodium hydroxide with and without 5% sodium sulphite or 7.5% sodium metabisulphite (freshly made up) as shown. Reextraction from pH 9 was then carried out and colour developed as usual. Typical results are shown in Table 30.

Table 30. Effect of reducing agents present during alkaline extraction of total phenols.

Urine	Na_2SO_3	$Na_2S_2O_5$	µg. Chromogen (as oestriol)
1	-	-	120
	+	-	78
2	-	-	94
	+	-	69
3	-	-	95
	+	-	83
4	-	-	70
	-	+	54
5	-	-	50
	+	-	40
6	-	-	85
	+	-	76
7	-	-	70
	+	-	58
	-	+	58

These experiments provide additional confirmation of the formation of chromogens by molecular oxidation in/

in alkaline solution of certain phenolic substances. It will be seen that the removal of such impurities is incomplete even by reextraction from pH 9, for less chromogen is found when the oxidation is prevented. It was believed, however, that this was more of academic than of practical importance, since experience suggested that it was preferable to allow these chromogens to form at a stage of the procedure where they can be mainly eliminated. If reducing agents are to be used at all they must be used throughout, but this does not seem feasible.

c. Chromogenic impurities due to amphoteric substances

In addition to the weakly acidic materials that result from molecular oxidation of dihydric phenols, evidence of the existence of a group of basic substances was found in this preliminary survey. Firstly it was found with ether as an extractant, that 25% more chromogenic impurities could be extracted from hydrolysed urine if this was neutralized. This suggested that the hydrochloric acid usually present at this stage was effective in preventing the removal by ether of a group of substances, and would partly explain why the chromogens were increased by ethyl acetate extraction, which, as already stated, was done from a neutral aqueous phase. In order to utilize this fact, the aqueous phase was made 10 N with sulphuric acid/

acid after hydrolysis and prior to ether extraction. Visible charring of the aqueous solution occurred and the phenolic chromogens were in fact increased by this modification. Since this group was present in the lipophilic phenols as well as the hydrophilic fraction, the existence of a phenolic or enolic group must be hypothesized. Moreover the behaviour described here is typical of the presence of basic groups. Accordingly this group of chromogens is considered to be amphoteric in nature. Nitrogen analyses carried out on the phenolic residues from four different male urines showed the presence of nitrogen to the extent of 3.3%, 2.8%, 4% and 4.1% in the oestriol fractions, and 2%, 2%, 1.6% and 2% in the lipophilic phenolic residues. It was thought that this group might include methylated xanthines, but attempts to demonstrate their presence by paper chromatography were unsuccessful.

2. Preliminary Purification of the Oestriol Fraction.

a. Removal of acids.

The preliminary experiments described above indicated the desirability of allowing molecular oxidation of labile material to occur in alkaline solution and of eliminating most of the acidic products by reextraction from pH 9 ± 0.5. Hydrophilic phenolic/

phenolic fractions of hydrolysed male urine (100 ml. aliquots) were obtained by the Clayton-Marrian fractionation procedure (see p. 78). The aqueous solutions obtained from the benzene partition were reextracted in the usual way after treatment as given below:

(1) Nothing added to the water.

(2) 2 ml. $K_2CO_3/KHCO_3$ buffer, pH 9.3 added.

(3) Solution taken to pH 13-14, shaken, then taken to pH 9.3 with CO_2.

Typical results are shown in Table 31.

Table 31. Purification of oestriol fraction by alkalinization and extraction from pH 9.3.

	μg. of chromogen as oestriol		
Urine	Ext.(1)	Ext. (2)	Ext. (3)
1	109	63	27
2	117	68	49
3	120	69	36
4	75	50	34
5	-	18	16

The results in the first four urine extracts clearly demonstrated the benefits obtained by extraction from pH 9.3, and moreover showed this purification to be enhanced by preliminary exposure of the urine residues to a higher pH. The latter effect was not seen in the fifth urine. This specimen was more than 24 hours old when hydrolysed and had intentionally been allowed to stand without a preservative. The water washes obtained from benzene in this case, but not in the others, were/

were brown. It is probable that bacterial oxidation in the aged urine fulfilled the function of strong alkalization in the fresh.

This simple treatment was incorporated into the final procedure. Experiments showed that the exact pH of the aqueous phase was not critical for purification, but since it was important not to exceed 9.5 for oestriol recoveries electrometric determinations were carried out on each sample. As will be seen, this was subsequently changed.

b. Removal of bases.

The amounts of chromogen shown in the preceding tables were found in 100 ml. aliquots of undiluted urine. Since it was desirable to use at least 1/5 of the daily output it was apparent that sufficient purification had not yet been attained. Moreover certain urines, even after the steps for the removal of acids had been taken, still contained large amounts of impurity. The frequent appearance of pink or purple, suggesting indigoids, pointed once more to the possibility of nitrogenous contaminants. The method of eliminating such compounds was obvious, but was arrived at only indirectly. The clue was furnished by an unsuccessful partition chromatogram that was actually being devised for this purification.

A /

A chromatogram with 4% butanol in ethylene dichloride as the mobile phase and N-NaOH as stationary phase showed marked retention of oestriol, but a pigment band moved exceedingly fast in this system. This immediately suggested a modification of the acid removal technique, in which the aqueous phase of the benzene partition, made normal by the addition of solid sodium hydroxide, was extracted by ethylene dichloride (2 x 1/2 volumes). The expected result followed: the oestriol was not extractable, but was recovered quantitatively from the aqueous phase on adjustment to pH 9-9.5 and ether extraction. The ethylene dichloride did however remove a considerable amount of material; this was precisely the behaviour one would expect of substances with basic groups. Rimington (1946) stated that indigoids could not be removed from chloroform by sodium hydroxide. The results of this purification step in urines with a high indican content are shown in Table 32.

Table 32. Effect of extraction with ethylene dichloride of the oestriol fraction in N-NaOH.

		μg. as oestriol		
Urine	Control	$EtCl_2$ Extd.	$EtCl_2$ Ext.	$EtCl_2$ Blank
1	44	29	19	2.0
2	81	48	35	4.5
3	116	100	42	3.3
4	40.4	27	22	3.3

The third urine represented 2/5 of a 24 hour specimen, the others 1/5. The controls were the aqueous extracts/

extracts from benzene, alkalized, adjusted to pH 9.5 and reextracted in the usual manner. Aliquots of the same urine ($EtCl_2$ extracted) treated as above were extracted with ethylene dichloride at the intermediate alkaline stage. The colours were developed by means of the hydroquinone sulphuric acid reagent (unmodified). In every case there has been a significant decrease in the non-specific chromogens.

3. Partition Chromatography of Oestriol Fraction.

From the beginning of this investigation it was realized that partition chromatography with columns would be required for the final purification. A search for the appropriate solvent system was begun in 1950, and the finalized system has been in continual use since early 1951. In this time minor inconsistencies in the elution behaviour of pure oestriol have been noted. On each occasion the cause of the inconsistency has been found and eliminated. The effect of possible variable factors has been investigated, and these factors eliminated by rigid standardization of the procedure at the appropriate stages. The approach to the problem has been almost entirely empirical. The specific effect of variable factors was established by experiment, and then consideration was directed to the/

the theoretical explanation. The matter presented here will be treated in the same way.

a. Materials and technique.

Evidence had previously been obtained (see p. 90) of the low adsorptive powers of kieselguhr for the oestrogens. Thus when Martin (1949) proposed its use as the inert support for partition chromatogram columns it was the obvious choice for the present investigation. Of the various types available, Celite 535 (Johns Manville Co., Ltd., London) was adopted because the particle size was such as to permit adequate flow of solvent. On the advice of Synge (1950) columns were designed as shown in Appendix III, p. 13 with no constriction at the outlet. The technique of adding a definite amount of equilibrated stationary phase to a weighed amount of Celite and packing with a perforated disc (Martin, 1949) was adopted. Full details are given in Appendix II, p. 11 , and descriptions of the special apparatus in Appendix III, p. 13 . Since the solvent systems selected for the final method were such as to permit ready solution in equilibrated mobile phase, this method of application to the column has been adopted. During this step, in order to minimize frontal analysis (Tiselius, 1947) with resultant spreading of the bands, the solute was introduced/

introduced into the column by three separate 1 ml. washes of the residue. Each wash was allowed to go into the top of the column before the next one was applied. Collection of eluate was not begun until the third wash had gone in.

b. Terminology.

The use of the terms stationary phase and mobile phase require no comment. Reversed phase chromatography (Howard and Martin, 1950) denotes that the less polar of the two phases is preferentially held by the inert support. Percolation rate (R_P), as used here, means the rate of flow of the solvent.

c. Search for a suitable solvent system.

There are three available methods of selection:

(1) When the partition coefficients of the solutes in various systems are known or obtainable, the optimum system is one in which the ratios are 9 and 19 (Synge, 1950). These ratios allow adequate separation with a minimum of band-spread from vertical diffusion in the second component. Accordingly this method has been adopted in selecting the system for oestrone and oestradiol.

(2) When the solutes are of known composition and can be demonstrated on paper, a variety of solvents can be run in paper chromatographic analysis and that/

that system selected which gives the widest separation.

(3) When the partition coefficients are unknown and unobtainable and the nature of the solute undetermined, selection must remain purely empirical.

In the particular case of oestriol purification, in view of the indeterminate nature of the contaminants, only the empirical approach was feasible. Since one component of this fraction (oestriol) was known, the range of solvents was restricted to those in which oestriol showed marked polaripetence so that the reversed phase technique could be avoided. Moreover, for routine purposes, the desired method of applying the solutes was to dissolve them in the mobile phase. Thus what was needed was a pair of immiscible solvents with oestriol partitioning markedly in favour of the more polar yet still being reasonably soluble in the less polar. Moreover for simplicity it was desirable to keep the number of components of the phases to a minimum.

By this reasoning the range of possible systems was definitely narrowed. The early investigations were carried on before the partial purification of the oestriol fraction, by removal of acidic and basic constituents, had been accomplished. Systems designed specifically for elimination of these groups were thus tried/

tried first. Some very interesting systems were tried, but disadvantages inherent in them all precluded their adoption. A summary of these investigations is given in Appendix V, p. 32 . The results are of interest not only in establishing the general behaviour patterns of oestriol in various solvent systems but also because the chromogens are clearly separable into two distinct groups behaving as acidic and basic substances.

After the development of the preliminary stages for the removal of acidic and basic substances of the oestriol fraction, it became clear that partition chromatography was required only to remove trace impurities. In view of the degree of purification which earlier workers had obtained by using aqueous methanol in separating funnel partitions, it was decided to try this as the stationary phase. Ethylene dichloride was chosen as the mobile phase because it seemed to be the least polar substance which was a reasonably good solvent for oestriol. Initially 50% methanol was tried as the stationary phase but oestriol appeared in the eluate before the complete removal of the residual chromogens. The partition of oestriol in favour of the stationary phase was increased by raising the methanol concentration to 70%, and this system proved to be highly effective.

d. Results of purification of oestriol by partition chromatography.

It was established in preliminary experiments that the elution of oestriol in this system began after 15 ml. Before proceeding, it was desirable to show the relation between the purification obtained by discarding the first 12-15 ml., and that obtained by removal of acidic and basic impurities. Accordingly chromatograms of the oestriol fraction were run collecting the appropriate cuts, with and without prior treatment. The results are shown in Table 33. The control urine represented the water washings from benzene taken to pH 14, re-extracted with ether at pH 9.3, washed with water and distilled to dryness. The ethylene dichloride purified extract was obtained by making the aqueous solution of hydrophilic components N-sodium hydroxide, washing with ethylene dichloride (2 x ½ vols.) and re-extracting from pH 9.3 as in the control. All extracts represented a 1/5 aliquot 24 hour male urine.

Table 33. Relationship between chromatogram and ethylene dichloride extraction.

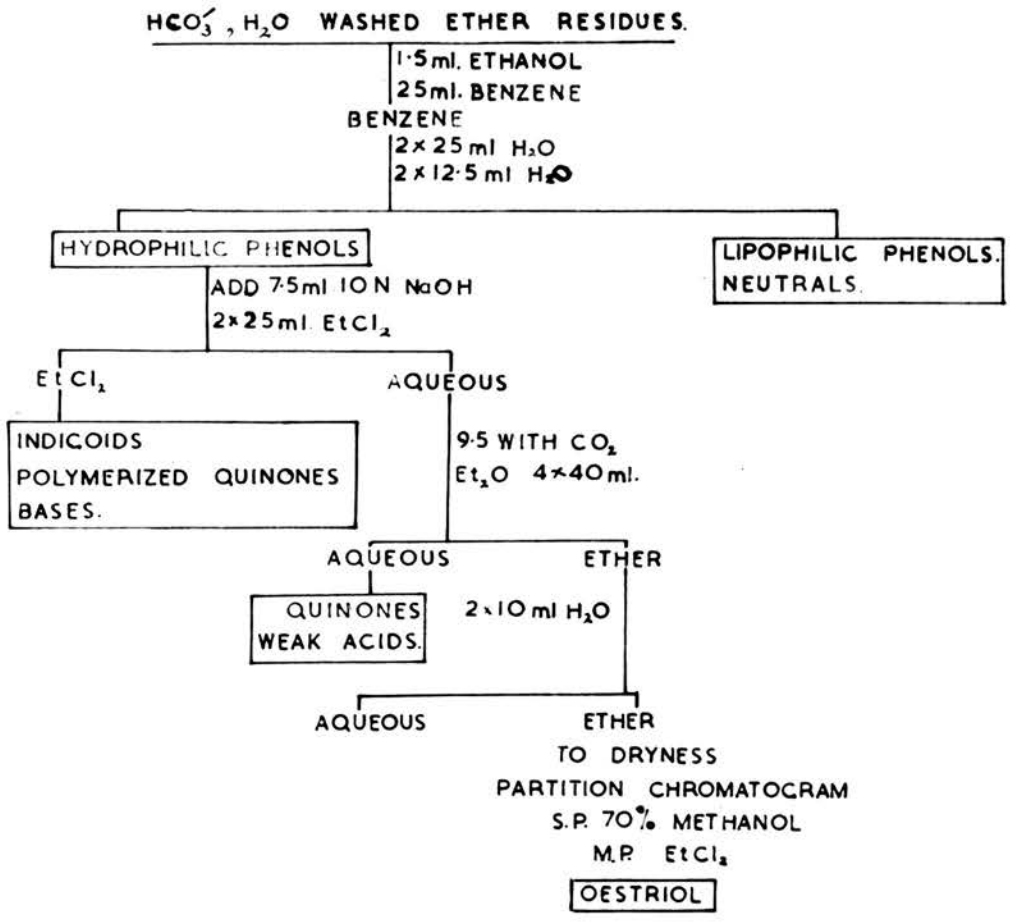
	μg. as Oestriol			
	Urine 1		Urine 2	
ml. of Eluate	Control	$EtCl_2$ Extd.	Control	$EtCl_2$ Extd.
0-14	80	33	138	34
14-40	24	12	37	17

The/

The second urine extract was heavily contaminated by a purple and red pigment (? indigoid). The purple band moved rapidly down the column being eluted by 5-6 ml. This was followed by a red band which was not completely removed until 15 ml. Neither of these bands appeared in the same urine after ethylene dichloride purification. It was obvious from both samples that this latter procedure was highly effective not only in decreasing the total chromogen content, as has already been seen, but also in removing the specific impurities accompanying oestriol in the chromatogram.

The method of reextraction with ether from 9.3 was by now so well established in the method that its relation to the chromatogram was not investigated. However, the procedure of purification in use at this time involved adjustment of the aqueous phase from pH 14 to 9.3 prior to reextraction with ether. The final pH was checked electrometrically and buffered by borate-sodium hydroxide. Occasionally in making this adjustment too much hydrochloric acid was added in the partial neutralization and the solution went to the acid side. This was always accompanied by a lightening of colour in the solution, which was not fully reversed on return to pH 9.3. It appeared that such extracts were more contaminated than those which/

FIGURE 13. SEPARATION AND PURIFICATION OF OESTRIOL.



which never went below 9.3 after strong alkalization. Table 34 shows the chromatographic behaviour of two such extracts, which were washed with ethylene dichloride in the strongly alkaline medium.

Table 34. Effect of acidification during adjustment of aqueous phase from pH 14 to 9.3.

ml. of Eluate	0-2	2-4	4-6	6-8	8-10	10-12	12-14
µg. as oestriol pH 14 to 2 to 9.3	5.9	12	3.9	4.7	2.6	1.9	1.7
µg. as oestriol pH 14 to 9.3	5.0	5	4.2	2.6	1.8	1.3	1.1

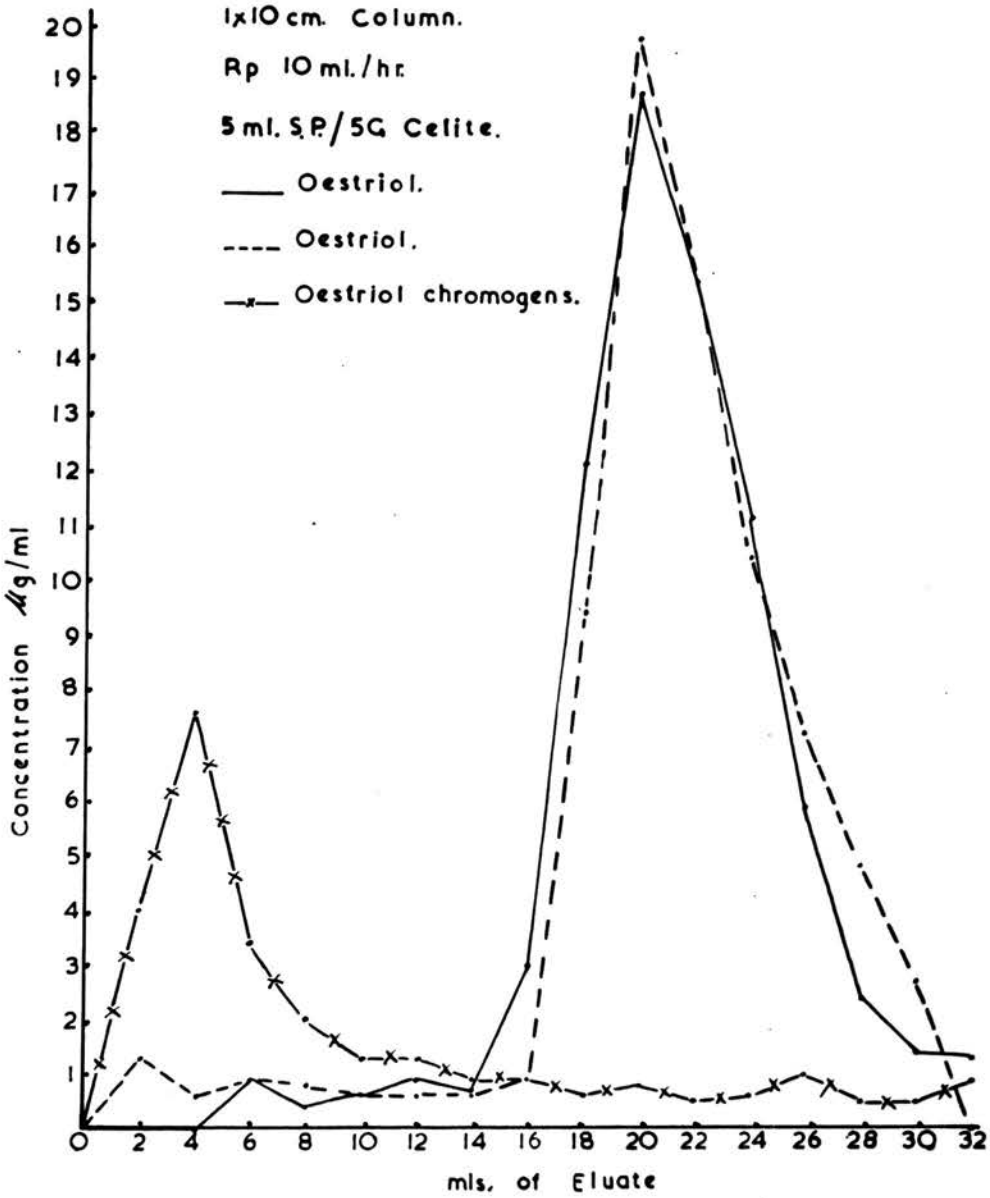
A significant decrease in impurities has been achieved by avoiding acidification during this stage. The procedure was modified by the use of carbon dioxide. A simple manifold was devised with five outlets, four for the urine fractions under test and the fifth for a blank containing water. A sodium hydroxide solution (7.5 ml. of 10 N) was added to each, and a few drops of thymolphthalein to the blank. CO_2 was passed into each until the indicator was decolorized in the fifth sample. A final check on the pH of the test solutions was made with accurate indicator paper (Johnsons, 8410). The details of the procedure are shown in Fig. 13 on the facing page.

When/

When attention was directed to recoveries of oestrogen from the chromatographic procedure, it was found that there was a steady loss of oestriol of 15-20%. For example, collection from 15-30 ml. of the eluate from a chromatogram of a purified oestriol fraction of male urine (1/5 aliquot, 24 hour sample) yielded 5.6μg. of endogenous oestriol and residual chromogens, whereas the same urine residue to which 144μg. of oestriol had been added gave in the 15-30 ml. cut 122.6μg. as oestriol. This represented a yield of 82% $\left(\frac{122.6 - 5.6}{144}\right)$. Since these results were obtained with the unmodified hydroquinone reaction (Brown, 1952b), it was suspected that residues from the column were interfering with the production of the colour. The suspicion became a certainty when the residue obtained from collection of the cut from 15-30 ml. from a blank column depressed the optical density of oestriol standards from 0.300 to 0.259 (i.e. 14%). The residues from 15 ml. of mobile phase on the other hand did not appreciably affect colour development (0.340, 0.335 for standards, 0.330 in presence of solvent residues). It seemed that something being eluted from the celite was interfering with colour development. Accordingly, the celite was partially calcined and treated with concentrated hydrochloric acid as suggested by

Martin/

Fig.14. Elution pattern of Oestriol and Oestriol chromogens using 70% Methanol (S.P.) Ethylene dichloride (M.P.)



Martin (1950). The details of the method are shown in Appendix I, p. 2.

When the above experiment was repeated the impurity had been removed (standards 0.340, 0.339; in presence of residues from blank chromatogram 0.330, 0.330). Subsequent recoveries of oestriol from the chromatographic analysis were satisfactory.

The overall purification achieved was demonstrated in a series of experiments, of which Fig. 14 shows a typical result. Three chromatograms were run under identical conditions, two with pure oestriol and the third with the partially purified oestriol fraction from male urine (1/5 aliquot, 24 hour collection). Similar aliquots, one by the Clayton-Marrian procedure (see p. 78), the other with reextraction from 9.3 (without the ethylene dichloride extraction), gave without chromatography residues corresponding to 152 and 79 μg. as oestriol respectively. Thus removal of the acids formed by oxidation in alkaline solution caused a 52% purification, but the complete three-stage procedure removed 96% of the chromogenic impurities.

A comparable degree of purification was found in other male urines, the final residue representing 5-20 μg. (as oestriol) per 1/5 aliquot of a 24 hour specimen/

specimen. Some of this undoubtedly represented endogenous oestrogen, but most of the residues showed very faint traces of brown. It was then shown that the residue from 50 ml. of ethylene dichloride dried over B.P. anhydrous calcium chloride during the purification procedure (see Appendix I, p. 4) gave an optical density corresponding to 5.6 μg. of oestriol. Substitution of A.R. calcium chloride as the drying agent improved this blank to 1.7 μg. The same sample on being redistilled through a column with cold finger, discarding large head and tail fractions, showed a residue corresponding to 4.5 μg. as oestriol. It thus appeared that the limits of purification from a practical standpoint had been reached, and that the uneliminable trace contaminants must be taken care of by the colour correction formula (Brown, 1952c) (see p. 26).

e. Control of variables in the partition chromatogram.

Since in the routine method it was essential that a definite portion of the eluate be taken as the true oestriol fraction, the behaviour pattern of the column had to be rigidly standardized. The most obvious variables were temperature, percolation rate and particle size of the slurry. It was decided to equilibrate the solvents and carry out the chromatographic analyses in air enclosures thermostatically controlled. The simple apparatus devised to this

end/

Fig. 15. Scatter diagram showing elution pattern of oestriol in the course of a year. using 70% methanol (S.P.), ethylene dichloride (M.P.). 1×10 cm. column.

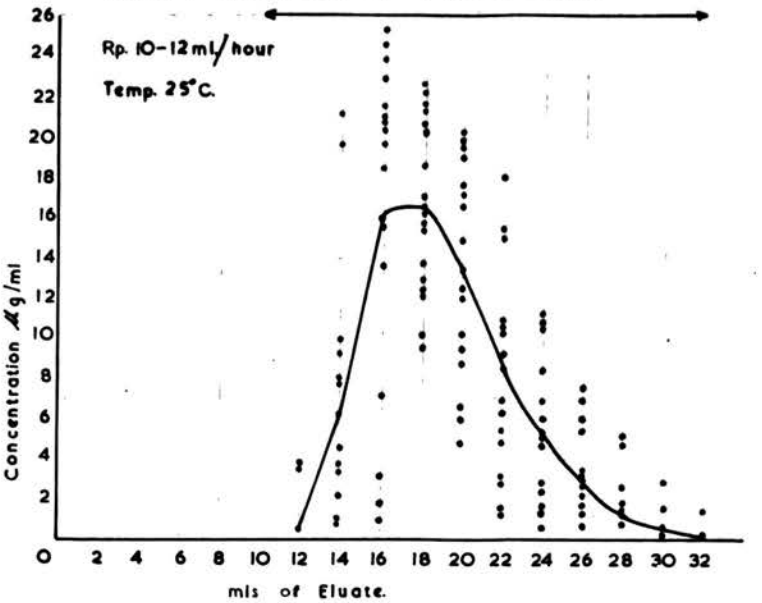
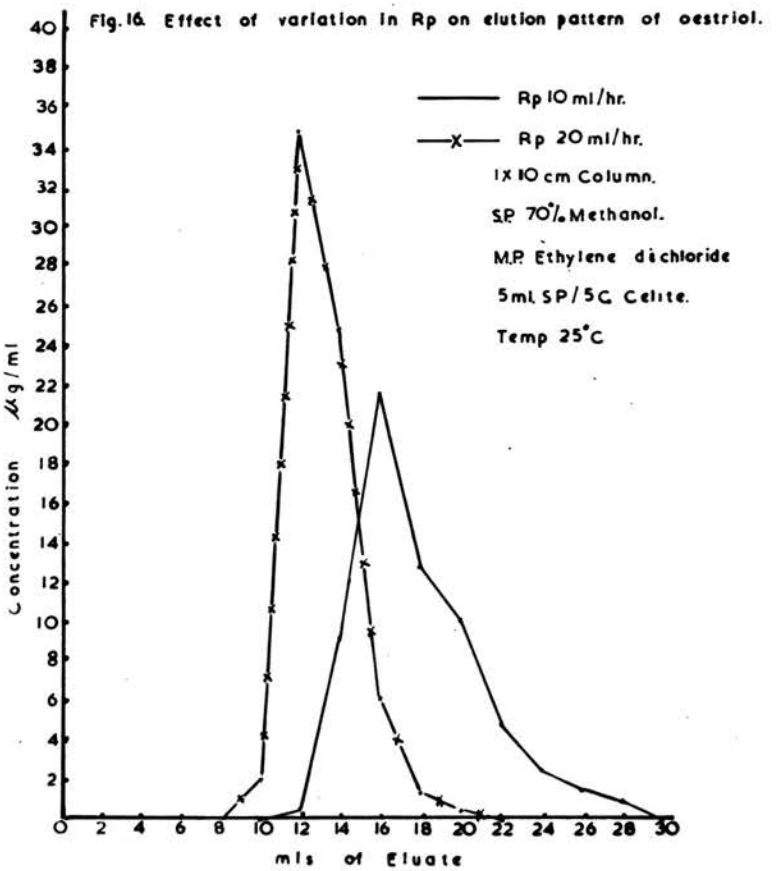


Fig. 16. Effect of variation in Rp on elution pattern of oestriol.



end is shown in Appendix III, p. 13. Preliminary experiments led to the adoption of 10 ml./hour in a 10 cm. column as the most satisfactory percolation rate (R_p) for the mobile phase. This rate allows time for adequate equilibration (improving the separation) without excessive vertical diffusion (and consequent spreading of the bands). It has been adopted in all the experiments, and by relating the volume to height can be expressed as 12.8 cm. per hour, which figure can be adapted to a column of any diameter. The homogenization of the slurry which is done in the packing procedure resulted in uniform particle size. It was found however that the celite after purification was quite deliquescent. Unless precautions were taken to protect it from moisture during storage, it absorbed sufficient water to alter the particle size of the slurry.

With these precautions the uniformity in elution patterns of the chromatograms was shown by experiments with different batches of material conducted over the course of a year. The results obtained, plotted in the form of a scatter diagram, are shown in Fig. 15. The solid line represents the mean of the various readings. In each case the standard procedure was adopted: the solutes were dissolved/

dissolved in the equilibrated mobile phase (3 x 1 ml.), and each 1 ml. wash allowed to go into the top of the column separately. Collection of the eluate was not begun until the third wash had gone in.

The effect of variation in R_p is shown in Fig. 16 on the facing page. It will be seen that with the faster rate of flow there has been less spreading of the band, but not enough time has been allowed for equilibration. This meant that the column was not operating at maximum efficiency and oestriol appeared earlier in the elution. This variable did not present a problem since with a little practice, columns were packed with a sufficient degree of firmness to attain a percolation rate of 10-12 ml./hour. The method of maintaining a constant level of mobile phase on top of the column is shown in Appendix III, p. 13 . The perforated disc used for packing originally had unnecessarily large concentric holes (S.W.G. 19). When a disc with smaller holes (S.W.G. 22) was substituted, the resultant decrease in particle size (giving more rapid equilibration) made the column less dependent on R_p. Thus the permitted variation was increased to 10-15 ml./hour.

Fig. /

Fig. 17. Effect of amount of Stationary Phase Applied to Celite.

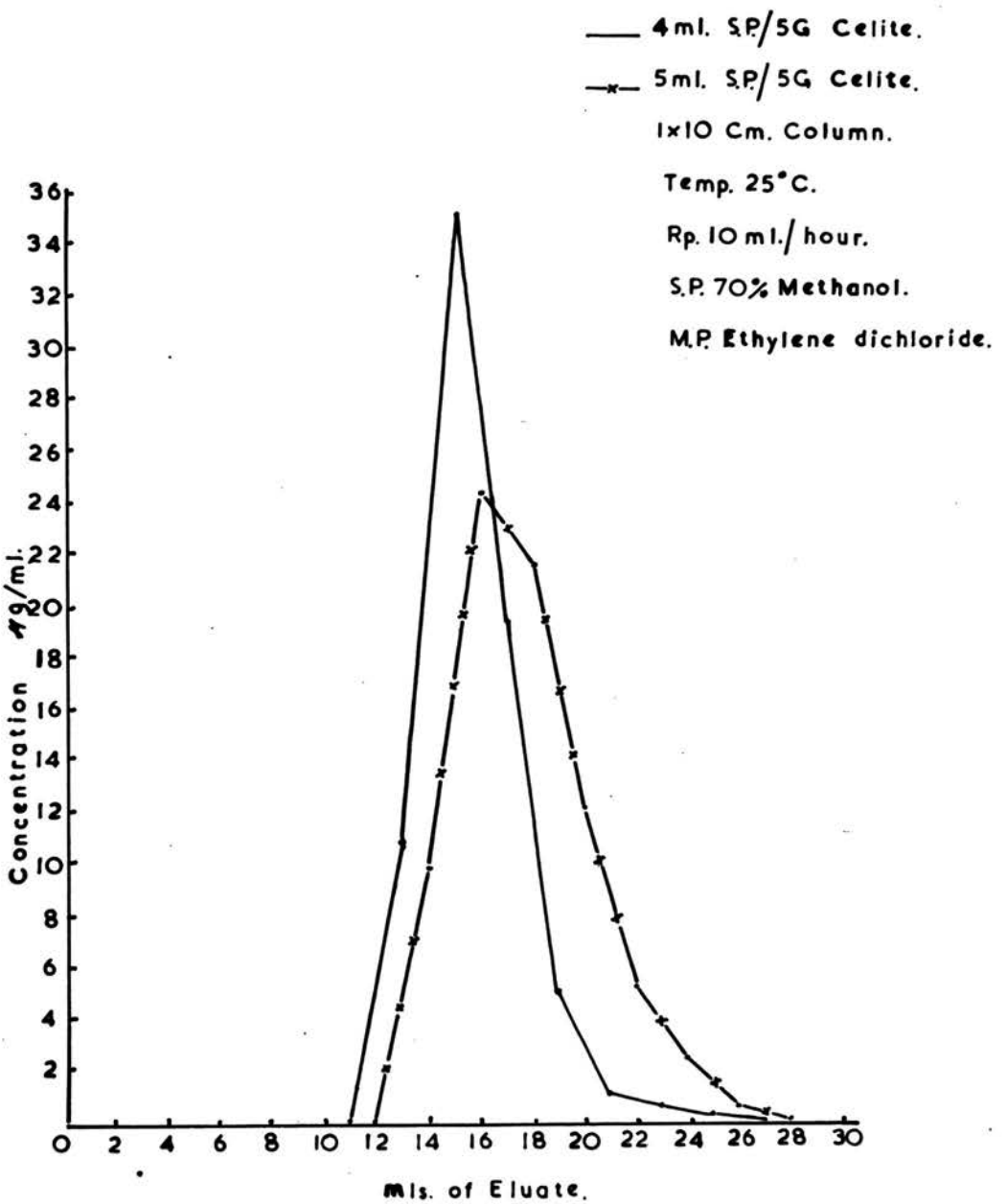


Fig. 17 demonstrates a comparison obtained by varying the amount of stationary phase applied to the celite. As was expected, a decrease caused slight increase in the rate of flow of the oestriol. An increase to 5.5 ml./5 g. of celite caused a slight broadening. This variable was effectively controlled by adding the 5 ml. of stationary phase from a bulb pipette.

One final theoretical variable that defied effective demonstration was the loss of stationary phase while it was being mixed with celite by stirring in a beaker. This should have been of importance since this phase contained three components of varying volatility. In any event to eliminate the possibility of variation, 5 ml. of stationary phase were added to 5 g. of celite in a weighing bottle (Gallenkamp, 12239) and stirred for a few seconds; the bottle with top in place was then placed in a microid flask shaker (Griffin & Tatlock, B45-460) for 15 minutes.

4. /

4. Purification and Separation of Oestrone, Oestradiol-17β.

a. Preliminary partition chromatograms.

From the outset of the investigation it appeared that the more difficult problem in the lipophilic phenols was the separation of oestrone and oestradiol-17β, and that by a suitable application of methods already described purification could be achieved. Initially a solvent system was tried which had been shown to separate the diazo-esters of oestrone and oestradiol-17β (Heftman, 1950). It was to be expected from theoretical considerations (Martin, 1949) that a system suitable for the separation of the derivatives of the oestrogens would also be effective in the separation of the free compounds. Heftman quoted R_f values of 0.91 and 0.85 which were not in the range suitable for partition chromatography (Synge, 1949). This in fact was soon found to be the case and the system was abandoned after a few simple modifications had been tried.

A more rational approach to the selection of a suitable solvent system was made possible because partition coefficients were known. Martin and Synge (1941) in their first paper on partition chromatography related the point of elution of a solute to its partition coefficient as follows:

R /

$$R = \frac{A}{A_L + \alpha A_S} \qquad (1)$$

where R = ratio of movement of position of maximum concentration of solute to simultaneous movement of surface of mobile phase in empty part of tube above chromatogram column.

A = area of cross section of the column.

A_S = area of cross section of stationary phase.

A_L = area of cross section of mobile phase.

α = partition coefficient, i.e. ratio of concentration of solute in stationary phase to concentration in mobile phase, at equilibrium.

Butt and co-workers (1951) modified the equation to

$$\alpha = \frac{V_R}{LA_S} - \frac{A_L}{A_S} \qquad (2)$$

where L = length of column

and V_R = volume of eluate in ml. to peak concentration (retention volume).

This may be rearranged to

$$V_R = \alpha L . A_S + A_L . L \qquad (3)$$

It follows that

$$V_{R_1} - V_{R_2} = C(\alpha_1 - \alpha_2) \qquad (4)$$

where $C = L A_S$.

Therefore, for any given proportional difference between α_1 and α_2, the ease of separation, resulting from the difference/

difference in movements of the solutes on a column, will be greater as the partition coefficients are further removed from unity.

Thus from the published data (Bachman and Pettit, 1941) solvent systems were selected with partition coefficients greater than unity and widely separated for oestrone and oestradiol-17β. For 'ordinary phase' partition chromatography, where the more polar of the two solvents is held stationary, two systems seemed to be suitable. These were:

(1) 30% ethanol/petroleum ether, in which the partition coefficients in favour of the stationary phase (30% ethanol) were 1.8 and 11.5 for oestrone and oestradiol-17β respectively.

(2) N-sodium hydroxide/benzene, in which the partition coefficients in favour of the stationary phase (N-sodium hydroxide) were 4 and 19 for oestrone and oestradiol-17β respectively.

Since the oestrogens are dissociated in alkaline solutions and should thus show a non-linear partition isotherm, attention was first directed to 30% ethanol/petroleum ether. Investigations were carried out at some length, and it was conclusively shown that with a 1 x 10 cm. column, separation could readily be effected. Oestrone was eluted from 15-35 ml., with oestradiol/

oestradiol not appearing in the eluate until 85 ml. A major difficulty, however, in connection with the quantitative application of the solutes to the column arose from the slight solubility of the oestrogens. It was found impossible to transfer the dry residues quantitatively with 3 x 1 ml. washes of the mobile phase. Even after dissolving the oestrogens in acetone, mixing the solution thoroughly with a small amount of dry celite, allowing the solvent to evaporate, and pouring the dried powder on top of the chromatogram, marked band spreading occurred and the oestrone was eluted from 11-85 ml. Moreover, it was found that this system was not effective in the purification of oestrone: the eluate fraction was not completely separated from a rapidly moving band of chromogenic material.

With the demonstration of the acidic and basic nature of some components of the impurities, it became desirable to use a system which would aid in their removal. At this time the use of a benzene/sodium hydroxide system in the chromatographic separation of various oestrogens was reported. This was therefore investigated. As has been pointed out (p.121) the partition coefficients are favourable for separation by/

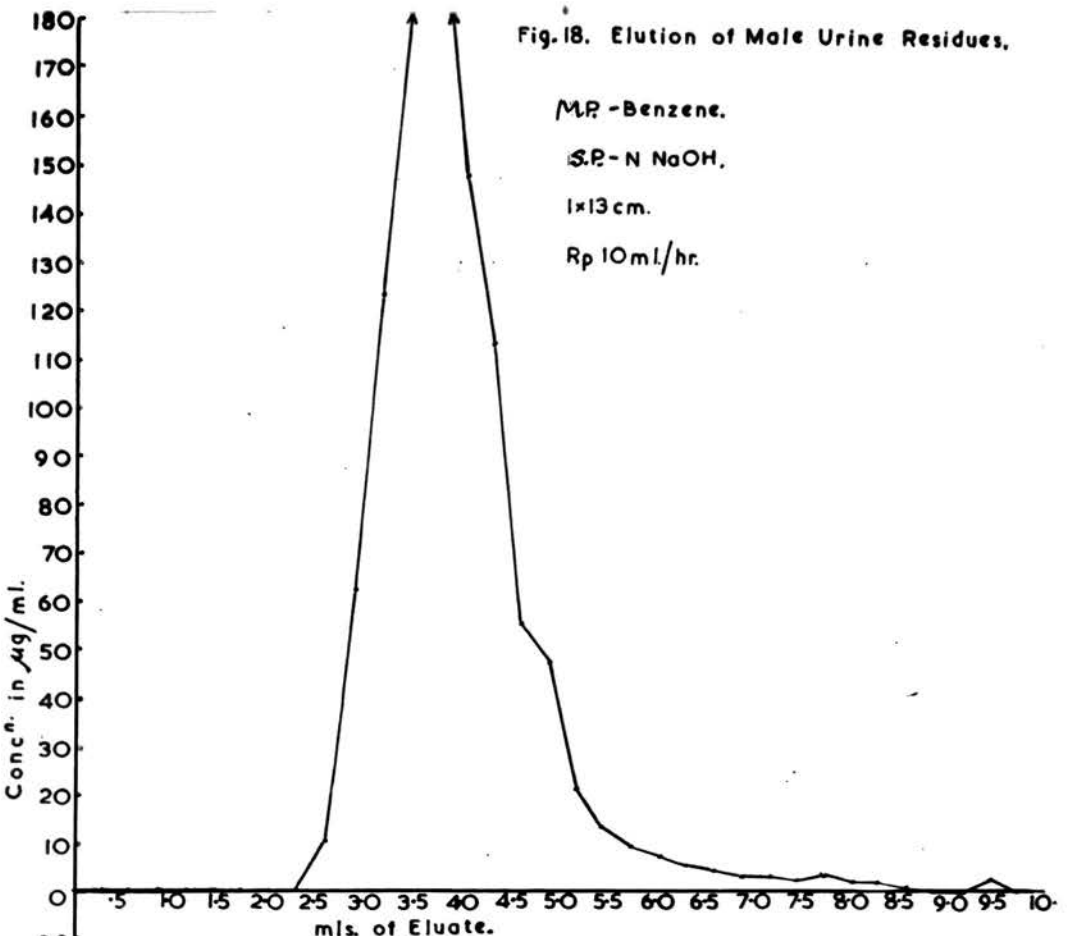


Fig. 18. Elution of Male Urine Residues.

M.P. - Benzene.

S.P. - N NaOH.

1×13 cm.

Rp 10 ml./hr.

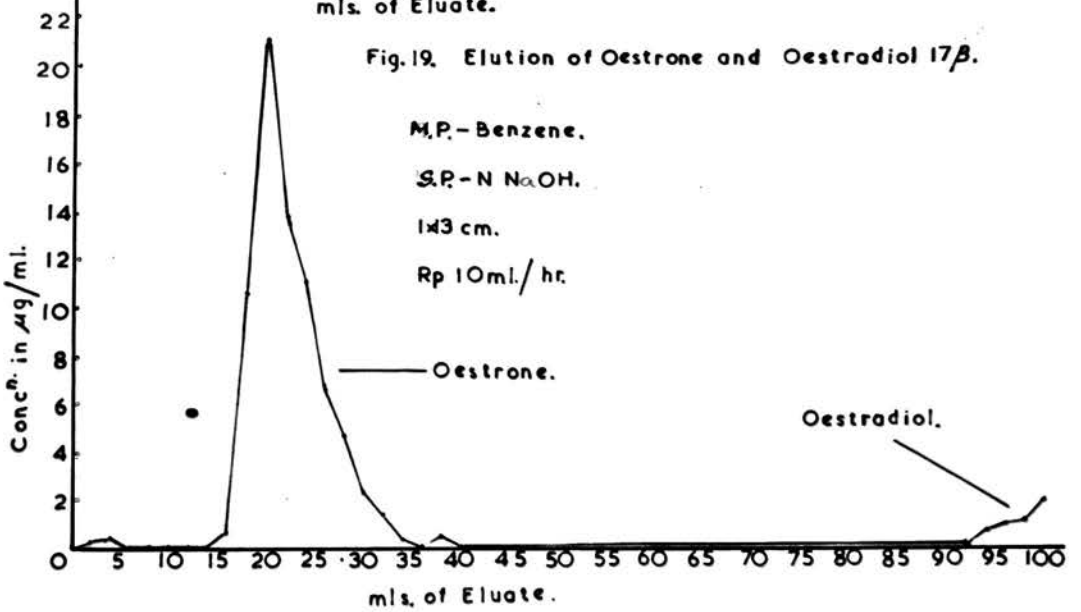


Fig. 19. Elution of Oestrone and Oestradiol 17β.

M.P. - Benzene.

S.P. - N NaOH.

1×13 cm.

Rp 10 ml./hr.

by chromatography. Moreover, as Professor Marrian noted, the use of such a system would eliminate the necessity of separating the lipophilic phenols and neutrals early in the procedure. 500 ml. (1/5 aliquot of a 24 hour specimen) of male urine were hydrolysed and taken through the extraction procedure to the stage of lipophilic phenols + neutrals (see p. 78). The residue was applied to a partition chromatogram with N-sodium hydroxide as the stationary, benzene as the mobile, phase. The elution pattern found is shown in Fig. 18. The results obtained with oestrone and oestradiol-17β are shown in Fig. 19. It will be seen that there was a remarkable purification. A large band of chromogens, presumably 'neutrals', were eluted well in advance of oestrone. A distinct brown residue was left on top of the column. This was presumably acidic material formed, by molecular oxidation, on contact with the strongly alkaline stationary phase. One difficulty with the pure oestrogens was the delayed elution of oestradiol-17β. The separation obtained was in fact too good. The elution pattern of this substance was confirmed several times, and it became obvious that a modification was required to increase the rate of its elution.

The/

The behaviour of the pure oestrogens was what one would expect on theoretical grounds. It will be remembered that partition coefficients of 9 and 19 have been quoted as the ideal values for separation and purification of solutes (Synge, 1950, see p. 107). As an examination of Martin and Synge's original equation (p. 120) shows, for any given ratio of partition coefficients maximal separation of two solutes will be obtained when the values are greater than unity. In the solvent system N-sodium hydroxide/ benzene, the partition coefficients for oestrone and oestradiol-17β are 4 and 19 in favour of the polar phase. The 1 x 13 cm. column used in the experiment was required in order to gain maximal efficiency in the separation of oestrone from the rapidly flowing contaminants of the neutral fractions, but such conditions resulted in a prolonged delay in the elution of oestradiol-17β. Thus with partition ratios of 4 and 19, adjustment of conditions to give optimal efficiency for the more rapidly moving component has given such a wide zero elution zone between the two that the second component has undergone marked vertical diffusion on the column. In this way, the efficiency of the column for the second component has been markedly decreased. If the partition coefficients were/

were somewhat closer together but still away from unity, the chromatogram could be used with a high degree of efficiency for both components. This seems to be the reason for selecting solvent systems with partition ratios of about 9 and 19 for the separation and purification of two known solutes as was recommended originally by Synge (1950).

On the other hand for the particular purposes of this investigation, the system under discussion presented certain definite advantages. The separation of the two oestrogens was so marked that a large safety factor was introduced into the method. Moreover, the chromogens had separated into two distinct bands. The neutral (and presumably also the basic) components were eluted well ahead of the oestrone. The acidic products of oxidation of non-oestrogenic phenols appeared to be firmly held on top of the column.

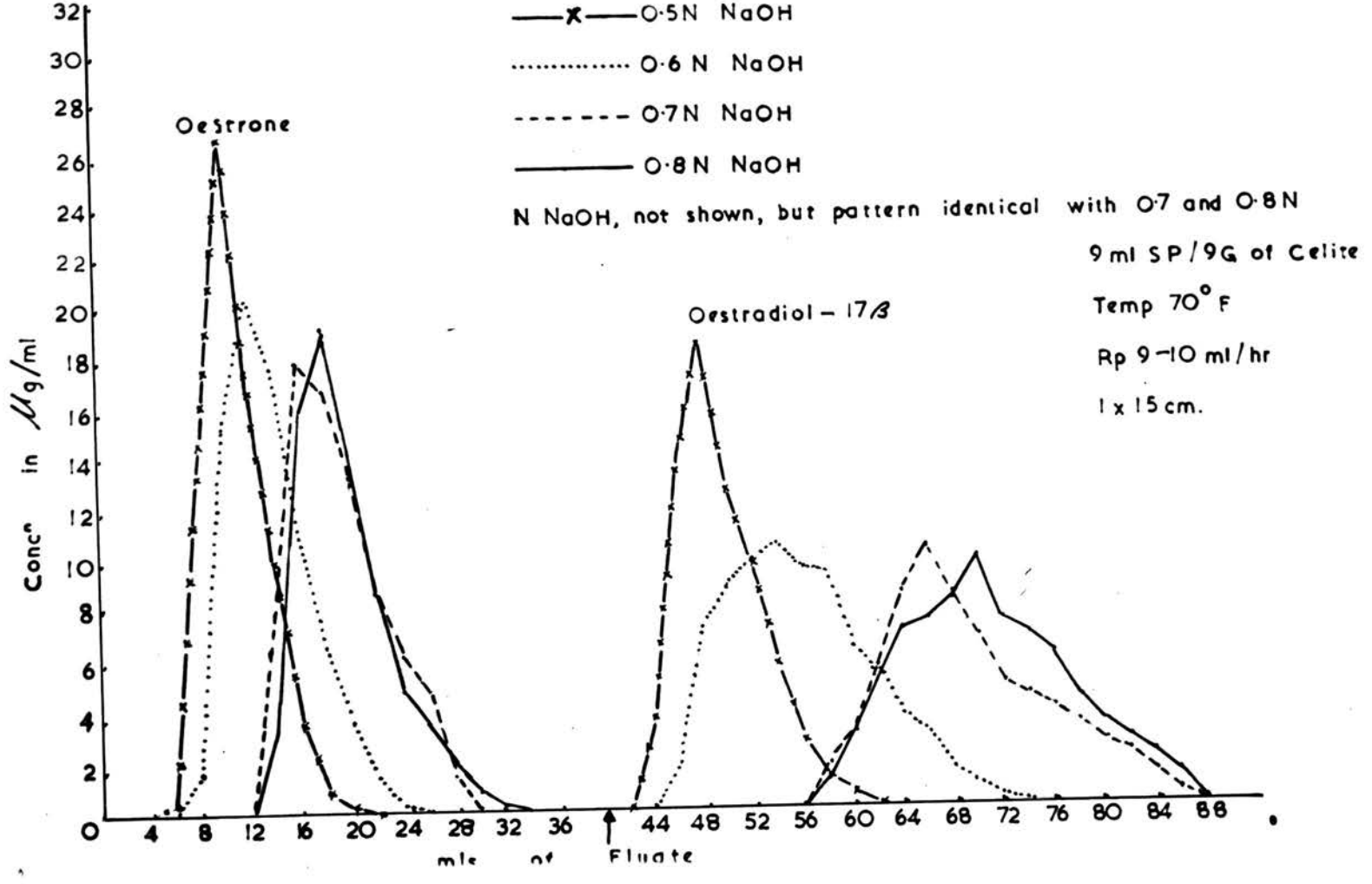
The real difficulty therefore lay in the inconvenience of continuing elution to the 200 ml. which would be required to ensure quantitative removal of the diffuse oestradiol-17β band. It was decided to elute with 50 ml. of the original mobile phase in order to ensure complete removal of oestrone and then to modify the procedure in order to sharpen the elution pattern of the second oestrogen.

Theoretically/

Theoretically it should be possible to do this by changing from elution analysis to displacement analysis. Accordingly traces of dilute mineral acid were introduced into benzene and this substituted for the original mobile phase. This was effective, the oestradiol-17β being displaced in front of the chromogens. This was a true displacement in that the acid added to the mobile phase acted by alteration of the partition coefficient (Martin, 1949). It was considered, however, to be a possible source of loss in that destruction of the oestrogen might occur due to the presence of traces of acid during removal of the solvent.

The problem was then approached by the technique of fractional elution which has been so widely used in adsorption chromatography (Reichstein and Shoppee, 1949). Immediate success was obtained by the use of 75% ethylene dichloride-benzene. This solvent was substituted for the original mobile phase when the eluate was 50 ml. Marked sharpening of the band occurred and quantitative elution was readily achieved. This procedure was used for more than a year. Never in this time was oestrone found in the eluate after 35 ml., and so for economy of time and material it was decided to change the solvent at 40 ml. All the data in the following Figures have been obtained by this procedure.

Fig 20 Effect of Variation in normality of stationary phase.



b. Effect of variables on the elution of oestrone, oestradiol-17β.

Repeated chromatography by the method outlined above soon demonstrated that there were rather serious variations in the elution patterns, particularly of oestrone. On occasion it began to appear in small amounts in the 8-10 ml. fraction. It was decided that a full investigation should be conducted. The important variables found are discussed below.

(1) Effect of variation in the normality of the stationary phase.

All other factors were kept constant, but the normality of the stationary phase was varied from 0.5 N to 1N-NaOH. The results are shown in Fig. 20 on the facing page. It will be seen that at this high operating temperature, the highest peaks and sharpest curves were obtained with 0.5 N-NaOH,but oestrone was eluted too quickly for the removal of the neutral and basic impurities. Increase to 0.6 N advanced the elution only very slightly. There was, however, a significant change at 0.7 N-NaOH and further increase to a normal solution did not alter the pattern of elution. Obviously therefore the best choice offering least possibility of variation was 0.8 N-NaOH. This has been adopted and to avoid excessive/

excessive changes due to carbonate formation, a standardized solution is prepared each week. Further increase in concentration was undesirable in that inorganic residues would be present in the benzene in quantities sufficient to lead to destruction of oestrone during the removal of the solvent.

In these experiments approximately 150 μg. of oestrone and oestradiol-17β were transferred to the column with 3 x 1 ml. of equilibrated stationary phase, allowing each wash to go into the top of the column. Fractions were collected every 2 ml. and analyzed for the appropriate oestrogen by the hydroquinone sulphuric acid colour reactions.

The results were highly interesting in that (in conjunction with the data of the next section) a complete explanation was obtained of the only important variations encountered during the past three years. When this solvent system was originally used, the practice was to mix Celite and a large excess of approximately N-NaOH (previously equilibrated with benzene) in a beaker with thorough stirring to secure a uniform dispersion/

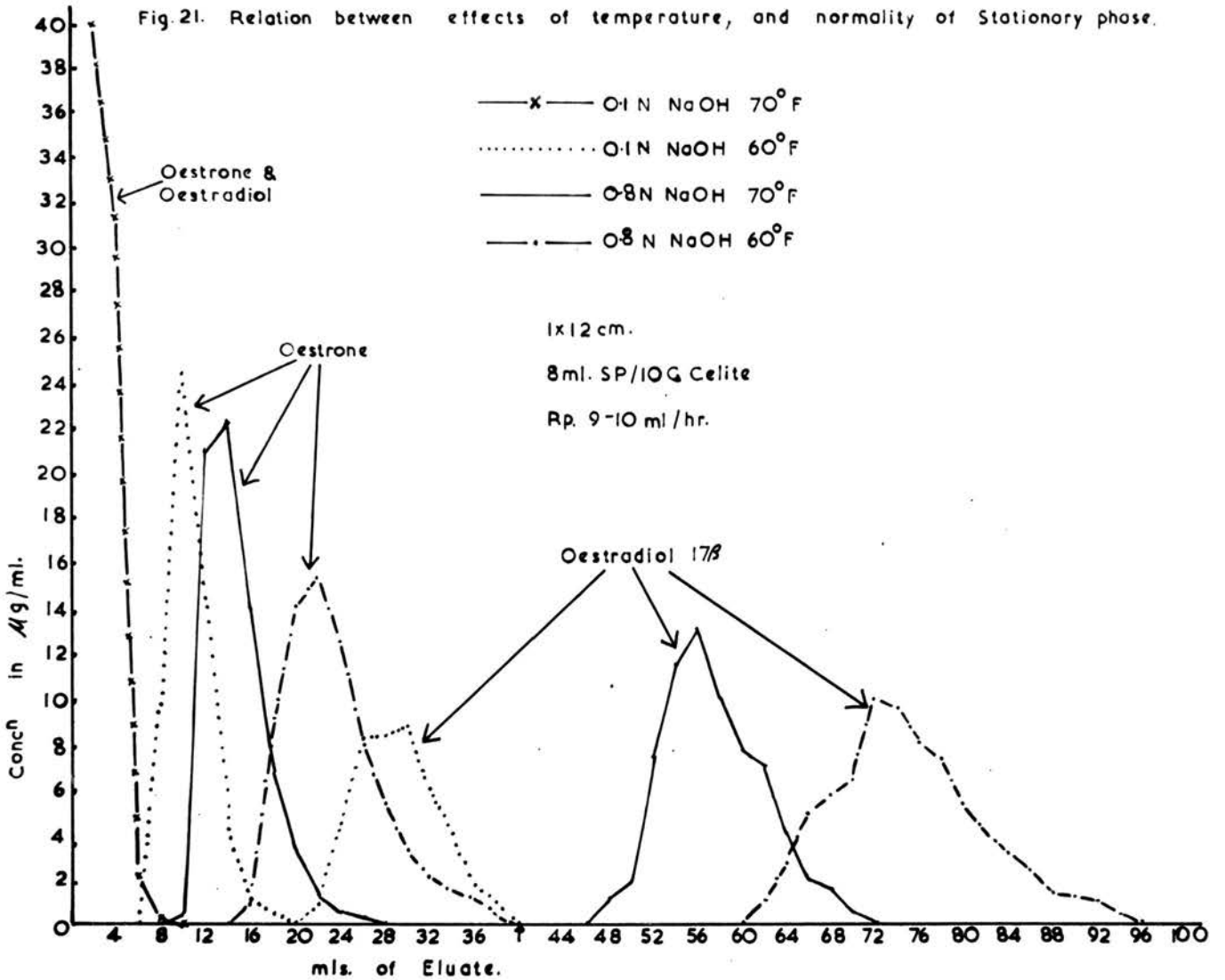
dispersion of the stationary phase in the Celite. The mixture was then poured on to a Buchner funnel to form a pad of about half an inch in depth, and sucked 'dry' with an efficient water pump. In this way the excess of stationary phase was removed and a uniform standardized amount left within the cellular matrix of the Celite, which could of course not be removed by suction. The 'dry' powder was then made into a slurry with the mobile phase and packed in the column in the usual way. Subsequently when variations in behaviour occurred this step seemed the most probable source of error. It was thought that the prolonged passage of CO_2-laden air through the Celite pad during the filtration was causing a partial neutralization of the sodium hydroxide. Addition of various standardized alkali solutions in the volume shown to be retained on the Celite after the filtration procedure, and obtaining uniform dispersion in the usual way by stirring in a beaker, proved that the original method gave columns comparable to those obtained by the addition of 0.5-0.6 N-NaOH. In order to achieve uniformity

with/

with earlier results, the stationary phase was changed to freshly prepared 0.58 N-NaOH. For a while very consistent results were obtained and it was considered that the variable had been removed. Subsequently, however, the difficulty reappeared. It was noticed that early elution of oestrone occurred during times when the laboratory was warm. With the standard separating funnel technique, an attempt was made to show a variation in partition coefficient with temperature. This was unsuccessful, presumably because the change was too small to be demonstrable with the colour reaction. It has already been seen in Fig. 20 that 0.6 N-NaOH at 70°F. allowed oestrone to appear in the cut from 6-8 ml., whereas experiments carried out over the course of the previous six months had established 12 ml. as the point at which oestrone ordinarily appeared. Intermediate values had occasionally been obtained and these invariably occurred on the warmer days. Preliminary experiments clearly indicated that the variations found with temperature and normality of the stationary phase were interrelated.

(2) /

Fig. 21. Relation between effects of temperature, and normality of Stationory phase.

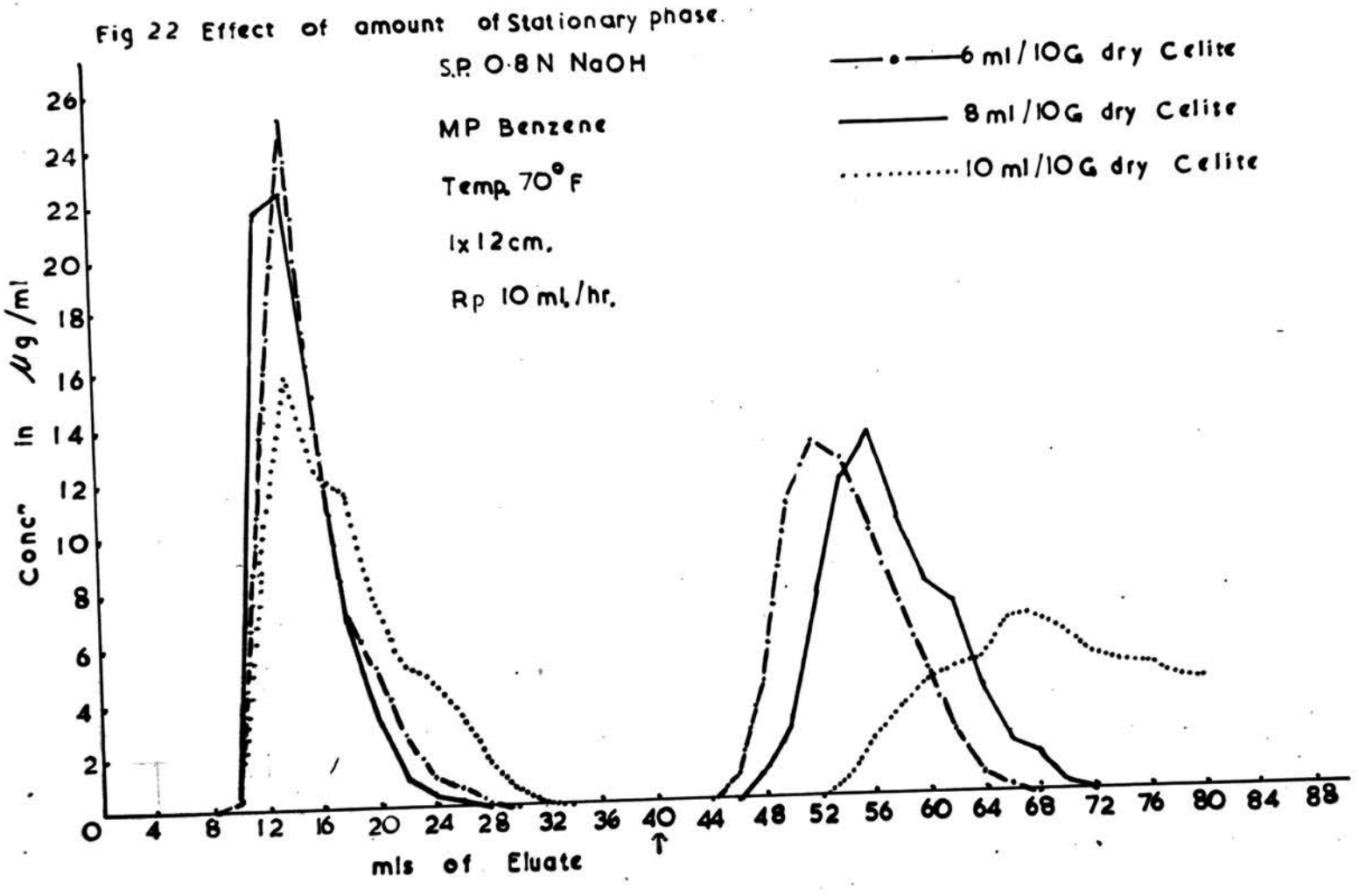


(2) Effect of variation in temperature.

A series was done varying both these factors and typical results are shown in Fig. 21 on the facing page. It will be seen that at 70°F. with 0.1 N-NaOH as the stationary phase no separation at all was obtained, both oestrogens being completely eluted by 8 ml. Cuts taken up to 100 ml. contained no oestrogen. At 60°F. with this stationary phase the two were just separable. When the normality was increased to 0.8 N-NaOH, variation in temperature still had an effect on the elution patterns of oestrone and oestradiol-17β, but to nothing like the same degree. Even so it was obvious that if 1 x 12 cm. columns were to be used the temperature should be below 70°F. to allow a zero elution zone between the chromogens and oestrone. In the finalized procedure the column length has been maintained at 12 cm. and the operating temperature fixed at 65°F.

The explanation of these marked effects is obscure. An increased temperature is desirable in that a closer approach to equilibrium is obtained (Tompkins, 1949). Moreover, on basic principles/

Fig 22 Effect of amount of Stationary phase.



principles one would expect less deviation from ideal solutions at high temperatures. On the other hand, more alkali would be taken up by the benzene and proportionately less left in the stationary phase on equilibration. This would explain the greater effect at the lower concentrations and is probably the correct explanation.

(3) Effect of variation in amount of stationary phase.

Indications were obtained in the course of this work of a slight variation in elution behaviour if the Celite after drying in an oven for 48 hours was exposed to moist air before use. An experiment was conducted keeping all other factors constant but varying the amount of stationary phase added. The results are shown in Fig. 22 opposite. It will be seen that 6 ml. and 8 ml. per 10 g. Celite were virtually identical for oestrone, but that oestradiol-17β was eluted somewhat earlier and more sharply at the lower concentration. Much more pronounced effects were seen with further increase in the amount of stationary phase added. Again the variation was more marked for the slower moving/

moving component and quite marked spreading of the band occurred.

From theoretical considerations, since

$$R = \frac{A}{A_L + \alpha A_S} \qquad (1)$$

one would expect that decrease in A_S (with increase in A_L) should increase R. In other words, with less stationary phase the solutes would run faster. Consideration of the general distribution law suggests a similar conclusion. The expected result was found as demonstrated in Fig. 22. There is, however, an upper limit to the amount of stationary phase. At a concentration of 10 ml./ 10 g. Celite, the advantage gained in delaying the elution of the oestrogens was lost by the marked band spreading which occurred. It was observed that the slurry obtained under these conditions was granular, whereas in the other two cases it was finely flocculent. Formerly 9 ml. had been added, and it was on these occasions that the necessity of keeping the Celite dry was seen. Even with this precaution irregularities were sometimes noticed in the elution curve for oestradiol-17β (cf. Fig.20). As Martin (1949) pointed out, the consistency of slurry obtained with the packing technique adopted here/

here depends very largely on the interfacial tension of the two liquids. With benzene-water, where this tension is high, it is necessary to use a disc containing large holes (S.W.G.19) in order to force the packer through the slurry. The resultant particle size therefore tends to be relatively large. An excess of stationary phase increases this effect and results in a granular slurry. Under these conditions a longer time is necessary for adequate equilibration so that percolation rate becomes more critical. Moreover, as Glueckauf (1949) has pointed out, the larger the particles the greater the tendency for the bands to be disrupted by channelling down the walls. It was decided to use 8 ml. stationary phase per 10 g. of dry Celite in the finalized method. To decrease this further seemed likely to result in too small a zero elution zone between oestrone and the group of polarifugal impurities of urinary extracts.

(4) Variation in size of column.

That variations in the height of the column will affect behaviour calls for no demonstration. The height of 12 cm. has been adopted because with the/

the other variables controlled the desired elution pattern is obtained. Where higher operating temperatures are used, column length must also be increased. The diameter of the column has been arbitrarily fixed at 1.0 cm. Partridge (1950) recommended maintaining a height-diameter ratio of approximately 10 to 1. Glueckauf (1949) pointed out that if 'the column is too short and fat, disturbances due to channelling and tilting of the boundaries become predominant, effects which cannot be assessed numerically. If the column chosen is too thin and long then the area of the larger pores at the wall becomes an appreciable fraction of the total cross-sectional area of the column and severe wall-channelling arises leading to a boundary form in the column of the type which causes mixing of the different separated solutes in the eluate'.

(5) Effect of mode of addition.

It will be recalled that an advantage of this solvent system is that the equilibrated mobile phase is an efficient solvent for both oestrogens. In the standardized procedure the residues in a round bottom flask are transferred to the top of the/

Fig. 23, Effect of mode of addition of solutes

S.P. 0·8N NaOH

M.P. BENZENE

TEMP. 60° F

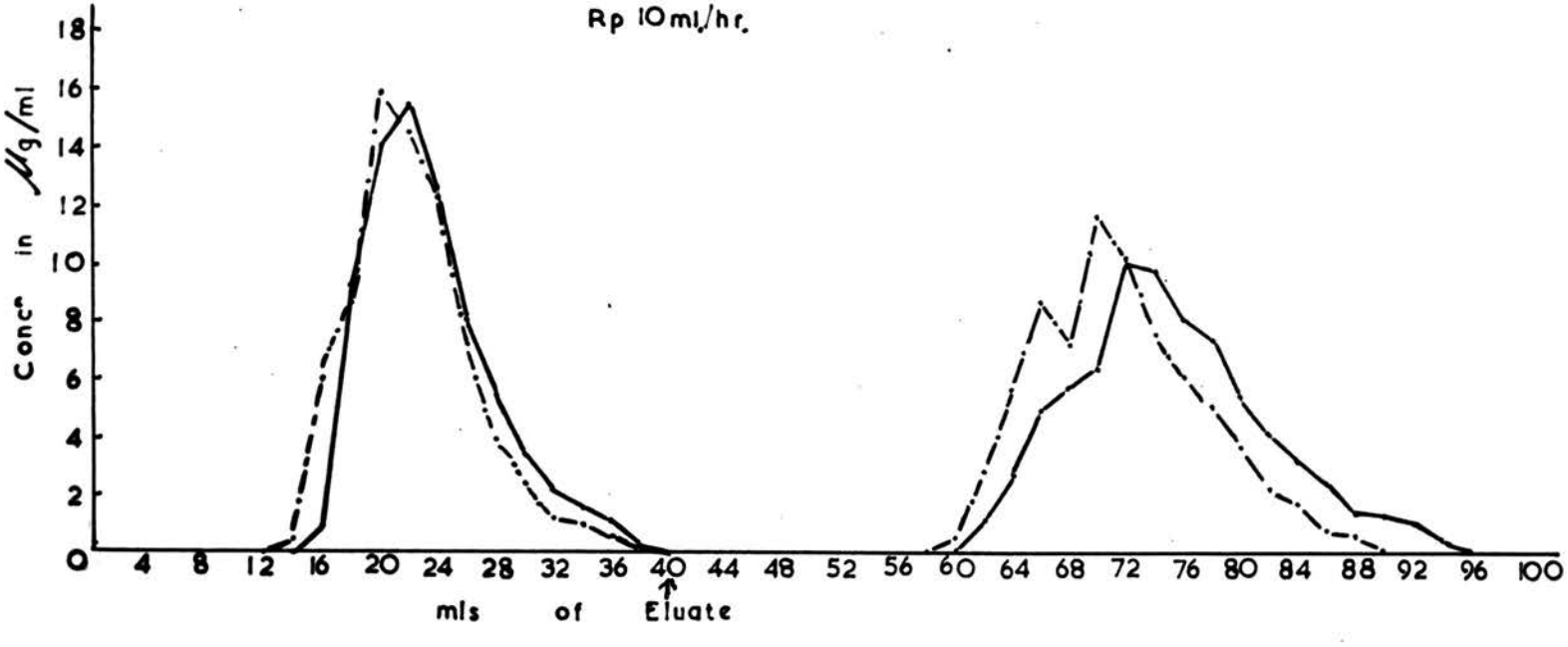
8 ml. S.P./10 G Celite.

1×12 cm.

Rp 10 ml./hr.

——— 3×1 ml. separately

—·— 3×1 ml. all at once



the column by three individual 1 ml. washes, each entering the column before the next is added. Where a larger volume is used the effect of frontal analysis is seen in a marked spreading of the band (Martin, 1949). The solutes are insufficiently soluble and spread over too large a surface to allow the use of an aliquot of a single 1 ml. wash. On the other hand, it was desirable in a routine method to add the three washes successively without waiting for each to enter the column. A comparison of these two modes of addition is shown in Fig. 23. It will be seen that for the faster moving component (oestrone) there is a slight tendency even at 60°F. for somewhat early elution. The effect is more marked and the curve more irregular for oestradiol-17β. It was concluded that the method of separate addition was preferable.

(6) Effect of variation in percolation rate of mobile phase.

Originally it was believed to be impossible to obtain the desired percolation rate with gravity feed to the column. As more experience

was/

Fig. 24, Effect of variation in Rp and Concentration

S.P. 0·8 N NaOH

M.P. Benzene

Temp. 60° F.

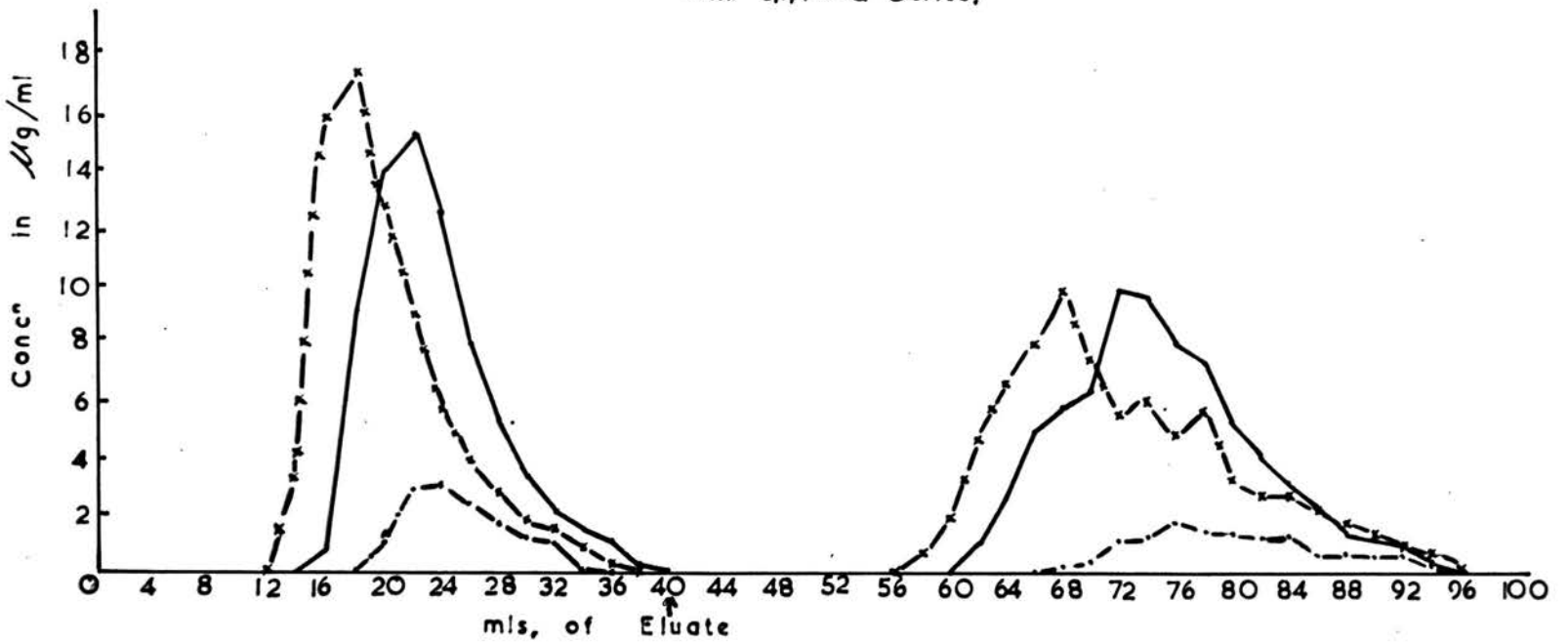
1x12 cm.

8 ml S.P./10 G Celite.

——— Rp 9-10 ml/hr. (150 µg)

—·— Rp 9-10 ml/hr. (25 µg)

—x— Rp 20 ml/hr.



was gained in packing chromatograms, the problem was largely eliminated. As has already been stated, the optimal R_p represents a compromise between the two opposing factors of adequate equilibration and vertical diffusion. Cook (1949) found the volume of the band to increase markedly when the rate of flow was decreased from 10 ml./hr. to 1 ml./hr. and ascribed it at least in part to vertical diffusion. Fig. 24 shows the significant advance in point of elution as the R_p was increased to 20 ml./hr.

(7) Effect of concentration.

Fig. 24 also shows a comparison between elution pattern at 25 and 150 µg. concentrations. It will be seen that qualitatively similar elution patterns were obtained at both concentrations. In elution analysis such as has been used here, the permissible loading of the columns is low. If the quantity of solutes applied is too great, the partition coefficient is affected and asymmetry of elution pattern results, characterized by steep leading edges and

decreased/

decreased zero elution zones (Tompkins, 1949; Martin, 1949). The range investigated here however is sufficiently wide that only in late pregnancy urine might the permissible concentration be exceeded and in such a case a suitable aliquot should be applied to the column.

The relative symmetry of the curves at the higher concentrations is rather unexpected. It will be recalled that doubts were felt about using this solvent system for fear of curved partition isotherms and resulting tendency to tailing in elution. Certainly Levi (1949) found isotherms of the Freundlich type when buffers were used as the stationary phase in elution analysis of various organic acids. In the system under investigation here, the large excess of alkali would ensure that the oestrogens are present in the aqueous phase as the ionized sodium salts. The results obtained suggest that this same ionic species is also present in the moist benzene, so that a linear not a curved isotherm results. Thus the partition coefficient of oestrone between benzene and N-sodium hydroxide may depend on the amount/

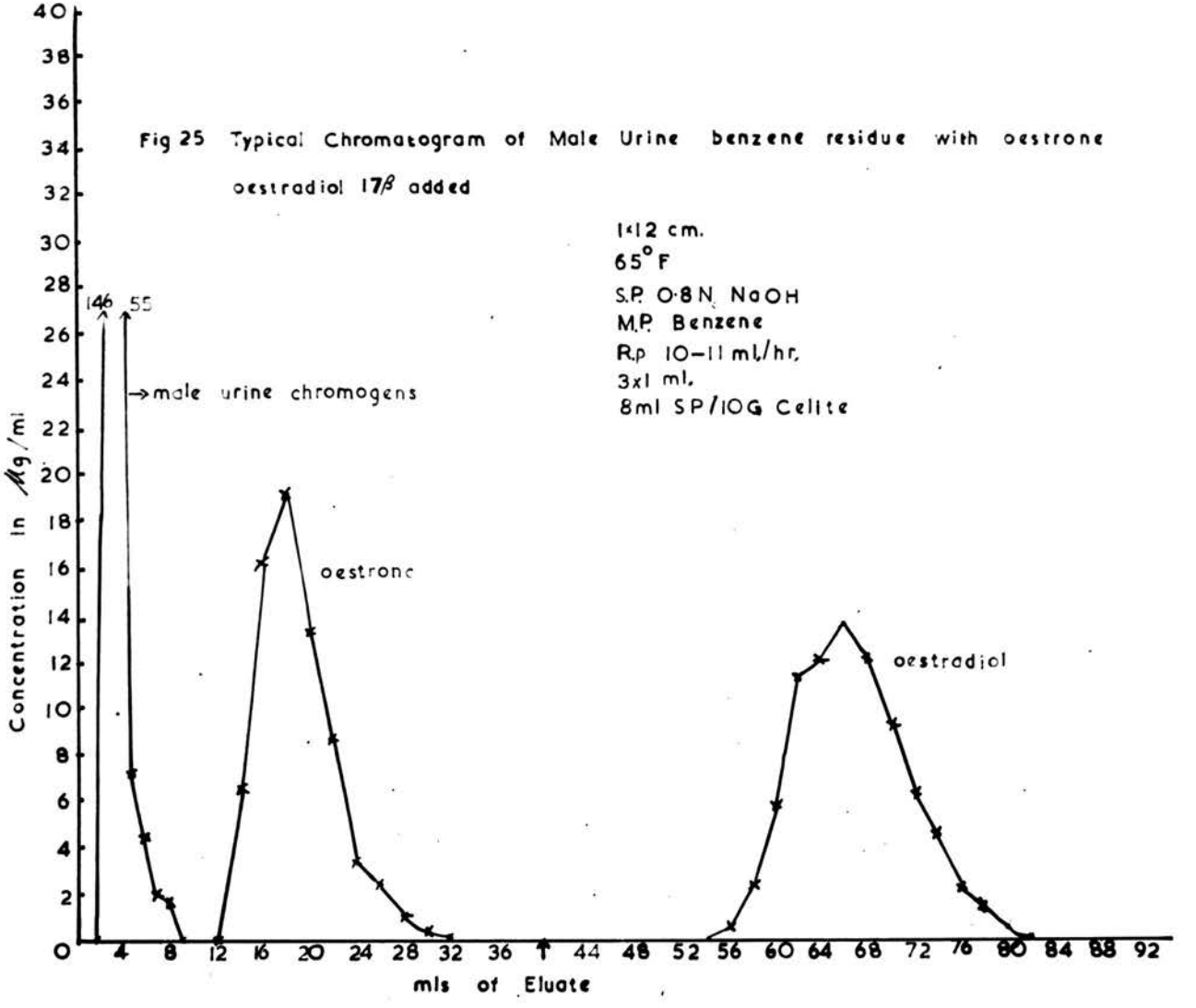


Fig 25 Typical Chromatogram of Male Urine benzene residue with oestrone oestradiol 17β added
1×12 cm.
65° F
S.P. 0·8 N. NaOH
M.P. Benzene
R.p 10–11 ml./hr.
3x1 ml.
8ml SP/10G Celite
146
55
→male urine chromogens
oestrone
oestradiol
Concentration in µg./ml
mls of Eluate
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
4
8
12
16
20
24
28
32
36
44
48
52
56
60
64
68
72
76
80
84
88
92

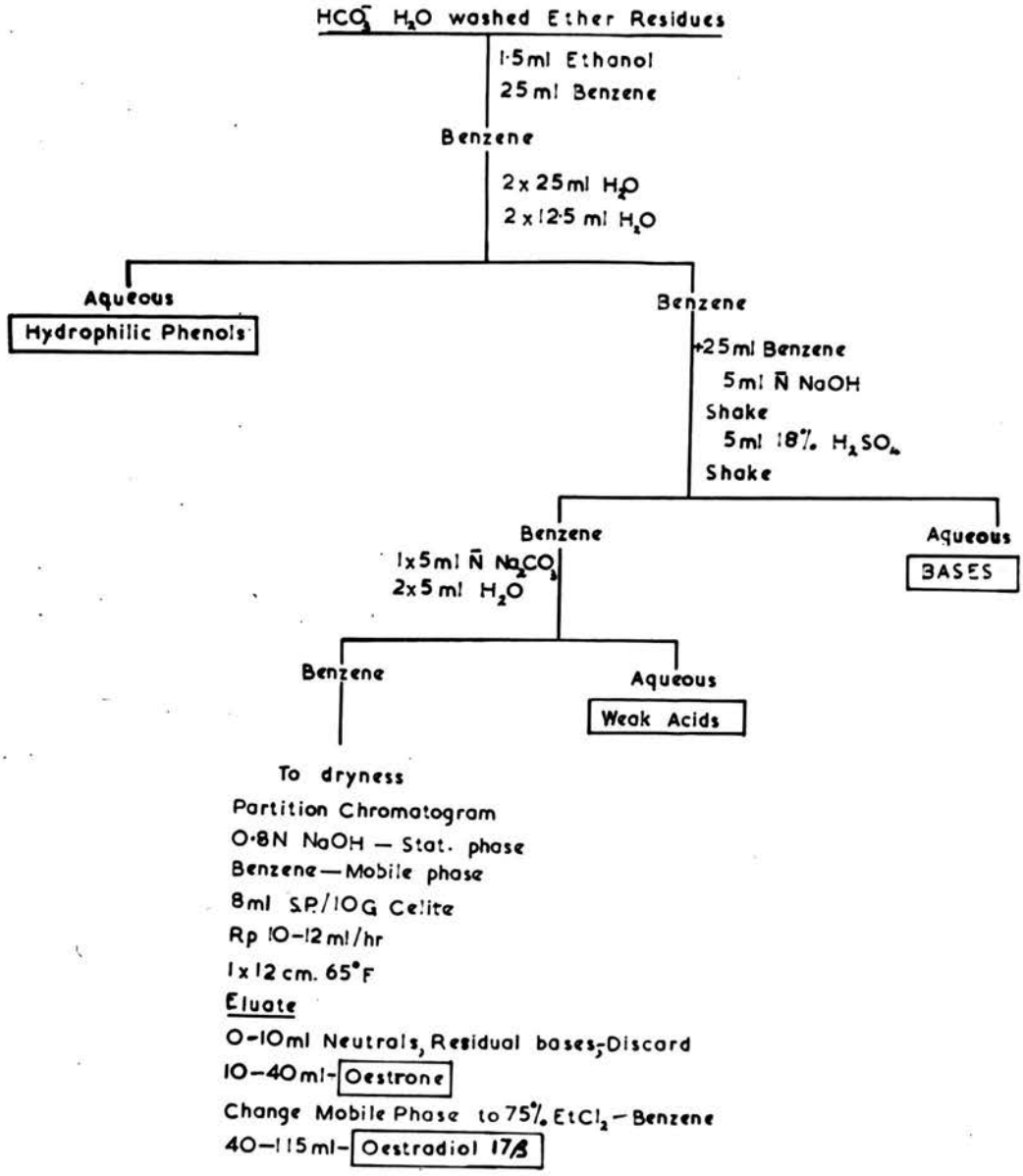
amount of sodium hydroxide taken up by the benzene, and on the partition of the ion. This would explain the marked dependence on temperature seen in the chromatogram, especially when the available alkali is small (0.1 N-NaOH stationary phase).

The efficiency of the chromatographic procedure in the purification and separation of oestrone, oestradiol-17β is shown in Fig. 25 on the facing page.

c. Preliminary Purification of Oestrone, Oestradiol-17β.

Difficulty was encountered in dissolving completely the residue of lipophilic phenols and neutrals in certain urines. Moreover in some cases no zero elution zone was found between the chromogens and oestrone. For these reasons it was considered desirable to carry out some preliminary purification. As has already been stated, when the concentrated residue was placed on the chromatogram a dark band formed on top of the column and was not eluted by 75% ethylene dichloride in benzene. This zone, which the author believes to arise from molecular oxidation of labile phenols, did not contaminate the final oestrogen fractions/

Figure 26. Separation and Purification of Oestrone, Oestradiol 17β.



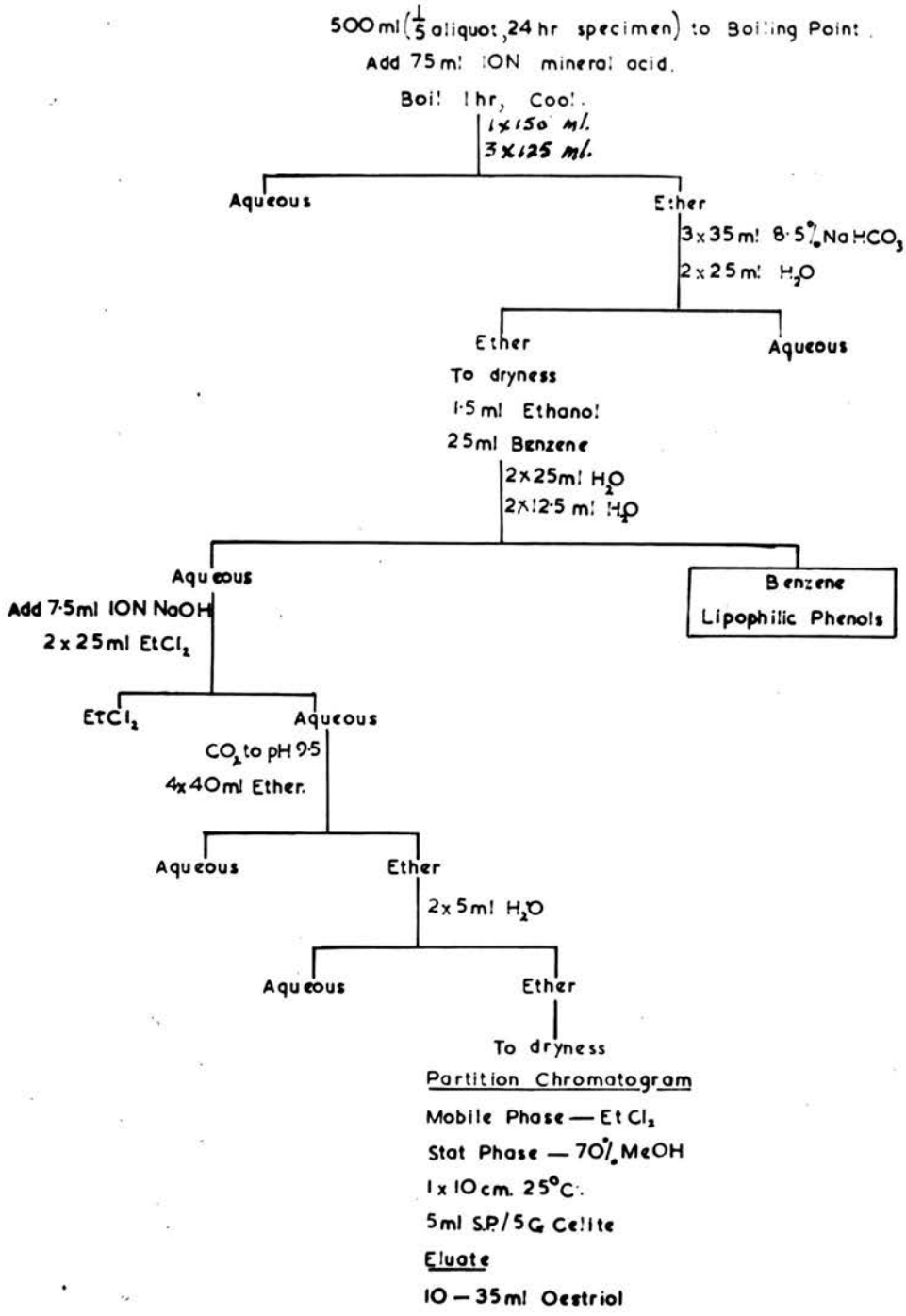
fractions. Partial removal of it beforehand, however, eliminated the difficulty of dissolving the residue in the mobile phase. This removal was accomplished by shaking the benzene solution with N-sodium hydroxide and washing with N-sodium carbonate after acidification.

The chromogens appearing in the eluate immediately prior to oestrone (4-10 ml.) were not lessened by separation from the neutral fraction as in the Clayton-Marrian procedure. It was thought that this band represented basic material and it was markedly decreased by washing the benzene with 15% (v/v) sulphuric acid as suggested by Bachman and Pettit (1941).

The modified procedure is shown in Fig. 26 on the facing page.

IV. SOURCES OF ERROR IN THE METHOD.

Figure 27. Procedure for Extraction and Purification of Oestriol.



IV. SOURCES OF ERROR IN THE METHOD.

The information set forth in Part III has been primarily concerned with the efficiency of various procedures in the purification of the oestrogens in acid hydrolysed urine. It will have been obvious however that in the selection of methods for the removal of impurities full consideration was given to the necessity for quantitative recovery of oestriol, oestrone and oestradiol-17β. This aspect of the problem is discussed below.

A. Loss of Oestriol on Extraction from Aqueous Acid.

1. General Discussion.

The method adopted for the extraction and purification of oestriol from urine after acid hydrolysis is shown in Fig. 27. Initially A.R. hydrochloric acid was used since this had been widely used by various workers since 1934. The exhaustive ether extraction was adopted in view of the discrepancy in the literature in regard to the partition coefficient of oestriol between this solvent and dilute mineral acid. Mather (1942) used bioassay and found a value of 6.1. This was confirmed by Brown (1952c). Bachman and Pettit (1941) however reported a ratio of over 20. Engel/

Engel and his coworkers (1950), and Diszfalusy (1953) are in agreement with this higher ratio. The varied results of the different investigators may possibly be due to contamination of commercial oestriol by a more polarifugal component. As has already been stated, Swyer and his coworkers (1952) reported an impurity in their oestriol which was intensely reactive in fluorimetric methods of determination. Experiments conducted by the present author showed a partition coefficient of 5.5-6.0, and this has been used to determine the procedure for the ether extraction.

Data published by all the investigators indicate that the bicarbonate washes will not remove oestriol. The small water washes were introduced to avoid possible destruction during distillation of the ether. The efficiency of water, in the amounts shown, in the removal of oestriol from a solution of benzene containing 6% ethanol was conclusively demonstrated by Clayton and Marrian (1949). The present author has shown that no loss of oestriol occurs on shaking in normal sodium hydroxide nor does ethylene dichloride extract any from this medium. Engel and his coworkers (1950) showed that the partition coefficient of oestriol between ether and an aqueous phase does not fall appreciably/

appreciably until the pH is over 9.5, and this has been confirmed by Brown (1952c) and by the present investigator.

The introduction of the partition chromatogram resulted in a small loss of 3-5%. This has been demonstrated repeatedly over the course of the past three years. Invariably a component giving the typical pink colour in the Kober reaction has appeared in the eluate from 0-3 ml. As yet this has not been fully investigated but it is believed that this merely indicates trace contamination of the oestriol being used in this department by a more polarifugal substance. This has been disregarded in the present investigation.

All of the procedures of the method were thus virtually quantitative and it was felt that recovery experiments would merely represent an assessment of the technical skills of the operator. Initially in order to avoid complications arising from endogenous metabolites, oestrogen was added in known amount to a mixture of 500 ml. of water and 75 ml. A.R. hydrochloric acid in the cold and treated as shown in the flow sheet in Fig. 27. Typical results showing the recovery of oestriol are listed in Table 35. A more complete series is shown in Appendix VI, p. 35.

Table/

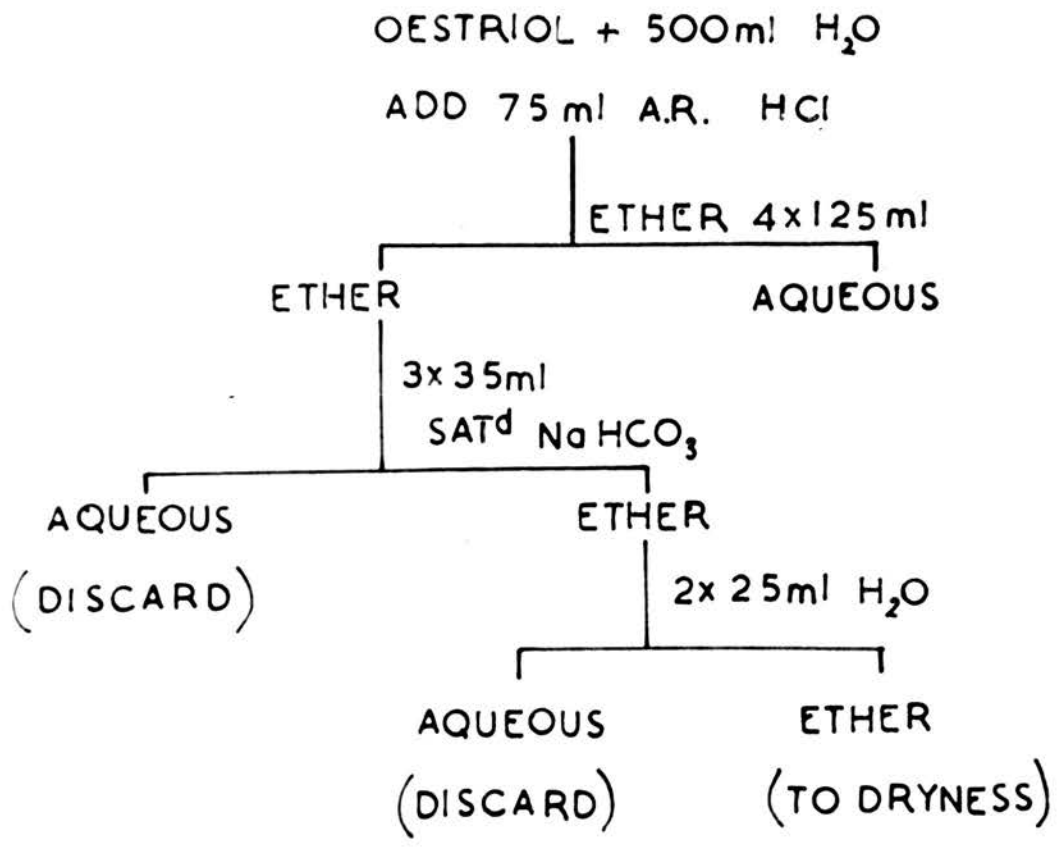
Table 35. Recovery of oestriol from 500 ml. of water and 75 ml. concentrated HCl.

Oestriol µg.	D_{604}	D_{601}	D_{606}	$D_{corr.}$	% Recovery
150	0.145	0.045	0.004	0.138	74
	0.141	0.045	0.006	0.132	71
	0.144	0.038	0.001	0.141	75
	0.138	0.037	0.001	0.135	72
25	0.104	0.051	0.014	0.085	45
	0.110	0.055	0.014	0.090	48
	0.150	0.060	0.014	0.131	70
	0.147	0.052	0.014	0.130	70
Stds.	0.186	0.038	0	0.186	
	0.190	0.038	0.001	0.189	

In each case the first 8 ml. of eluate from the chromatogram was shown to contain only the trace contaminant already mentioned and continuing the elution beyond 40 ml. did not increase the yield. The results include experiments in which the ether volumes at all stages were increased, equilibration times prolonged, and distillations carried out under nitrogen. None of these measures was in any way beneficial.

The results clearly indicated that the recovery of oestriol from pure solution was completely unsatisfactory. Not only were the yields low in all the experiments but some showed markedly decreased values for no apparent reason. It was clear that the causes of these effects had to be found. The individual steps were investigated and it was soon found that loss was occurring/

FIG. 28 EXTRACTION OF OESTRIOL FROM AQUEOUS MINERAL ACID.



occurring in the initial extraction, which is reproduced in Fig. 28 on the facing page. Attention was therefore directed to this procedure.

2. Colour Reaction

Much of this investigation was carried out prior to the demonstration of Type II inhibition and fading, which have been discussed in Part II. In all the experiments, however, the reagent was stabilized by the addition of ethanol or m-cresol so that the major defect in the estimation of oestriol in the presence of solvent residues had been eliminated. Investigation of these faults was carried out over a period of months with different reagents and under different conditions of illumination in the laboratory, but never were quantitative recoveries obtained from the simple procedure shown in Fig. 28. Definite proof that the hydroquinone colour reaction when stabilized with m-cresol or ethanol (see Appendix II, p. 8) was not the cause of the loss was obtained by carrying through a blank extraction and adding pure oestriol to the residues obtained on distillation of the ether. Typical results are shown in Table 36. 'Oestriol extracted' is the residue obtained by extracting 25μg. of oestriol from a mixture of 500 ml. of distilled/

distilled water and 75 ml. A.R. hydrochloric acid with ether as shown in Fig. 28, p. 145, and transferring to a Kober tube with ethanol. 'Blank extraction + oestriol' is obtained by adding 25μg. of oestriol to a Kober tube containing the residues obtained from a similar extraction from aqueous acid containing no oestriol.

Table 36. Stability of m-cresol modified hydroquinone colour reaction in the presence of ether residues.

Expt.		D_{604}	D_{601}	D_{606}	$D_{corr.}$	% Recovery
1	Oestriol extracted	0.162	0.086	0.034	0.119	72
	Blank extract + oestriol	0.212	0.100	0.036	0.166	100
	Blank extract	0.032	0.048	0.022	0.003	
	Standard	0.169	0.028	0	0.169	
		0.163	0.025	0	0.163	
2	Oestriol extracted	0.183	0.089	0.034	0.141	83
	Blank extract + oestriol	0.219	0.105	0.038	0.168	99
	Standard	0.172	0.029	0.004	0.168	
		0.175	0.029	0.005	0.173	
3	Oestriol extracted	0.191	0.085	0.026	0.158	85
	Blank extract + oestriol	0.206	0.070	0.021	0.182	98
	Blank extract	0.022	0.046	0.017	-	
	Standard	0.182	0.028	0.003	0.182	
		0.192	0.032	0.003	0.192	
4	Oestriol extracted	0.170	0.088	0.036	0.126	72
	Blank extract + oestriol	0.201	0.098	0.036	0.156	89
	Standard	0.175	0.026	0	0.175	
		0.175	0.026	0	0.175	

These results have been selected to show that at least on occasion type II inhibition and fading did not interfere with the colour reaction. At no time however was quantitative recovery of oestriol from dilute hydrochloric/

hydrochloric acid achieved. This was taken as a definite indication of loss of oestriol during this simple procedure and investigation was directed toward finding the source of this loss.

3. Loss on Transferring Residues to Kober Tubes

It was considered possible that a slight loss might be occurring during the transfer from a 1 litre round bottomed flask to the Kober tube. This was done by introducing 3 ml. of ethanol from a bulb pipette, washing down the walls of the flasks in the process, and transferring the solution with another pipette. The procedure was repeated twice and the ethanol removed in a fine jet of compressed air, the tube being immersed in a boiling water bath. The air was cleaned by passing through cotton wool and then through a fine sintered disc. The results obtained with the ethanol stabilized hydroquinone colour reaction are shown in Table 37.

Table 37. Transfer of 25μg. of oestriol from 1 l. flasks to Kober tubes.

	D_{604}	D_{601}	D_{606}	$D_{corr.}$
Oestriol transferred	0.186	0.072	0.021	0.160
	0.187	0.054	0.020	0.167
	0.178	0.054	0.013	0.163
Standard	0.172	0.032	0.002	0.172

The/

The findings suggested a slight loss of oestriol and the introduction of extraneous material during the transfer process. At this time the 1 litre round bottomed flasks were cleaned by standing overnight in chromic acid, rinsed with water to remove most of the cleaning solution and then given an alcohol rinse to remove the final traces of oxidant. They were then rinsed twenty times with tap water and three times with distilled water. No particular attention was being paid to the ground glass joints however. The procedure was modified so that the flasks were filled to the top with cleaning solution and the joints immersed in ethanol for two or three minutes during the rinsing process. Repetition of the transfer procedure from flasks cleaned in this manner gave qualitatively and quantitatively more satisfactory values for optical density as shown in Table 38.

Table 38. Transfer of 25μg. of oestriol from 1 l. r.b. flasks (modified cleaning procedure) to Kober tubes.

	D_{604}	D_{601}	D_{606}	$D_{corr.}$
Oestriol transferred	0.178	0.048	0.009	0.168
do.	0.183	0.041	0.008	0.176
do.	0.181	0.043	0.008	0.173
Standard	0.172	0.032	0.002	0.172

In/

In all subsequent work, glassware with ground glass joints was treated as described above. It will be obvious however that this modification did very little to improve the recoveries. At the same time it was shown that prolonged heating of the oestriol in a stream of air during the removal of the ethanol being used at that time was not harmful even in the presence of ether residues. These results are shown in Table 39.

Table 39. Effect of heating oestriol (25μg.) in the presence of solvent residues (ethanol-hydroquinone colour reaction).

	D_{604}	D_{601}	D_{606}	$D_{corr.}$
Oestriol + 10 ml. ethanol; heated for additional 30 min.	0.162	0.039	0.003	0.158
	0.168	0.045	0.006	0.160
Standard	0.166	0.039	0.003	0.162
	0.169	0.040	0.002	0.166
Extracted oestriol in 10 ml. ethanol just to dryness	0.145	0.058	0.028	0.115
Extracted oestriol in 10 ml. ethanol; heated for additional 30 min.	0.145	0.060	0.023	0.118
Standard	0.164	0.023	0.005	0.160
	0.155	0.020	0.005	0.152

The evidence cited above clearly indicated that no significant loss or destruction of oestriol was occurring during the transfer procedure. It was clear then that the low recoveries were due to processes occurring/

Table 40. Destruction of oestriol by Fe^{+++}

Extracted from 75 ml. HCl and:	D_{604}	D_{601}	D_{606}	$D_{corr.}$	% Recover
500 ml. tap water	0.148	0.072	0.023	0.119	69
500 ml. distilled water	0.149	0.084	0.031	0.111	65
500 ml. glass dist. water	0.142	0.073	0.021	0.114	66
500 ml. dist. water + 0.5 mg. Fe^{+++}	0.035	0.063	0.022	0.003	2
500 ml. dist. water + 0.5 mg. Cu^{++}	0.143	0.074	0.021	0.114	66
Standard	0.173	0.030	0.003	0.172	
	0.173	0.032		0.172	

occurring either while the oestriol was in contact with the dilute hydrochloric acid or during the extraction-distillation procedure.

4. Loss of Oestriol on Contact with Fe^{+++}

Rosenmund (1948) demonstrated the destruction of oestrogens by acid hydrolysis in presence of traces of ferric chloride. It was thought possible that this ubiquitous contaminant might be contributing to the losses encountered during extraction. In order to test this hypothesis, 25 μg. of oestriol were added to 500 ml. of water of different degrees of purity and 75 ml. of A.R. hydrochloric acid. Ferric chloride (0.5 mg. Fe^{+++}) and cupric sulphate (0.5 mg.) were added to two of the flasks. The solutions were allowed to stand at room temperature for 48 hours. The oestriol in all cases was extracted as shown in Fig. 28. The results obtained using the ethanol stabilized hydroquinone colour reaction are shown in Table 40 on the facing page. The results clearly indicated that rather large amounts of Fe^{+++} added to the aqueous phase caused complete destruction of oestriol on standing at room temperature. Cu^{++} caused no destruction. The experiments with tap water showed the same recoveries as that obtained/

obtained with water distilled in an all glass apparatus, though the former would undoubtedly contain more iron.

Further experiments were carried out in an attempt to establish the mechanism of the reaction, and the most important results are shown in Table 41.

Table 41. Characteristics of Fe^{+++} -induced destruction of oestriol.

Expt.	Conditions	D_{604}	D_{601}	D_{606}	$D_{corr.}$	% Recovery
1	Immediate extraction	0.144	0.116	0.033	0.096	50
	4 hr. standing	0.061	0.048	0.015	0.040	11
	Standard	0.183	0.028	0	0.185	
		0.193	0.029	0	0.195	
2	12 hr. standing	0.050	0.068	0.027	0.013	7
	12 hr. standing + 100 mg.Vit. C	0.186	0.118	0.035	0.137	76
	Standard	0.181	0.031	0.003	0.180	
		0.183	0.031	0.003	0.182	
3	1 hr. standing	0.098	0.096	0.043	0.043	27
	1 hr. standing + 1.4 mg. hydroquinone	0.195	0.089	0.037	0.151	95
	Standard	0.155	0.018	0	0.156	
		0.163	0.021	0.002	0.162	

In all cases 0.5 mg. Fe^{+++} was added to the aqueous phase along with the oestriol and hydrochloric acid. The solution was extracted immediately, or allowed to stand at room temperature for the time shown. The results show that prolonged standing was not required, there being 50% destruction even with immediate extraction. Vitamin C in a concentration of 1/50,000 was effective in preventing the massive destruction.

Hydroquinone/

Hydroquinone (1/50,000) protected in a similar manner and moreover allowed quantitative recovery of the oestriol. It thus appeared that this antioxidant was able to prevent not only the iron-induced oxidation but also the loss which up to this time invariably occurred.

The results shown in Tables 40 and 41 suggested that the destruction caused by Fe^{+++} was not related to the low recoveries of oestriol which occurred in the routine ether extraction. Similar recoveries were obtained from water samples of different iron content. Relatively large amounts of Fe^{+++} were required to cause additional loss. There was a definite difference between the hydrophilic antioxidant Vitamin C and the lipophilic antioxidant hydroquinone, although they would be equally effective in reduction of Fe^{+++}. Dr. Ramsay in this Department was unable to demonstrate the presence of any iron in the glassware used or in the final extraction residues, by means of the sensitive 2-2'-dipyridyl reagent. Moreover, 1 mg. of Fe^{+++} added to the aqueous phase was removed during the washing procedure, none being demonstrable in the final extracts. It was concluded that this contaminant did not present any problem provided A.R. reagents were used throughout.

5. Antioxidants

It was noted that hydroquinone was effective not only in preventing the massive destruction by Fe^{+++} but also gave quantitative recovery. This fact was confirmed on several occasions and other antioxidants were tried. Typical results are shown in Table 42, and the complete set of investigations is recorded in Appendix VI, p. 35.

Table 42. Effect of antioxidants in protecting against destruction of oestriol during extraction.

Antioxidant	D_{604}	D_{601}	D_{606}	$D_{corr.}$	% Recovery
7 mg. hydroquinone	0.184	0.074	0.024	0.153	95
	0.225	0.169	0.049	0.152	95
Standard	0.165	0.033	0	0.163	
	0.164	0.033	0.003	0.160	
100 mg. Vitamin C	0.188	0.159	0.045	0.121	68
	0.188	0.109	0.040	0.138	77
	0.187	0.154	0.052	0.116	65
Standard	0.186	0.035	0.007	0.181	
	0.182	0.038	0.008	0.176	

It will be seen that hydroquinone prevented the apparent destruction of oestrogen during the extraction process, whereas Vitamin C even at a higher concentration was ineffective. As shown in the complete series in the Appendix, amino naphthol sulphonic acid was also incapable of increasing the yield. This suggested that the loss was occurring when the oestriol was in the ether phase, since the lipophilic agent was effective/

effective and the hydrophilic agents ineffective. The disadvantage of hydroquinone was obvious in that as has been seen in Part III it is a source of non-specific chromogens. This is evident in Table 42, by the large difference in the D_{606} readings in the duplicates. Moreover, since the purification method has been designed to eliminate hydroquinone it would be necessary to add it at each stage and accept the brown residues which so readily form. The primary usefulness of this series of experiments therefore was that they indicated the ether phase as the site of the destruction.

6. Peroxides

Long before the indication from the hydroquinone, the possibility that peroxides in the ether were the cause of the destruction had been considered. Proof of this has, however, been difficult to obtain. On the other hand, it was readily demonstrated that residues resulting from distillation of the ether caused a non-specific component in the colour developed in all modifications of the Kober reaction. Thus without a correction formula it would be believed that ether was enhancing the colour. A typical experiment is shown in Table 43.

Table/

Table 43. Stability of oestriol during distillation of ether.

Expt.	D_{604}	D_{601}	D_{606}	$D_{corr.}$
1	0.180	0.072	0.012	0.161
	0.190	0.085	0.014	0.166
2	0.168	0.048	0	0.164
	0.188	0.069	0.008	0.173
Standard	0.162	0.028	0	0.163
	0.168	0.028	0	0.169

Expt. 1 - 25μg. oestriol was dissolved in 500 ml. of ether (water saturated), and the ether distilled almost to dryness. The solution was then transferred to a Kober tube with 3 x 3 ml. ethanol, and the solvents 'puffed down' at 100°C. in a current of air.

Expt. 2 - As for Expt. 1, but the distillation was taken to dryness and the dry residue heated an additional 5 min. in a boiling water bath.

The results shown are typical of many similar experiments carried out. The ether used was A.R., purified by washing with a concentrated solution of ferrous sulphate in dilute sulphuric acid, and distilled through a column (4 ft.). It gave a negative vanadium pentoxide test like all other ethers which have been used in this investigation. There is no evidence of destruction or of enhancement provided the/

Table 44. Effect of hydrogen peroxide and acetaldehyde on the colour produced by oestriol in the m-cresol stabilized hydroquinone colour reaction.

Conditions	D_{sol}	D_{sol}	D_{sus}	$D_{corr.}$
25μg. oestriol + 5 mg. H_2O_2	0.160	0.036	0.008	0.152
25μg. oestriol + 5 mg. H_2O_2 + 25μg. AcH	0.175	0.080	0.008	0.156
25μg. oestriol + 5 mg. H_2O_2 + 50μg. AcH	0.192	0.135	0.011	0.157
25μg. oestriol + 5 mg. H_2O_2 + 75μg. AcH	0.202	0.182	0.010	0.156
Standard	0.150	0.018	0	0.151

the correction formula is applied. Excessive heating of the residue after distillation caused no loss, but diminished the nonspecific background colour as evidenced by a decrease in the D_{606} readings. This suggested that volatile substances were responsible for the effect on the Kober reagent.

Ether peroxides are readily decomposed into hydrogen peroxide and acetaldehyde (Reimers, 1945, 1946a,b,c). It was found that addition of small amounts of these substances to the hydroquinone sulphuric acid reagent (Brown, 1952b) caused the appearance of a brown cloudy precipitate. This effect was markedly decreased by the addition of 2.5%(v/v) of m-cresol to the reagent as has already been discussed in relation to Type I inhibition of colour development. Various experiments were carried out in an effort to show destruction of oestriol on standing in the presence of these contaminants. Typical results are shown in Table 44 on the facing page.

The results show no evidence of destruction of oestriol even though the contaminants were in contact with the oestrogen for one hour. Allowing contact overnight gave similar results. The effect of the contaminants/

contaminants on the nonspecific colour is however apparent.

This failure to obtain destruction by adding aqueous solutions of hydrogen peroxide and acetaldehyde to a dry residue of oestriol did not exclude the possibility that loss might occur during distillation of ether solutions. Accordingly, oestriol was dissolved in ether of differing degrees of purity, the solutions shaken and the solvent was removed by distillation. The residues were transferred to Kober tubes with ethanol in the usual manner and colours were developed with the m-cresol stabilized colour reaction. The results are shown in Table 45.

Table 45. Effect of acetaldehyde and peroxides on oestriol (25 μg.) in ethereal solution.

Expt.	Conditions	D_{604}	D_{601}	D_{606}	$D_{corr.}$	% Recovery
1	50 ml. peroxide free ether containing 50 μg. AcH	0.168	0.047	0.014	0.154	100
	Standard	0.154	0.021	0.004	0.154	
2	50 ml. technical ether (+ V_2O_5 test)	0.026	0.028	0.012	0.010	6
	Standard	0.160	0.023	0	0.160	
3	45 ml. peroxide free ether + 5 ml. tech. ether (+ V_2O_5 test)	0.168	0.045	0.009	0.157	98
	Standard	0.158	0.022	0.001	0.160	

Again/

Again no destruction was obtained in the presence of small amounts of contaminants. Grossly contaminated ether did however completely destroy the oestriol. This is in agreement with earlier workers (cf. Cohen and Marrian, 1934) who stressed the necessity for the use of peroxide-free ether. The results of both Table 45 and Table 43 seemed to indicate that peroxides were not formed during the distillation process in quantities sufficient to account for the low recoveries. It was possible however that even these trace amounts could over a long period cause destruction. In order to check this possibility six uncontaminated oestriol residues and six residues obtained from distillation of 500 ml. of ether solutions, were left in the desiccator for two weeks. Colours were then developed using the m-cresol stabilized hydroquinone sulphuric acid reaction. The results are shown in Table 46.

Table 46. Effect of prolonged contact between oestriol and residues obtained by distillation of 500 ml. of peroxide-free ether.

D_{604}, Standards	0.174,	0.172,	0.172,	0.173,	0.171,	0.170
D_{604}, Distillation Residues	0.090,	0.125,	0.169,	0.156,	0.167,	0.165
% Recovery	52	73	98	90	97	96

At/

At this stage in the investigation of the effect of ether residues it was clear that the use of peroxide-free ether caused no loss of oestriol during distillation under ordinary operating conditions. Residues left from the ether distillations did however cause an appreciable increase in the background colour developed in the Kober reaction and thus were a source of nonspecific chromogens. It was shown that more background colour was produced by carrying through a complete blank extraction than from distillation alone. This was assumed to be indicative of formation of more peroxides at some earlier stage of the procedure. Since the low recoveries were found only on complete extraction from aqueous acid (see Fig. 28, p. 145), and not on distillation alone, peroxides were still considered to be the cause of the difficulty. It was felt that the vanadium pentoxide test was too insensitive, and that the so-called 'peroxide-free' ether might in fact contain peroxides which during the extraction procedure would be increased to levels capable of destroying oestriol. Over a period of three months new and different methods of purification of ether were tried. These involved treatment with sodium hydroxide, sodium wire, sulphuric acid, potassium permanganate/

permanganate, ferrous sulphate in neutral and acid solution and silver oxide, singly and in various combinations. In retrospect the fallacy in the reasoning is obvious. The problem was however complicated by the use of an unsatisfactory colour reaction. Thus, as has already been stated, the day to day variation in Type II inhibition and fading contributed largely to the low recoveries of oestriol on extraction from aqueous acid. Superimposed on the inevitable loss which occurred when oestriol was extracted from aqueous hydrochloric acid, there was very frequently an apparent loss due to interference with colour development. When conditions of illumination and second stage sulphuric acid concentration were right, recoveries were improved. On the assumption that the colour reaction was satisfactory, it was considered that the better yields were due to the particular grade of ether being used. Typical experiments illustrating these points are given in Appendix VI, p. 37.

In the same period possible methods of decreasing peroxide formation were investigated. Passing nitrogen into the ether and aqueous phases before use was of no benefit, nor was distillation in a/

a stream of nitrogen. Washing the ether extract with an aqueous solution of sodium dithionite did not improve the yields. During this period Dr. Brown in the Clinical Endocrinology Research Unit was experiencing difficulties in the quantitative recovery of oestrone methyl ether which he was able to rectify by removing the solvent (petroleum ether) under reduced pressure in an atmosphere of nitrogen. A modification of his apparatus was devised (see Appendix III, p.14) and was used in the latter experiments listed on p. 144. No significant improvement in the ether extraction recoveries resulted. A comparison of the two methods was carried out with the m-cresol stabilized reaction. The results are shown in Table 47.

Table 47. Effect of mode of removal of ethanol from oestriol in the presence of ether residues.

m-cresol stabilized reaction

	D_{604}	D_{601}	D_{606}	$D_{corr.}$	% Recovery
Solvent removed in air	0.162	0.038	0.015	0.149	80
	0.151	0.028	0.008	0.145	77
Solvent removed in N_2	0.182	0.052	0.021	0.162	86
	0.166	0.031	0.013	0.156	83
Standard	0.185	0.019	0.003	0.187	
	0.188	0.019	0.003	0.190	

Evidently/

Evidently the modified form of removal of the solvent has not significantly improved the recoveries. In order to avoid chance contamination during the removal of the solvent the closed system was adopted. It is however believed that a clean stream of air is just as effective. There remained the possibility that the low recoveries consistently obtained over a six month period were due to some technical error. Dr. Brown very kindly arranged to have the procedure carried out in his laboratory using his glassware and reagents. Recoveries of 68, 72, 78, 75% were obtained using the ethanol stabilized hydroquinone colour reaction.

At this stage of the investigation it was clear that definite loss of oestriol was occurring during ether extraction from dilute aqueous hydrochloric acid. It was equally clear that neither the cause nor the cure was known.

7. Hydrochloric Acid as the Damaging Agent.

Early in the investigation extraction from water rather than hydrochloric acid had been tried. Recoveries of 80-85% were obtained. Subsequently as/

as it became increasingly clear that the hydroquinone reaction, whether stabilized with m-cresol or not, was subject to Type II inhibition and fading, these experiments were repeated. The results obtained with the m-cresol stabilized reaction are shown in Table 48.

Table 48. Extraction of oestriol (25μg.) from H_2O and dilute HCl.

Conditions	D_{604}	D_{601}	D_{606}	$D_{corr.}$	% Recovery
Extraction from H_2O	0.178	0.055	0.025	0.153	92
	0.189	0.063	0.025	0.163	98
Extraction from dil. HCl	0.150	0.080	0.032	0.112	67
	0.175	0.085	0.034	0.135	81
Standard	0.172	0.027	0.005	0.170	
	0.165	0.024	0.004	0.164	

The improvement in recovery in the absence of hydrochloric acid was evident. The colour reaction showed Type II inhibition but successive readings of optical density were taken until the maximum value in all cases was obtained. The cause of the low recoveries seemed at last to be found. Experiments were then carried out to check recoveries after neutralization of the acid. These gave very low and irregular values possibly due to contamination in the B.P. sodium/

sodium hydroxide used for the neutralization. The decisive experiments which established the cure are shown in Table 49.

Table 49. Recovery of oestriol (25μg.) from different aqueous media.

Extracted from:				% Recovery $D_{corr.}$
575 ml. H_2O	0.204	0.073	0.023	107
500 ml. H_2O, 75 ml. HCl	0.159	0.070	0.030	76
500 ml. H_2O, 75 ml. HCl (neutralized with NH_3)	0.195	0.081	0.032	96
500 ml. H_2O, 75 ml. 30% H_2SO_4	0.195	0.067	0.025	101

Evidently oestriol was not being destroyed by hydrochloric acid when in the aqueous phase, since recoveries were satisfactory if neutralization was carried out prior to extraction. This was in agreement with the failure of hydrophilic antioxidants to overcome the loss as has already been discussed. Moreover, it was not merely a question of hydrogen ion concentration during the extraction since sulphuric acid was not harmful. These results have been confirmed on many occasions, and some of these are shown in Table 50.

Table/

Table 50. Extraction of oestriol from dilute sulphuric acid.

				% Recovery
Extract	0.190	0.063	0.025	99
"	0.192	0.065	0.028	99
"	0.192	0.066	0.027	99
"	0.193	0.067	0.028	99
Standard	0.168	0.024	0.004	
	0.164	0.028	0.004	
Extract	0.185	0.049	0.011	100
"	0.170	0.039	0.001	95
Standard	0.171	0.021	0	
Extract	0.198	0.084	0.019	94
"	0.185	0.051	0.013	94
Standard	0.182	0.030	0.003	
	0.182	0.030	0.001	

This extraction procedure was used as the guide to the investigation of Type II inhibition and fading. The reason for the loss found with hydrochloric acid is not readily apparent. It is unlikely that it is due to contamination of the reagent by heavy metals. During this investigation hydrochloric acidsof several different batches from two suppliers were used. Possibly the monovalent acid forms an oxonium salt with ether of greater stability than/does the divalent acid. If this were so, one could hypothesize that sodium bicarbonate is/

is able to remove sulphuric acid but not hydrochloric acid from ether. The significance of the finding is apparent. If hydrochloric acid is used for the hydrolysis, loss of oestriol will occur during distillation when only the standard washing procedure is used.

B. Loss of Oestrone and Oestradiol-17β on Extraction from Aqueous Acid.

Throughout this investigation the recovery of oestrone and oestradiol-17β from pure solutions has been much more satisfactory than in the case of oestriol. Typical results for the complete method (see Fig. 26) are shown in Table 50.

Table 50. Recoveries of oestrone and oestradiol-17β extracted from aqueous HCl (complete procedure).

Oestrone μg.	% Recovery	Oestradiol-17β, μg.	% Recovery
150	90, 95, 90	150	83,100,91,89
	98, 90, 90		90, 96,88
	91,95,91,95		99,98,85,89
50	81,99,87,71	50	89,106,93,84
25	72,72,95	25	95,93,92,89
	93,86,82,83		70,87,87
	100,86,94,97		77,67

The series was carried out prior to the standardization of the chromatogram temperature, but the results shown include only those in which zero elution zones were demonstrated before the collection of the oestrogen fractions. Optical densities were read at three filters and corrected values are shown. It is evident that/

Table 51. Recovery of oestrone and oestradiol-17β (25 μg.) from aqueous H_2SO_4.

Extraction procedure: Fig. 28, p.145
Colour reaction: Appendix II, p. 8
Transferring solvent removed in closed system of nitrogen: see Appendix III, p.14

a. Typical results.

	D_{604}	D_{601}	D_{606}	% Recovery
Oestrone				
Extract	0.445	0.108	0.009	98
	0.452	0.110	0.013	98
Standard	0.444	0.087	0.002	
	0.442	0.090	0.003	
Oestradiol-17β				
Extract	0.362	0.087	0.010	100
	0.365	0.092	0.014	100
Standard	0.345	0.070	0	
	0.349	0.071	0	

b. Transference with aldehyde containing ethanol (oestrone)

Extract	0.445	0.139	0.018	86
	0.442	0.134	0.020	86
	0.428	0.131	0.019	83
	0.465	0.165	0.032	86
Standard	0.490	0.115	0	
	0.485	0.110	0	

c. Transference with aldehyde-free ethanol (oestrone)

Extract	0.498	0.152	0.029	95
	0.505	0.170	0.035	94
Standard	0.495	0.112	0.011	
	0.505	0.111	0.010	

that on the whole the results were better than those obtained at the same time with oestriol (see p. 144). On occasion quite large losses did occur, but the much greater problem of oestriol led to the investigation of the colour reaction and the possible causes of destruction which have already been discussed. Oestrone and oestradiol-17β were destroyed to the extent of 40% and 75% by standing at room temperature in aqueous hydrochloric acid in the presence of 0.5 mg.% Fe^{+++}.

When a satisfactory colour reaction had been devised, the recovery of oestrone and oestradiol-17β on extraction from aqueous acid (Fig. 28, p. 145) was investigated. Typical results are shown in Table 51a. On one occasion, when the transferring was done with ethanol not freed of aldehydes, an appreciable loss occurred as demonstrated in Table 51b. Repetition of the experiment with aldehyde-free ethanol gave a more satisfactory recovery (Table 51c). This suggested that, in the presence of ether residues, aldehydes were able to bring about a chemical change in oestrone. More will be said of this effect in the next section.

C. Destruction of Oestrogen in the Presence of Solvent Residues.

The simple procedure of removal of 10 ml. of purified rerectified spirit from 25 µg. of oestriol in a closed system of nitrogen caused 10-15% destruction with certain batches of alcohol. This effect is possibly due to thiophene introduced into the ethanol from azeotropic distillation with benzene during manufacture. Substitution of purified absolute spirit eliminated this difficulty whether the solvent was removed in air or nitrogen. Even with this type of ethanol for transfer, losses of oestriol occurred on extraction from aqueous acid (see Fig. 28, p. 145) unless precautions were taken to render the alcohol aldehyde-free. A typical experiment is shown in Table 52. In each case, oestriol (25 µg.) was added to a 1 litre flask from which ether, taken through a blank extraction (Fig. 28, p. 145), had been distilled. The contents of the flask were transferred to Kober tubes with ethanol (3 x 2 ml.) and the solvent removed either in a stream of air or in a closed system of nitrogen as shown.

Table 52. Destruction of oestriol by heating in ethanol in the presence of ether residues.

Ethanol	Solvent removal	D_{604}	D_{601}	D_{606}	% Recovery
AcH+	N_2 Puffer	0.313	0.137	0.032	83
AcH+	Air stream	0.327	0.137	0.031	87
AcH-	N_2 Puffer	0.351	0.149	0.035	94
AcH-	Air stream	0.335	0.123	0.022	93

Colour/

Colour Reaction: Appendix II, p.8.

The results suggested that in the presence of ether residues traces of aldehyde are able to cause an appreciable destruction of oestriol. Subsequently transference was done with aldehyde-free absolute spirit; the solvent was removed by heating in a boiling water bath in a stream of air with the hydroquinone required in the first stage of the Kober reaction.

Under these conditions it was shown (see Table 53) that heating oestriol with ether residues alone caused no destruction. Oestriol (10 μg.) was added to the ether from a blank extraction (see Fig. 28, p.145) and the solvent distilled almost to dryness. In two cases the flask was heated on the surface of the water bath for an additional 15 min., and in the other two cases the residual solvent removed in vacuo. It will be seen that no destruction of oestriol occurred.

Table 53. Stability of oestriol on heating in the presence of ether residues.

	D_{480}	$D_{512.5}$	D_{545}	Corrected D^x
Standard	0.296	0.472	0.098	0.275
	0.244	0.422	0.071	0.264
Dry residue baked	0.295	0.466	0.098	0.270
	0.294	0.456	0.092	0.263
To dryness in vacuo	0.317	0.480	0.117	0.263
	0.413	0.550	0.149	0.269

Colour reaction: Appendix II, p. 10.

In/

x See p.171.

In this experiment, the optical densities were measured with the Unicam spectrophotometer S.P.600.

The addition of acetaldehyde to the ether before distillation caused destruction under these conditions and for this reason the Ag_2O method of purification has been retained. Washing an ether extract of oestriol with aqueous bisulphite or dithionite caused a loss, unless the ether was subsequently exhaustively washed with bicarbonate. This suggests that the effect is due to formation of a relatively stable oxonium salt as in the case of hydrochloric acid. It was also found that other solvents destroyed oestriol to a similar degree, whether heated in air or nitrogen. This led to the rigorous methods of solvent purification given in Appendix I and to the addition of hydroquinone during removal of solvents from the purified oestrogens. The disadvantage of this latter practice was that frequently there was an increase in the non-specific background colour from the residue in the Kober reaction but this was corrected by the use of Allen's (1950) equation.

D. /

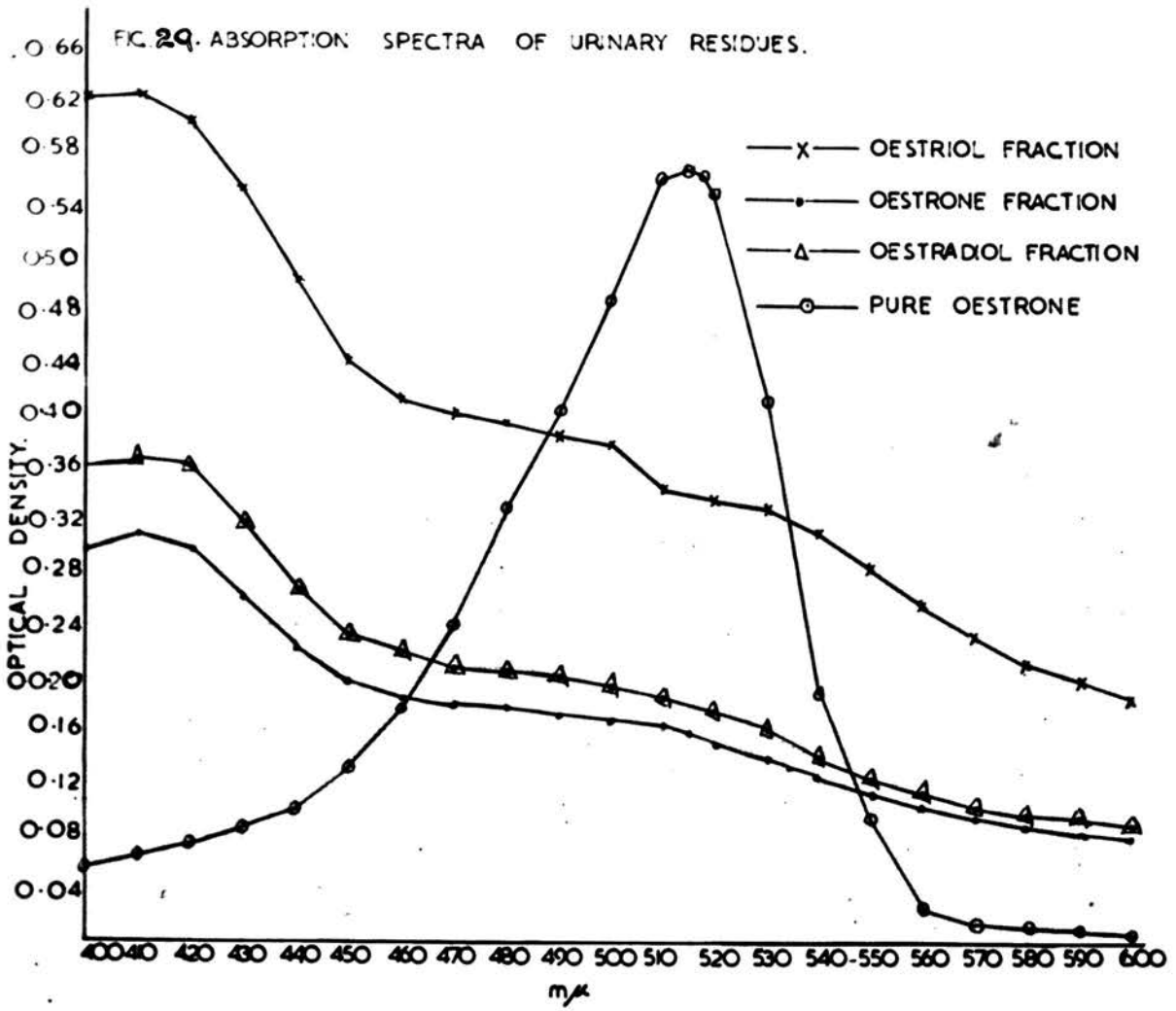


FIG. 29. ABSORPTION SPECTRA OF URINARY RESIDUES.
OESTRIOL FRACTION
OESTRONE FRACTION
OESTRADIOL FRACTION
PURE OESTRONE
OPTICAL DENSITY.
0·66
0·62
0·58
0·54
0·50
0·48
0·44
0·40
0·36
0·32
0·28
0·24
0·20
0·16
0·12
0·08
0·04
400 410 420 430 440 450 460 470 480 490 500 510 520 530 540 550 560 570 580 590 600
mμ

D. Overestimation of Endogenous Oestrogen due to Defect in the Colour Correction

The absorption spectra of the oestriol, oestrone and oestradiol-17β fractions of male urine residues taken through the modified hydroquinone colour reaction (Appendix II, p. 8) are shown in Fig. 29. The colour correction devised by Brown (1952c), which has been used throughout the pure solution experiments, is obviously unable to deal with this non-specific colour since a linear relationship does not hold throughout the range covered by the 601 and 606 filters. Very high values for endogenous oestrogen were calculated, particularly in the oestriol fraction, which in a series of normal male urines averaged 40-60 μg./24 hr. It was found that the use of a pure reagent or omission of the hydroquinone added in the colour procedure, did not improve the linearity of the residual background absorption.

It was therefore necessary to measure optical densities over a narrower range of frequency. This was done with the Unicam S.P.600 spectrophotometer. The equation of Allen (1950) was used for correction of the non-pink component:

$$CDX_X = OD_X - \frac{OD_a + OD_b}{2}$$

where OD_X = the observed density at wave length X, the absorption maximum (512.5 mμ. for oestrone and oestriol, 515 mμ. for oestradiol-17β).

Fig 30 Absorption Spectra of Oestrogens (10 µg.) in the Finalised Kober Reaction.

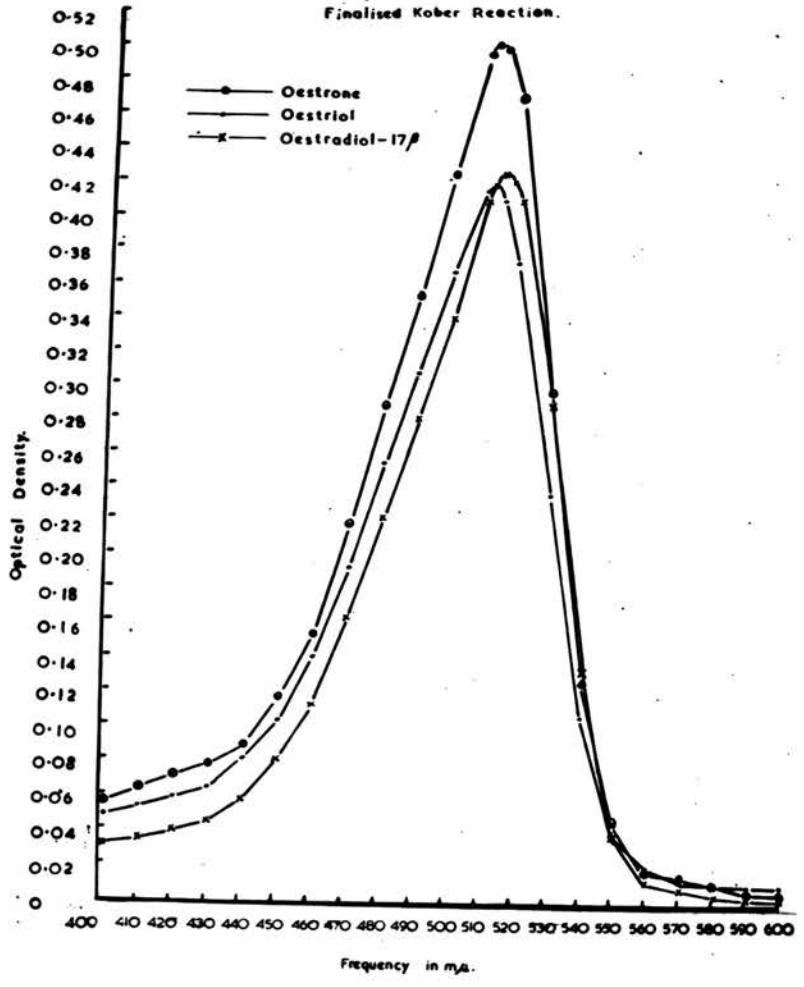
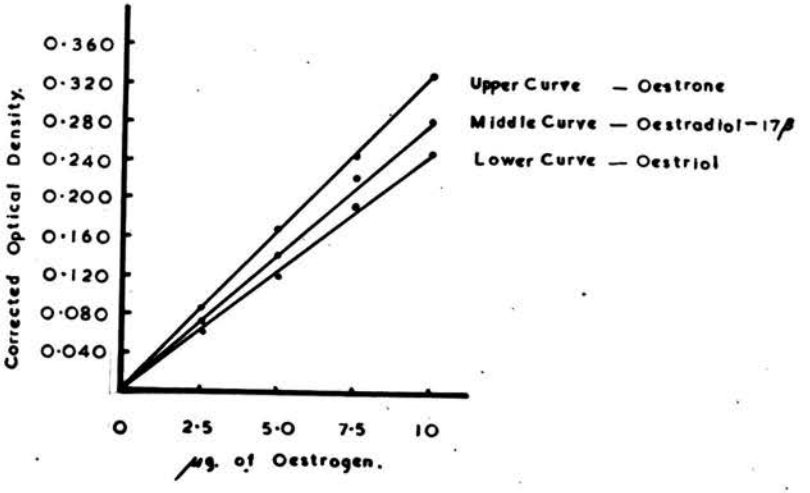


Fig. 31. Relation Between Concentration and Corrected Optical Densities in the Finalised Kober Reaction.



OD_a and OD_b = the observed densities at wave lengths a and b which are equidistant from X (480 and 545 mμ for oestrone and oestriol, 480 and 550 mμ for oestradiol-17β).

CDX_x = the calculated density for the particular oestrogen; this represents only a part of the total density at wave length X but the actual amount of oestrogen is directly related to this value.

Since the Unicam cuvettes require only 3.3 ml., a micromodification of the colour reaction was developed (see Appendix, p.10). The absorption spectra of the purified oestrogens (10 μg.) under these conditions are shown in Fig. 30. The $E^{1\%}_{1\ cm.}$ is approximately 1700 for oestrone and 1400 for oestriol and oestradiol-17β. By use of the colour correction, satisfactory conformity with Beer's law was obtained as shown in Fig. 31.

E. Destruction of Oestrogen by Urinary Residues

That urinary residues on being heated in ethanol can increase their chromogenicity and destroy oestriol is shown in Table 54. Portions (1/30) of the residue from the oestriol fraction of male urine (see /

(see Fig. 27, p. 141), were heated in a stream of air with 10 ml. of aldehyde-containing ethanol with and without oestriol as shown.

Table 54. Effect of heating urinary residues in ethanol, with and without oestriol (25 μg.)

	Optical Density			
	480 mμ	512.5 mμ	545 mμ	C.D.
Unheated residues	0.091	0.073	0.052	0.001
Residue heated with EtOH	0.114	0.092	0.068	0.001
Residue in EtOH, heated with oestriol	0.275	0.405	0.171	0.182
Oestriol standard	0.247	0.425	0.125	0.239

Colour reaction: 7.5 ml., Appendix II, p. 8.

When aldehyde-free ethanol was used no significant increase in the uncorrected optical densities from the urinary residues occurred, but the loss of oestriol was not diminished. A similar loss occurred when oestrone and oestradiol-17β were heated with the corresponding fractions of male urine. Clearly the diminished colour production could be due to either destruction of the oestrogen or interference with the colour reaction. Addition of hydroquinone to the tubes during the removal of the solvent prevented the loss as shown in Table 55.

Table 55/

Table 55. Prevention of destruction of oestrogens by urinary residues by the addition of hydroquinone during removal of transferring EtOH

	Optical Density			
	480 mμ	512.5 mμ	545 mμ	Corr.
Oestriol blank	0.345	0.327	0.229	0.015
Oestriol blank + oestriol (10 μg.)	0.726	0.791	0.330	0.263
Oestriol (10 μg.)	0.260	0.425	0.071	0.259
Oestrone blank	0.185	0.159	0.115	0.009
Oestrone blank + oestrone (10 μg.)	0.460	0.645	0.179	0.325
Oestrone (10 μg.)	0.258	0.483	0.076	0.316
Oestradiol blank	0.125	0.105	0.076	0.005
Oestradiol blank + oestradiol (10 μg.)	0.366	0.530	0.125	0.284
Oestradiol (10 μg.)	0.209	0.404	0.056	0.271

In each case the appropriate fraction of male urine (corresponding to 1/12.5 of the 24 hr. output) was heated in a stream of air with 10 μg. of oestrogen in 6 ml. of aldehyde-free ethanol. The colours developed by the residues in the micro-modification of the Kober reaction (Appendix II, p.10) were compared with those developed by pure oestrogen and fractions to which no oestrogen was added. It is apparent that the addition of hydroquinone (50 mg.) to the Kober tubes before removal of the transferring ethanol prevented the loss of oestrogen seen in the previous experiment.

F. Destruction of Oestrogens during the Extraction and Purification Procedure.

When the accuracy and reproducibility of the method were tested by recovery experiments, in which oestrogens were added to 1/5 aliquots of 24 hr. male urine specimens after hydrolysis, evidence was obtained of definite losses. At the start of these experiments, the initial extraction procedure (see Fig. 28, p.145) was modified so that after one wash with 35 ml. of 8.5% $NaHCO_3$ (to remove most of the mineral acid from the ether), the extract was shaken with 2 ml. of N-NaOH to effect oxidation of phenolic contaminants, then shaken with 35 ml. of 8.5% $NaHCO_3$ to buffer the NaOH to pH 8.5, and the combined washing discarded. This was repeated and followed by two washings with 25 ml. of water. In this way considerable purification was obtained so that the benzene-water partition was much less susceptible to emulsion formation. The subsequent alkalization procedures of the separated fractions (see Fig. 26, p. 140; and Fig. 27, p.141) still caused the formation of brown coloured substances. The losses which were encountered during the extraction of the individual oestrogens will be discussed below. All recoveries quoted are corrected for the non-pink component by the equation of Allen (1950) as described on p. 171, and for endogenous oestrogen by analysis of aliquots/

aliquots of urine to which no oestrogen was added.

1. Oestradiol-17β.

A series of thirty-three analyses of 25μg. of oestradiol-17β added to a 1/5 aliquot of a 24 hr. specimen of male urine after hydrolysis, gave recoveries of 61%, S.D. 8.5. Alteration of the procedure so as to buffer the sodium hydroxide shaking to a pH of only 10.5 gave in seven determinations 62%, S.D. 6.7. Since these losses were not encountered in recovery experiments from pure solution (see p.204), it appeared that urinary residues were in some way causing a loss, either real or apparent, of oestradiol-17β.

The chromatogram cuts were taken to dryness in vacuo and transferred to Kober tubes with aldehyde-free ethanol, and 50 mg. of hydroquinone were added before removing the solvent by heating in a stream of air. This procedure had already been shown to cause no significant loss of oestrogen (see Table 55, p. 174). Experiments conducted in which oestradiol (25μg.) was added to male urine residues immediately before chromatography, gave recoveries of 100, 96%; 88, 92%. It therefore appeared that the major losses were occurring during the earlier purification stages.

Brown (1952c) markedly improved his recoveries of oestrogen added to hydrolysed urine by the following procedures. The ether extract of hydrolysed urine

was/

was:

a. Washed with concentrated NaOH/$NaHCO_3$ buffer pH 10.5 (1 x 1/5 vol.);

b. Shaken with 1/20 vol. of 2N-NaOH;

c. Shaken again with 8.5% $NaHCO_3$ (1/5 vol.), and the combined washing discarded;

d. Washed with 8.5% $NaHCO_3$ (1 x 1/20 vol.);

e. Washed with water (1 x 1/40 vol.).

When the author's procedure was altered as above, in a series of twenty-one determinations with oestradiol-17β (25 µg.) added to 1/5 aliquots of 24 hr. male urine specimens after hydrolysis, recoveries of 71%, S.D. 13.5 were obtained. The improvement in yield is significant ($P < 0.01$). As has been noted above, with alkalization before the pH 10.5 wash, no improvement in the yield resulted. It therefore appeared that some urinary contaminant, largely removable from ether by washing with buffer of pH 10.5, could cause destruction of oestradiol-17β when the ether extract was shaken with N-NaOH. It is considered possible that a semi-quinoid system was acting as a potential mediator in the oxidation of oestradiol. Such phenomena are well known (cf. Waters, 1946). Suggestive evidence that such was actually occurring is given in Table 56.

Table /

Table 56. Apparent destruction of oestradiol-17β when hydroquinone was added before the alkalization step.

Expt.	Oestradiol extracted from	HQ added (5 mg.)	Recovery
1	Hydrolysed urine	Before alkalization	29%
	Hydrolysed urine	After alkalization	72%
2	500 ml. H_2O, 75 ml. 30% (v/v) H_2SO_4	Before alkalization	87;76%
		After alkalization	98;94%

It was clear, however, that appreciable losses were still occurring with the modified procedure. In fact, when the level of added oestradiol was dropped to 10μg. per 1/5 aliquot of the 24 hr. output, in a series of fifteen determinations, recoveries of only 51%, S.D. 8.2 were obtained.

In order to find the point of loss, an experiment was done in which oestradiol (10μg.) was added to a 1/5 aliquot of a 24 hr. urine specimen at different stages of the purification procedure. The results are shown in Table 57.

Table /

Table 57. Investigation of point of loss of oestradiol-17β during extraction from hydrolysed urine.

Expt.	10μg.oestradiol added	Recovery (%)
1	To hydrolysed urine	59
2	Before distillation of ether	71
3	Before benzene-water partition	79
4	Before chromatogram	111

Endogenous oestradiol in this specimen of urine was not measured,so the recoveries were not corrected for this. These results suggested that the principal loss was occurring during the washing of the benzene. This procedure in pure solution gave quantitative recoveries,but an appreciable loss occurred in the presence of urinary residues as shown in Table 58.

Table 58. Loss of oestradiol-17β during alkalization of the benzene solution (10μg.).

Expt.	Treatment of Benzene (50 ml.)	Recovery
1	a. None	91%
	b. Shaken with 2.5 ml. N-NaOH, acidified with 2.5 ml. 18% H_2SO_4, combined wash discarded; washed with N-Na_2CO_3 (1 x 5 ml.), H_2O (2 x 5 ml.)	68%
2	a. None	80%
	b. Shaken with 3 ml. N-NaOH, 4 ml. of 8.5% $NaHCO_3$ added and shaken, combined wash discarded; washed with 2 x 5 ml. H_2O	66%

When/

When the washes of the benzene were omitted, recoveries of 85%, S.D. 5.8, were obtained in a series of ten determinations of 10μg. of oestradiol-17β added to 1/5 aliquots of 24 hr. specimens of male urine after hydrolysis. This was a very significant improvement ($P < 0.001$). As noted on p. 139, the purification of the water-washed benzene was introduced to minimize the residues to be applied to the column. With the introduction of the 10.5 wash and alkalization of the original ether extract, the necessity for this step largely disappeared. The chromatograms of male urine residues with no further washing of the benzene after removal of the oestriol, show after complete elution of the oestradiol-17β a brown zone (1-1.5 cm.) on top of the column and a yellow band (1-2 mm.) 1-2 cm. below.

2. Oestrone.

Recovery experiments with oestrone gave results qualitatively similar to those obtained with oestradiol-17β. These are summarized in Table 59.

Table /

Table 59. Summary of recoveries of oestrone added to 1/5 aliquots, 24 hr. specimens of male urine, after hydrolysis.

n.	Method	Level µg.	Recovery Mean	S.D.	t	P
40	Alkalization of ether without prior 10.5 wash	25	73	(8.5)	4.2 <	0.001
20	Alkalization of ether after 10.5 wash	25	82	(7.4)		
14	Alkalization of ether after 10.5 wash	10	79	3.6	4.0 <	0.001
12	Alkalization of ether after 10.5 wash; no treatment of benzene	10	87	6.0		

For basic method, see Fig. 26, p. 140.

n = number of determinations

Level = amount of oestrogen added to a 1/5 aliquot of 24 hr. urine specimen, after hydrolysis.

t = difference between means/Standard error of difference between means. Burn (1937) & Hill (1950).

P = probability

It will be seen that washing the initial ether extracts with a buffer of pH 10.5 prior to the alkalization, as suggested by Brown (1952c) and described on p. 177, has significantly improved the yield of oestrone. Still higher recoveries resulted from the second modification to the procedure: the elimination of the further treatment of the water-washed benzene (which involved a second alkalization).

3. /

3. Oestriol

The basic method for the extraction and purification of oestriol is shown in Fig. 27, p.141. This procedure gave recoveries of approximately 90% of oestriol added to 1.5 N-H_2SO_4, but further losses were encountered on extraction from hydrolysed urine. In attempts to improve these recoveries, the following three types of modification in the procedure for washing the initial ether extract were tried:

a. The combined ether extracts were: washed with 8.5% $NaHCO_3$ (1 x 35 ml.); shaken with 10 ml. of 2 N-NaOH, 30 ml. of 8.5% $NaHCO_3$ were added, shaken, and the combined wash (pH 10.5) discarded; washed with 8.5% $NaHCO_3$ (1 x 35 ml.) and water (2 x 25 ml.).

b. The combined ether extracts were: washed with 8.5% $NaHCO_3$ (1 x 35 ml.); shaken with 2 ml. of N-NaOH, 35 ml. of 8.5% $NaHCO_3$ were added, shaken, and the combined wash (pH 8.5) discarded; washed with 8.5% $NaHCO_3$ (1 x 35 ml.) and water (2 x 25 ml.).

c. The combined ether extracts were: washed with 100 ml. of pH 10.5 buffer (13 ml. of 5 N-NaOH diluted to 100 ml. with 8.5% $NaHCO_3$); shaken with 25 ml. of 2 N-NaOH, 100 ml. of 8.5% $NaHCO_3$ were added, shaken, and the combined wash (pH 10.0) discarded; washed with 8.5% $NaHCO_3$ (1 x 25 ml.) and water (1 x 12.5 ml.). (Brown, 1952c).

A /

A comparison between the recoveries of oestriol from hydrolysed urine obtained with the basic method and those found with these modifications is given in Table 60. All urine samples were stored during collection at room temperature without preservative, except where noted, in which case the urine was kept at -5°C. The symbols used are defined in Table 59.

Table 60. The recovery of oestriol from hydrolysed urine: effect of modification in the procedures for the initial ether extract.

Type	Washing Procedure	n	Level µg.	Recovery Mean	Recovery S.D.	t	p
Original	HCO_3^- and H_2O only	8	25	70	12		
a	Alkal^n, buffering to 10.5; no prior 10.5 wash	8	25	64	9.1		
b	Alkal^n, buffering to 8.5; no prior 10.5 wash	40	25	68	11.4	2.4	< 0.01
e	Alkal^n, buffering to 10.5; prior 10.5 wash	16	25	75	9.4	3.5	< 0.001
c	Alkal^n, buffering to 10.5; prior 10.5 wash (preserved urine)	10	25	61	10.1		
c	Alkal^n, buffering to 10.5; prior 10.5 wash	14	10	76	6.7		

It will be seen that of the various modifications tried, the only one which significantly increased/

increased the recoveries was Type c, in which the total ether extract was washed with buffer of pH 10.5 prior to being shaken with strong alkali. Under these conditions similar recoveries were obtained with 10 µg. added to 1/5 of the 24 hr. urine specimen. Evidently, however, preserved urine contains substances capable of destroying oestriol during the extraction procedure. Mitchell (1953) has noted a similar effect with fresh placentae, the damaging agent apparently diminishing when the tissue was heated in air. Preliminary experiments conducted to determine the point at which the remaining losses are occurring suggest that oestriol is being partially destroyed on removal of the solvent from the chromatogram fractions, which still remain contaminated with non-specific chromogenic material. It therefore appears that further purification is required. In addition there seems to be a loss of about 10% in the various extraction stages, as shown by the recovery experiments from pure solution (see p. 191, p.202).

V. /

V. A DETAILED DESCRIPTION OF THE FINAL METHOD WITH THE RESULTS OF RECOVERY EXPERIMENTS

V. A DETAILED DESCRIPTION OF THE FINAL METHOD WITH THE RESULTS OF RECOVERY EXPERIMENTS

A. Apparatus and Materials.

Distillations were carried out in round-bottomed flasks fitted with ground glass standard taper (B.24) joints. Glass beads were used to prevent bumping during the boiling of liquids. All glassware except that used for extraction was cleaned as described in Appendix II, p.10; separating funnels and conical flasks were rinsed with solvents and washed with water. Stopcocks were lubricated with water only.

All reagents were of analytical reagent purity except where otherwise noted. Purification of materials is described in Appendix I, pp.1-5. The urine used throughout this investigation was collected from normal males.

B. Collection of Urine.

All urine excreted from the second sample on the day of collection up to and including the first specimen of the following morning was collected in a W/qt. with a glass stopper. During this period the urine was stored at room temperature without preservative. Determinations were carried out immediately after the collection was completed.

1. /

1. Notes. It is important that the urine container be given the routine cleaning procedure (Appendix II, p. 10) since a profuse growth of coliforms and cocci was demonstrated when only water washing was used.

C. Hydrolysis of Urine.

The urine was diluted to 2500 ml. with distilled water and duplicate 500 ml. samples were added to 1 litre flasks containing 100 mg. ± 5 (calibrated measure) of 1-amino-2-naphthol-4-sulphonic acid (B.D.H. Laboratory Reagent Extra Pure). The solution was brought to the boiling point by heating over a flame and under an efficient reflux condenser; 75 ml. of hydrochloric acid (ca 36% w/w) were added through the top of the condenser. The boiling was continued for 1 hr. and the flasks then cooled under running water (ca 15°C.).

1. Notes.

a. During the hydrolysis an oily liquid with a pungent odour collects on the upper part of the condenser surfaces. This frequently turns brown and is a potential source of contamination of subsequent determinations. In order to avoid this variable factor, it is important to rinse the inside of the condenser with a stream of water at the conclusion of the hydrolysis.

b./

b. The dilution of the urine to a definite volume was introduced by Stevenson and Marrian (1947) in order to standardize the concentration of urinary solutes during hydrolysis. It also facilitates the extraction of a fixed aliquot of the 24 hr. output in that the same capacities of glassware and standard volumes of extractants may be used regardless of the volume of the 24 hr. excretion. The use of a 1/5 aliquot allows the determination to be carried out in duplicate even when other analyses are to be carried out on the same urine.

c. Preliminary boiling of the urine, prior to acidification, was introduced by Callow and co-workers (1939). This has been followed here because it not only facilitates accurate timing of the hydrolysis, but also removes atmospheric oxygen from the solution before the acid is added.

d. As discussed on pp. 165-6, when the ether extract is washed only with bicarbonate and water, the use of HCl for hydrolysis results in the loss of oestriol during the subsequent distillation. The earlier recovery experiments were therefore done with 30% (v/v) H_2SO_4. With the wash of pH 10.5, which has been incorporated into the final procedure, losses do not occur on extraction from 1.5 N-HCl. In the final method, HCl is used as the /

the hydrolytic agent because there is considerably less emulsification during the ether extraction, and the original ether extract and final oestriol fractions are less contaminated, than when H_2SO_4 is used for hydrolysis. Van Bruggen (1948) noted that H_2SO_4 was less effective than an equivalent amount of HCl in the hydrolysis of oestrogen conjugates in urine. The present investigation has demonstrated that there is less charring and more ether extractable impurities when 30% (v/v) H_2SO_4 is substituted for HCl.

e. The experiments carried out to check the recovery of oestrogens in the extraction and purification stages were done with urine which had been hydrolysed in the presence of 1-amino-2-naphthol-4-sulphonic acid (1/5000). The necessity for the use of such a reducing agent was demonstrated by Van Bruggen (1948). It was used in the recovery experiments because urines hydrolysed in this way showed definite violet ether extracts as compared with yellow brown extracts when no reducing agent was added. The characteristic coloration passed into the neutral fraction and possibly was due to indigoids. It may be that reducing agents present during hydrolysis alter the oxidation-reduction/

reduction potential of certain ether-extractable materials. For this reason the recovery experiments were conducted under the conditions probably best suited for the hydrolysis of urinary oestrogen conjugates (cf. Marrian and Bauld, 1951).

D. Initial Extraction and Purification.

The cooled urine was extracted in a 1 litre separating funnel with purified ether (see Appendix I, p. 3), 1 x 150, 3 x 125 ml. The combined ether extracts, including the charred material collecting at interfaces and on the glassware, were washed once with 100 ml. of pH 10.5 buffer, (13 ml. of 5 N-NaOH, diluted to 100 ml. with 8.5% $NaHCO_3$). The charred material largely dissolved in the aqueous phase and the remainder was discarded. The ether was then shaken 100 x with 25 ml. of 2 N-NaOH; this caused a brownish-red coloration in most urines. A solution of 8.5% $NaHCO_3$ (100 ml.) was then added, shaken, and the combined wash discarded. Washing with 8.5% $NaHCO_3$ (1 x 25 ml.) and water (1 x 12.5 ml.) was then carried out, and the ether distilled almost to dryness, the final 3-5 ml. being removed in vacuo.

1. Notes.

a. The necessity for removing the residual traces of ether at diminished pressure was never satisfactorily demonstrated; it has been retained/

retained because it appears to be good practice in view of the lability of the oestrogens. No benefit was found from drying the ether with sodium sulphate or passing N_2 in during distillation.

b. The reasons for the modified washing procedure (Brown, 1952c) have been discussed on pp. 177, 181,184 The final bicarbonate wash, which is always colourless,is to lower the pH of the wet ether before the water wash lowers the ionic strength.

c. The degree of browning which occurs on shaking with 2N-NaOH varies widely from urine to urine.

2. Recovery of Oestriol at this Stage.

Quantitative extraction of oestriol from 500 ml. of water and 75 ml. of 30% (v/v) H_2SO_4 with bicarbonate and water washing (see Fig. 28, p.145) has already been demonstrated (see p. 165). With the wash at pH 10.5 one would expect appreciable losses since the pK of oestriol is 9.1-9.4 (Butenandt and Westphal, 1934). Brown (1952c) demonstrated, however, that when buffers of high ionic strength are used that the washing procedure noted above caused no loss.

Table /

Table 61. Extraction of oestriol from 1.5 N-HCl (pH 10.5 wash and alkalization).

Expt.	Type of Oestriol	D_{480}	$D_{512.5}$	D_{545}	$CD_{Aliq.}$	CD_{Sample}	% Recovery
1	Standard (9.6μg.)	0.217	0.374	0.043	0.244		
		0.216	0.372	0.048	0.240		
	Extract (2/5 aliquot)	0.260	0.390	0.084	0.218		90
		0.200	0.339	0.050	0.214		89
	In this experiment, 24μg. of oestriol were added to 575 ml. of 1.5 N-HCl, and a 2/5 aliquot of the washed extraction residue taken for colour development.						
2	Standard (12.7μg.)	0.356	0.550	0.095	-	0.325	-
		0.388	0.570	0.105	-	0.323	-
	Extract (2/5 aliquot)	0.263	0.308	0.124	0.114	0.285	88
		0.282	0.330	0.144	0.118	0.295	91
		0.256	0.314	0.134	0.119	0.298	92
		0.214	0.275	0.106	0.115	0.288	89
	In this experiment, 12.7μg. of oestriol were added to 575 ml. of 1.5 N-HCl, and a 2/5 aliquot of the washed extraction residue taken for colour development.						

E. Separation of Oestriol from Oestrone-Oestradiol-17β.

The distillation residue in the 1 litre flask was dissolved in 1.5 ml. of aldehyde-free ethanol and transferred to a 50 ml. separating funnel with 25 ml. of purified benzene (see Appendix I, p.2). The solution was washed with water (2 x 25, 2 x 12.5 ml.), the 1 litre flask being rinsed with each wash, and the aqueous phase drawn off into 250 ml. separating funnels supported/

supported on the lower shelf of the double rack (see Appendix III, p. 14).

1. Notes

a. Because of the small volumes of solution at this stage the stoppers were rinsed with benzene after each extraction.

b. In view of the critical nature of the separation of oestriol and oestradiol, care was taken to ensure that the solvents were kept at 15-18°C.

c. The ethanol is required to facilitate solution of the distillation residue.

d. In pure solution experiments, both phases were clear during this separation but in the presence of urinary residues the upper phase remained cloudy. The alkalization of the ether extract (see p. 177) markedly diminished the tendency to emulsification at the interface. Addition of hexane to the benzene (1/1 or 1/4 Hexane-Benzene) decreased the cloudiness of the upper layer but allowed oestradiol to enter the aqueous phase; (15%, 6% respectively).

2. Recovery at this Stage.

Table/

Table 62. Typical experiment showing removal of oestriol (25μg.) from benzene.

	D_{604}	D_{606}	D_{601}	Corr. Recovery %
Standard	0.160	0.024	0	-
"	0.160	0.024	0	-
Extract	0.170	0.044	0.010	97
"	0.178	0.057	0.017	97

Colour Reaction - Appendix II, pp. 7-8.

F. Re-extraction and Further Purification of Oestriol.

The aqueous phase from the benzene-water partition was shaken with 7.5 ml. of 10 N-NaOH, extracted with ether (1 x 150 ml.); and taken to pH 9.3-9.5 with gaseous carbon dioxide. The latter procedure was carried out with a manifold, to which was attached capillary tubing leading to the bottom of the separating funnels (see Appendix III, p.14), the end one of which contained 75 ml. of water, 7.5 ml. of 10 N-NaOH and a few drops of thymolphthalein, thus acting as an indicator. The pH of the extractions was finally checked (9.3-9.5) with hydrion paper (Johnsons, 8410) and the aqueous phases were extracted with ether (4 x 40 ml.). The combined ether solutions were washed with water (1 x 5 ml.) and distilled to dryness, the residual fumes being blown in a stream of air at room temperature. The residue was stored overnight at this stage.

1. /

1. Notes.

a. The reasons for these procedures are given on pp. 101-5. With the use of the pH 10.5 wash and alkalization in the first ether extraction, there is much less browning when the alkali is added to the aqueous phase, than in the original procedure. The step is retained, however, since it is necessary for the extraction of impurities from pH 13.5-14.0.

b. Since a considerable amount of chromogenic impurities arose when certain batches of ethylene dichloride contacted N-NaOH, ether was substituted. The partition coefficient for the nitrogenous impurities of this fraction of urine is not as favourable as for the halogenated hydrocarbon, but this was overcome with the minimum of effort by a single extraction with a large volume; such a procedure was feasible in view of the ease with which ether may be recovered.

2. Recovery of oestriol from steps E and F.

Table 63. Typical experiment showing recovery of oestriol (25 μg.) from benzene-water partition, alkalization, washing with ether, and re-extraction from pH 9.5.

	D_{604}	D_{606}	D_{601}	Corr. Recovery %
Standard	0.160	0.024	0	
"	0.165	0.026	0	
Extract	0.170	0.050	0.008	97
"	0.173	0.043	0.008	100

G. /

Table 64. Recovery of oestrone and oestradiol-17β from 500 ml. of water and 75 ml. of 30% (v/v) H_2SO_4 through the complete original extraction procedure (Fig. 28, p. 145; Fig. 26, p. 140).

Oestradiol-17β (25 μg.)	D_G	D_V	D_Y	D_{corr}	Recovery%
Standard (25 μg.)	.186	.022	.001	.189	-
"	.192	.024	.001	.195	-
Extract	.196	.032	.003	.195	101
	.189	.034	.003	.187	98
	.204	.036	.005	.201	104
	.200	.034	.004	.197	102
Oestradiol-17β (50 μg.)					
Standard (25 μg.)	.206	.035	.002	.206	-
"	.216	.037	.004	.214	-
Extract (½ aliquot)	.224	.042	.007	.219	104
	.206	.035	.005	.204	97
	.218	.039	.005	.215	102
Oestrone (25 μg.)					
Standard (25 μg.)	.247	.037	.002	.249	-
"	.240	.035	0	.243	-
Extract	.248	.058	.008	.240	98
	.240	.051	.008	.233	95
	.252	.062	.012	.240	98
	.247	.048	.007	.242	99
Oestrone (50 μg.)					
Standard (25 μg.)	.242	.038	0	.245	-
	.242	.038	.002	.243	-
Extract (½ aliquot)	.234	.035	.003	.235	97
	.246	.041	.004	.245	100
	.239	.039	.004	.238	98
	.240	.040	.004	.239	98

Colour reaction Appendix II, p. 7.

G. Removal of Benzene from Oestrone-Oestradiol.

The water-washed benzene was transferred to a 250 ml. round-bottomed flask and sucked to dryness by a water pump on warming in a boiling water bath, a glass bead being used to facilitate removal of the solvent. The residues were stored overnight at this stage.

1. Recovery.

Elimination of the washing of the benzene (see Fig. 26, p.140) was done only at a late stage of the investigation (see p.177), so that the recoveries from pure solution by the earlier procedures are of importance and are shown in Table 64 on the facing page.

H. Partition Chromatography of Oestriol.

Equal volumes of purified ethylene dichloride and 70% methanol, maintained at 25°C. in the thermostatically controlled bath (Appendix III, p. 13), were shaken together and allowed to stand until clear. Separated 70% methanol (upper, stationary) phase and dry Celite (5 ml./5 g.) were shaken together in a closed container for 5 min. (see p.118), until the liquid was uniformly dispersed throughout the solid. Excess ethylene dichloride (lower, mobile) phase was mixed with the dampened Celite to make a uniform slurry, and the column packed to a height of 10 cm., as described in Appendix II, pp.11-12. The holes in the/

homogenizing disc (Appendix III, p.13) were S.W.G.22. Sufficient pressure was applied during packing to give a percolation rate (R_p) of 10-15 ml./hr. as the mobile phase flowed through the column by gravity.

The distillation residue from para. F. was transferred with mobile phase (3 x 1 ml.) to the packed column, supported by a clothes pin in the air enclosure of the water bath (Appendix III, p.13). These washes were delivered by a dropping pipette to the walls of the column immediately above the surface of the chromatogram, which was allowed to dry momentarily, each separate portion being allowed to enter the column before the next was applied. When the third wash disappeared below the surface of the chromatogram, mobile phase (enough to give a head of 1-2 cm.) was added down the walls of the column, a separating funnel containing 40 ml. of mobile phase connected (Appendix III, p. 13), and the complete chromatogram unit supported inside the air enclosure by the soft zinc collar affixed to the top of the separating funnel. Collection of the eluate was then begun.

Routinely the first 8 ml. were discarded and fractions collected as follows: 8-10,10-30,and 30-40 ml. The residues from the first and third cuts were taken through the colour reaction to prove the absence of oestriol. The middle fraction was taken to dryness with/

with 2-3 mg. of hydroquinone in the nitrogen puffer (Appendix III, p. 14) and 'Kobered' either directly or after a suitable aliquot was taken.

1. Notes.

a. The Celite, after purification (Appendix I, p.2), should not be dried at 110°C. for more than 48 hr. It may be stored in tightly stoppered containers.

b. Uniform R_p was obtained by the above procedure provided the air bubble was removed from the capillary inlet to the column.

c. Colorimetric estimations of the fractions from 8-10 ml. and 30-40 ml. provide a convenient method of checking the operation of the chromatogram. As experience was gained in the packing of the columns, these fractions were almost invariably oestrogen-free. Latterly, therefore, determinations of these fractions have only been done as a control procedure at weekly intervals. Recently a column was used with a very slight narrowing of the bore in the middle portion; this impeded free movement of the packing disc in this region and made the preparation of the column more difficult. It was soon found that this chromatogram allowed traces of oestriol into the first and third cuts.

d. The remainder of the 70% methanol phase was not used for the re-equilibration of a second volume of ethylene dichloride.

2./

2. Recovery at this stage.

In a series of 11 experiments in which 150 µg. of oestriol were applied to the column, recoveries of 95%, S.D.2, were obtained. In all instances a small amount of material, giving a typical colour in the Kober reaction, appeared in the first 2 ml. of eluate. This amounted to 2.0-2.5% of the oestriol applied and is possibly due to contamination of the Parke-Davis oestriol used in this investigation.

I. Separation and Purification of Oestrone and Oestradiol-17β by Partition Chromatography.

Equal volumes of 0.78-0.82 N-NaOH and purified benzene were shaken together at 65°F. ± 2 and allowed to stand until clear. Separated NaOH (lower, stationary) phase and dry Celite (8 ml./10 g.) were stirred together in a beaker for 5 min., until the liquid was uniformly dispersed throughout the solid. Excess benzene (upper, mobile) phase was mixed with the dampened Celite to make a uniform slurry, and the column packed to a height of 12 cm., as described in Appendix II, pp.11-12. The holes in the homogenizing disc were S.W.G.19. Sufficient pressure was applied during packing to give a percolation rate (R_p) of 10-12 ml./hr. as the mobile phase flowed through the column by gravity. The temperature of the room was controlled to 65°F. ± 2.

The distillation residue from Para.G. was transferred/

transferred with mobile phase (3 x 1 ml.) to the packed column. These washes were delivered by a dropping pipette to the walls of the column immediately above the surface of the chromatogram which was allowed to dry temporarily, each separate portion being allowed to enter the column before the next was applied. When the third wash disappeared below the surface of the chromatogram, mobile phase (enough to give an R_p of 10-12 ml./hr.) was added down the walls of the column, a separating funnel containing 45 ml. of mobile phase connected (Appendix III, p.13), and collection of the eluate begun.

Routinely, the first 8 ml. were discarded and fractions collected as follows: 8-10 ml., 10-30 ml., and 30-40 ml., all in Kober tubes. The mobile phase remaining on the top of the column was poured off, and replaced by unequilibrated ethylene dichloride-benzene (3/1 v/v). The next 40 ml. (i.e. 40-80 ml.) were collected in a 250 ml. round-bottomed flask, and finally 80-90 ml. in a Kober tube. The residues from fractions 8-10 ml., 30-40 ml., and 80-90 ml. were taken through the colour reaction to prove the absence of oestrone from the first two, and of oestradiol from the third. The 10-30 ml. cut containing oestrone, was taken to dryness with 3-5 mg. of hydroquinone in the nitrogen puffer (Appendix III, p.14), and 'Kobered' either directly or after a suitable aliquot was taken. A

stream/

stream of carbon dioxide was passed into the 40-80 ml. cut for 1-2 min. and the solvent sucked to dryness under diminished pressure by warming in a water bath. The residue was transferred to a Kober tube with aldehyde-free ethanol either in toto or after a suitable aliquot was taken. Hydroquinone (3-5 mg.) was added and the ethanol removed in the nitrogen puffer (Appendix III, p.14).

1. Notes.

a. The NaOH phase was not used again for equilibration of a second batch of benzene. It was convenient to standardize enough alkali to last a week (ordinarily 1 l.).

b. It is important that the slurry be flocculent since irregular elution patterns result from the use of a granular slurry (cf. p.134). This necessitates rigid precautions to keep the Celite dry; in practice this was met by storing it at 110°C. and cooling in a vacuum desiccator over P_2O_5 immediately before use. As already noted, prolonged heating is not desirable for the Celite used for the oestriol chromatogram.

c. There is a tendency for the eluate to creep up the outside of the column at the bottom; this is overcome by rinsing with benzene as the fractions are changed.

d./

d. The larger oestradiol cut is treated with CO_2 to neutralize any alkali removed from the column by the mobile phase.

2. Recovery at this stage.

Recoveries of 93, 100, 99, 100, 94% were found when 150 µg. of oestrone were added to the chromatogram and 93, 102, 99, 99% in a similar series for oestradiol-17β.

J. Colour Reaction.

The colour reaction used was the modified hydroquinone reaction (micro-method), the technique of which is described in Appendix II, p.10. As pointed out on p. 171 , the intensity of the colour produced was measured in the Unicam Spectrophotometer SP-600 (band width 3 mµ) at three frequencies, viz.,

For oestrone and oestriol - 480, 512.5, 545 mµ.

For oestradiol-17β - 480, 515, 550 mµ.

The non-pink component of the colour was eliminated by the use of the correction formula of Allen (1950), already described on p. 171.

K. Recovery of Oestrogens from Aqueous Acid Through the Complete Extraction Procedure.

1. Oestriol.

The recovery of oestriol (150 µg., 25 µg.), added to 500 ml. of water and 75 ml. of 30% (v/v) H_2SO_4 and taken through the extraction procedure described/

described above, is shown in Table 65.

Table 65. Recovery of oestriol from aqueous acid.

Oestriol added μg.	Aliquot	D_{604}	D_{606}	D_{601}	$D_{corr.}$	% Recovery
150	1/6	0.338	0.100	0.019	0.324	92
		0.315	0.070	0	0.322	91
Standard(25μg.)		0.350	0.084	0.002	0.355	-
"		0.343	0.080	0.001	0.352	-

Colour reaction - Appendix II, pp.8-9.
Colour correction - p.62.

		D_{480}	$D_{512.5}$	D_{545}	$D_{corr.}$	
25	2/5	0.313	0.409	0.097	0.204	86
		0.443	0.515	0.158	0.214	90
		0.349	0.447	0.132	0.206	87
Standard(10μg.)		0.234	0.380	0.051	0.237	
		0.234	0.382	0.051	0.239	

Colour reaction - Appendix II, p.10.
Colour correction - p.171.

2. Oestrone.

The recovery of oestrone (150μg., 25μg.), added to 500 ml. of water and 75 ml. of 30% (v/v) H_2SO_4 and taken through the extraction and purification procedure shown in Fig. 28, p. 145 and Fig. 26, p.140, is shown in Table 66. The method is as described above but also includes the original washing of the water-washed benzene.

Table/

Table 66. Recovery of oestrone from aqueous acid.

Oestrone added μg.	Aliquot	D_{604}	D_{601}	D_{606}	$D_{corr.}$	% Recovery
150	1/6	0.470	0.118	0.014	0.456	96
		0.440	0.104	0.019	0.432	91
		0.470	0.107	0.009	0.463	97
		0.465	0.105	0.012	0.456	96
	Standard (25μg.)	0.480	0.100	0.006	0.478	-
25	1/1	0.490	0.146	0.031	0.456	98
		0.485	0.142	0.026	0.456	98
		0.470	0.128	0.021	0.447	97
		0.485	0.132	0.025	0.459	99
	Standard (25μg.)	0.468	0.105	0.009	0.462	-

Colour reaction - Appendix II, pp.8-9.

Colour correction - p.62.

3. Oestradiol-17β.

The recovery of oestradiol-17β (150μg., 25 μg.), added to 500 ml. of water and 75 ml. of 30% (v/v) H_2SO_4 and taken through the complete extraction and purification procedure shown in Fig. 28, p.145 and Fig. 26, p.140, is shown in Table 67.

Table/

Table 67. Recovery of oestradiol-17β from aqueous acid.

Oestradiol added μg.	Aliquot	D_{604}	D_{601}	D_{606}	$D_{corr.}$	% Recovery
150	1/6	0.380	0.078	0.010	0.380	94
		0.385	0.084	0.015	0.380	94
		0.395	0.085	0.015	0.390	97
	Standard	0.400	0.075	0.008	0.404	-

Colour reaction - Appendix II, pp.8-9.

Colour correction - p.62.

		D_{480}	D_{515}	D_{545}	$D_{corr.}$	
25	2/5	0.227	0.400	0.066	0.253	96
		0.250	0.408	0.076	0.245	93
	Standard	0.215	0.408	0.054	0.273	-
	(10μg.)	0.215	0.385	0.047	0.259	-

Colour reaction - Appendix II, p.10.

Colour correction - p.171.

L. Recovery of Oestrogens added to Hydrolysed Urine

The results obtained when 10μg. of the three oestrogens were added to 1/5 aliquots of urine (hydrolysed as in Para. C) and taken through the procedures described in Para. D-J are shown in Table 68. The yields obtained with higher levels have been given in the previous part on sources of error.

Table /

Table 68. Recovery of oestrogen (10μg.) on extraction and purification from hydrolysed urine (1/5 aliquots, 24 hr. specimens).

Oestrogen	No.of Determinations	Recovery (%) Mean	S.D.
Oestriol	14	76	6.7
Oestrone	16	88	5.4
Oestradiol	10	85	5.8

M. A Critical Appraisal of the Final Method.

The investigations reported in this thesis were undertaken as a preliminary to a study of oestrogen metabolism in cardio-vascular disease. It was necessary that the final method be capable of separating oestriol, oestrone and oestradiol-17β and of estimating these oestrogens at the level of 100-400μg./day, the expected urinary excretion following administration of 1-2 mg. of [redacted] oestradiol (Clayton, 1949). It was desirable that the method be capable of measuring the excretion of endogenous oestrogen by normal males. The final method may be assessed in terms of these two objectives by the standards proposed by Borth (1952).

1. Reliability.

a. Sensitivity.

Table 69 lists the details of a typical experiment in which the recovery of 10μg. of oestriol, oestrone and oestradiol-17β, added to two 1/5 aliquots of/

Table 69. Details of recovery of oestrogens from hydrolysed urine.

	D_{480}	$D_{512.5}$	D_{545}	D_{corr}	% Recovery
Oestriol					
Standard 10.1 µg.	0.268	0.406	0.089	0.227	-
Urine + 10.1 µg. oestriol	0.954	0.927	0.518	0.191	66
	1.102	1.035	0.580	0.194	67
Urine	1.125	0.940	0.628	0.063	(13.5 µg/day)
	0.863	0.732	0.488	0.056	
Oestrone					
Standard (10.3 µg.)	0.324	0.531	0.114	0.312	-
	0.313	0.517	0.114	0.303	-
Urine + 10.3 µg. oestrone	0.659	0.828	0.329	0.334	93
	0.694	0.840	0.356	0.315	88
Urine	0.509	0.465	0.317	0.052	(9 µg/day)
	0.435	0.408	0.270	0.055	

Oestradiol-17β	D_{480}	D_{515}	D_{550}	D_{corr}	
Standard (9.7 µg.)	0.222	0.384	0.064	0.241	-
	0.230	0.398	0.076	0.245	-
Urine + 9.7 µg. Oestradiol	0.782	0.850	0.444	0.236	94
	0.804	0.850	0.440	0.238	91
Urine	0.676	0.563	0.434	0.008	(2 µg/day)
	0.770	0.640	0.490	0.010	

of hydrolysed urine, was measured, with a simultaneous analysis of the endogenous excretion of the same specimen. At the wavelength corresponding to maximum absorption of pink, the ratio of the optical density of non-specific urinary chromogens to that of oestrogen (10 μg.) is ca 1.9, 0.7, 1.5 for oestriol, oestrone and oestradiol respectively. While Allen (1950) did not limit the accuracy of his equation in this way, one does not feel justified in utilizing a colour correction when ratios greater than those quoted obtain. It is felt then that in this particular urine, reliable estimations could not be obtained when the oestrogen level was below 50 μg. per 24 hr. specimen. The figures quoted for the endogenous levels should therefore be regarded as approximations. Thus in so far as sensitivity is concerned, the original objective (100-400 μg./day) has been met, but the secondary objective (endogenous excretion of normal males) has not.

It is worth noting that a good portion of the extraneous colour results from solvent residues, acting either directly or through oxidation of the hydroquinone added as a protective agent during removal of the solvent. This may be seen for example in Table 65, p.202, in which the background colour in the pure solution experiment corresponded to an optical density at 512.5 m μ of 0.335 (2.5 x aliquot). The sensitivity

of/

of the method can thus be increased by more rigorous solvent purification. In addition the oestriol fraction still remains contaminated with trace amounts of urine residues; further purification stages are being tried.

b. Specificity.

Similar considerations apply to the specificity. Unquestionably separation from the major urinary contaminants has been obtained, but the final specificity of the method still depends to a large extent on the colour reaction in urines of low oestrogen titre.

c. Accuracy.

This factor, which signifies the closeness with which measurement approaches its true value, is apparent from the S.D. quoted in Table 68, p.205. It is satisfactory for oestrone and oestradiol-17β, and approaches the theoretical for oestriol (cf. Table 69, p.206 and Table 65, p. 202).

d. Precision.

The precision or reproducibility of measurements by the method is somewhat better than indicated by the standard deviations quoted in Table 68. The spread in these is at least in part due to variations in urines. The duplicates found in the last five oestrone recovery experiments were: 79, 80; 95, 90; 93, 98; 91, 88%.

2. /

2. Practicability.

a. Speed.

Four determinations can be carried out in 9-10 man hours, but for convenience the author has so arranged the method that extraction, chromatography, and colour development are done on separate days, with four analyses being begun on each of the first four days of the week. If necessary an analysis could be completed within 24 hours.

b. Expenditure.

A high resolving spectrophotometer is the only expensive apparatus needed for the method. Materials required include only the common laboratory reagents.

c. Skill.

It is felt that a technician, with a basic training in laboratory work, could become thoroughly competent in the method with 2-3 weeks further instruction. There is, however, a definite requirement for close supervision and 'quality control' by a research worker thoroughly familiar with all the sources of error in the method.

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APPENDICES I-VI

APPENDIX I

PURIFICATION OF MATERIALS

The fractionating columns used for purification of solvents were 2 ft. long, lagged with asbestos rope, and filled with Raschig rings. They were usually used with a cold tap condenser, giving a reflux ratio of at least 30/1.

Acetone - Technical grade acetone was refluxed for 4 hr. with $KMnO_4$ (15 g./l.) and either anhydrous K_2CO_3 (100 g./l.) or KOH (5 g./l.), (depending upon whether it was necessary to have the solvent anhydrous) and fractionally distilled twice. The redistillation was required since traces of MnO_4^- were carried over. Even with this purification, residues were left in flasks after drying which interfered with the phenol sulphonic acid colour reaction.

Alumina - The 'acid washed' variety was obtained by shaking 50 ml. A.R. HCl with a suspension of 200 g. of Al_2O_3 (Savory and Moore, or Peter Spence) in 500 ml. of H_2O for 15 min., and washing with H_2O (12 x 500 ml.) by stirring and filtration until the filtrate attained maximum pH (Cambridge meter), usually 4.4 - 4.5. Sufficient MeOH soluble material was present to cause an appreciable background colour in the Kober reaction, so the washed Al_2O_3 was refluxed 2x for 10 min. with

with 250 ml. of purified MeOH. The material was then heated at 100°C. under reduced pressure (water pump) until the evolution of gas had ceased. To attain Activity I (Brockmann) 2 hr. at 300°C. under reduced pressure (oil pump) were required.

When the pretreatment with acid was not required, the Al_2O_3 was washed with water until the supernatant dropped to pH 8 and given the treatment with MeOH, etc. as described above.

Benzene - A.R. reagent was refluxed for 1/2 hr. with A.R. H_2SO_4 (54 ml./l.), and washed with water (1 x 1/10 vol.) and NaOH (3 x 1/10 vol.), the third alkaline wash being left in contact for 1 hr. The benzene was then washed with distilled water (1/10 vol.), ensuring that the first wash was alkaline, the last neutral; partially dried with Na_2SO_4, and distilled.

Butanol - Technical grade reagent was refluxed for 10 hr. with NaOH (15 g./l.) and distilled.

Celite 535 - The technical grade was partially calcined at 400°C. for 4 hr., and then allowed to stand overnight in A.R. HCl. The yellow supernatant was poured off and additional acid added until the aqueous layer was almost colourless. The solid was then washed with distilled H_2O by stirring, decantation and filtration until the washings were free of Cl^- ($AgNO_3$ test) and of Fe^{+++}

(NH_4SCN test), and the Celite did not change the pH of distilled water (Cambridge meter). The solid was then spread in a thin layer in evaporating dishes, dried at 110°C. for 48 hr., and stored in glass stoppered bottles. For benzene-aqueous alkali chromatograms, it was necessary to keep the Celite in a desiccator over P_2O_5.

Ethanol - Ethanol was refluxed for 16 - 24 hr. with Zn dust (25 g./l.) and NaOH (100 g./l.), and fractionally distilled. Initially 'rectified spirit' was used but heating with certain batches caused 15 - 20% destruction of oestriol. This may possibly be due to thiophene introduced into the reagent during azeotropic distillation with benzene. Present indications are that 'absolute spirit' which is purified during manufacture by simple distillation does not contain the damaging agent. To obtain aldehyde-free reagent, ethanol, purified as above, was allowed to stand over m-phenylene diamine for 1 wk. and doubly distilled prior to use. It was found that samples which did not turn yellow on heating 0.2 ml. for 5 min. with the oestriol reagent, gave low blanks with the dinitro phenyl hydrazine test of Gornall and MacDonald (1953). The former test has been adopted.

Ether - A.R. ether was purified by shaking 2 l. for 6 min. with 16 g. of $AgNO_3$ in 120 ml. of H_2O and 200 ml.

of N-NaOH, washing with H_2O (1/10 vol.) to neutrality, and distilling. Originally a 4 ft. fractionating column was used, but unless the glass rings were cleaned daily they increased the formation of substances interfering with the Kober reaction. The distillate was collected in amber W/qt. and used within 3 days.

Ethyl Acetate

Type I - B.P. reagent was dried by standing over anhydrous $CaCl_2$ (B.P.) for 24 hr., thereby removing both water and EtOH. The solvent was poured off and fractionally distilled twice.

Type II - B.P. reagent was washed with 5% Na_2CO_3 (1 x 1 vol.), with a saturated solution of $CaCl_2$ (3 x 1/4 vol.), and with H_2O (3 x 1/4 vol.). The solvent was dried over anhydrous K_2CO_3 (12 hr.), and fractionally distilled twice.

Ethylene dichloride - B.P. reagent was washed with N-NaOH (3 x 1/5 vol.) and H_2O (1/5 vol. to neutrality), dried over $CaCl_2$, and distilled through a 2 ft. fractionating column into an amber bottle. This procedure was very effective in the early part of this investigation when material manufactured by I.C.I. was used. B.D.H. Laboratory Reagent Grade when purified in this way leaves a residue on distillation. This material was washed with H_2SO_4 (3 x 1/50 vol.), H_2O

(1 x 1/25 vol.), N-NaOH (1/15 vol. until basic), 10% HSO_3^- (1 x 1/15 vol.), 8.5% $NaHCO_3$ (1 x 1/15 vol.), and H_2O (to neutrality). It was partially dried over anhydrous Na_2SO_4 and distilled, the second distillation being within 24 hr. of use.

Methanol - B.P. reagent was refluxed for 15 - 20 hr. with 5% Zn dust and 10% NaOH, and doubly distilled. MeOH purified in this way gave lower blanks in the DNPH test of Gornall and MacDonald (1953) than when purified by Mg and I_2. Samples purified with furfural were contaminated with this substance even after 6 distillations. The water content was determined by the method of Rising and Hicks (1926).

APPENDIX II

METHODS IN GENERAL USE

A. Colour Reactions

1. Colour reaction using phenol sulphonic acid reagent

a. Preparation of reagent - Analar H_2SO_4 (60 ml.) was added dropwise from a burette to 40 g. of redistilled phenol in a conical flask, with continuous shaking, and at a rate insufficient to cause heating. The reagent was allowed to stand for 24 hr. at room temperature in a glass stoppered flask before use, and was discarded at the end of the third day.

b. Procedure - To the dry residue of oestrogen in a 'Kober tube' (see Appendix III, p. 13) were added (from a bulb pipette with the tip removed) 3 ml. of reagent, warmed to 37°C. for ease of pipetting. The tube was fitted with a top and immersed in a boiling water bath for exactly 20 min., shaking vigorously twice in the first 10 min. The tube was then immersed in an ice-salt mixture for at least 5 min. Water (3 ml.) was added to the tube, stirring with a glass rod with flattened end, and the tube was transferred to a boiling water bath for exactly 3 min., stirring 15 sec. of every min. The tube was cooled for 9 min. in an ice-salt mixture and allowed to stand at room temperature for 1 min. H_2SO_4 (10% v/v) was added to a final volume of 15 ml. and the solution was

thoroughly/

thoroughly stirred. The intensity of the colour with the 604 filter (Ilford) was read on a Spekker colorimeter within 30 min. All the heating procedures were carried out in a darkened part of the laboratory.

2. Modified phenol sulphonic acid colour reaction

All steps were carried out as above except for substitution of 2 ml. for 3 ml. of H_2O as the second stage diluent and increasing the heating time from 3 to 5 min.

3. The hydroquinone aqueous sulphuric acid colour reaction

a. Preparation of reagents - Solutions of hydroquinone (2% w/v) were prepared in 76%, 66% and 60% H_2SO_4 (v/v) for the oestriol, oestrone and oestradiol-17β reagents respectively.

b. Procedure for colour reaction - The appropriate reagent (4 ml.) was added to the dry residues of oestrogens in Pyrex test tubes (20 mm. x 150 mm.) and heated for 20 min. in a metal rack immersed in a boiling water bath, shaking once during the 4th - 5th min. The rack was then removed and cooled in cold water. 1 ml. of water was added to the oestriol tubes, 0.5 ml. of water to the oestrone tubes and the oestradiol tubes were not diluted. The contents were mixed thoroughly by shaking, heated in a boiling water bath for 5 min., then placed in cold water to cool. H_2SO_4 (30% v+v) was added to the tubes to a total

volume/

volume of 15 ml., and the contents mixed by a footed stirring rod. The optical densities of the test solutions were read after 5 min. in a Spekker colorimeter with the 604 filter.

The colour reaction for oestriol was stabilized where shown in the text by the addition of 0.1 ml. EtOH or m-cresol to each 4 ml. of reagent immediately before use.

4. Modified hydroquinone-aqueous sulphuric acid colour reaction

a. Preparation of reagents -

i. Oestradiol reagent - Quinone (20 mg., redistilled) was heated with 1 l. of 60% (v/v) H_2SO_4 (Hopkins and Williams or B.D.H.), containing 10 mg. $NaNO_3$ (A.R.), until the solution became pale green and slightly cloudy. Hydroquinone (20 g. B.D.H. Laboratory Reagent) was added and dissolved by shaking and heating on a boiling water bath. The solution lightened in colour and became more opalescent. With certain batches of B.D.H. H_2SO_4, traces of brown oxides of N_2 appeared. These were removed along with the opalescence by filtration through Por. 4 sinter glass. Occasionally the reagent crystallized out, requiring reheating. The final solution was a definite pink colour and non-opalescent.

ii. Oestrone reagent - This was prepared in the same manner as the oestradiol reagent and contained 20 g. of/

of hydroquinone, 20 mg. quinone and 10 mg. $NaNO_3$ in 1 l. of 66% (v/v) H_2SO_4. The final solution was yellow with a trace of pink.

iii. Oestriol reagent - This was prepared in the same manner as the oestradiol reagent and contained 20 g. of hydroquinone, 20 mg. of quinone and 10 mg. $NaNO_3$ in 1 l. of 76% (v/v) H_2SO_4. Less cloudiness appeared during its preparation and the final solution was yellow with no trace of pink.

b. Procedure for colour reaction - To a dry residue of oestrogen and 100 mg. $\pm$ 5 (calibrated measure) of hydroquinone, the appropriate reagent, in volume varying with the particular oestrogen (5.8 ml. for oestriol, 6.5 ml. for oestradiol, 6.8 ml. for oestrone), was added. The tube, with top in place, was heated in a boiling water bath for 20 min., shaking twice during this period to ensure solution of the hydroquinone. The tube was then immersed in a bath of cold water (ca 15°C.), 100 mg. $\pm$ 5 hydroquinone added and diluted as follows: 1.7 ml. H_2O for oestriol, 1.0 ml. reagent for oestradiol, 0.7 ml. H_2O for oestrone. The solution was mixed by lateral shaking (8-10x) and reheated for 15 min., with top in place, shaking twice during this period to ensure solution of the hydroquinone.

5. Modified/

5. Modified hydroquinone-aqueous sulphuric acid colour reaction (micro method).

a. Preparation of reagents - as for previous section

b. Procedure for colour reaction - To a dry residue of oestrogen and 50 mg. ±3 (calibrated measure) of hydroquinone in a Kober tube was added the appropriate reagent in volume varying with the particular oestrogen (2.6 ml. for oestriol and oestradiol, 3.0 ml. for oestrone). The tube, with top in place, was heated in a bath of boiling water for 20 min., shaking twice during this period to ensure solution of the hydroquinone. The tube was then immersed in a bath of cold water (ca 15°C.), 50 mg. ±3 hydroquinone added and diluted as follows: 0.7 ml. of H_2O for oestriol, 0.7 ml. of reagent for oestradiol and 0.3 ml. of H_2O for oestrone. The separate additions of oestradiol reagent were to wash the added hydroquinone to the bottom of the tube. The solution was mixed by lateral shaking (8-10x) and reheated for 15 min. with top in place, shaking twice during this period to ensure solution of the hydroquinone.

B. Cleaning of glassware

Glassware was rinsed with tap water and allowed to stand in chromic acid for 12 - 24 hr. It was then rinsed with water and ethanol, washed with a vigorous stream of tap water for 2 - 3 min., and finally rinsed with/

with distilled water.

C. Standard solutions.

Oestrogens (5 mg.) were dissolved in 100 ml. of aldehyde-free ethanol and stored at 4°C.

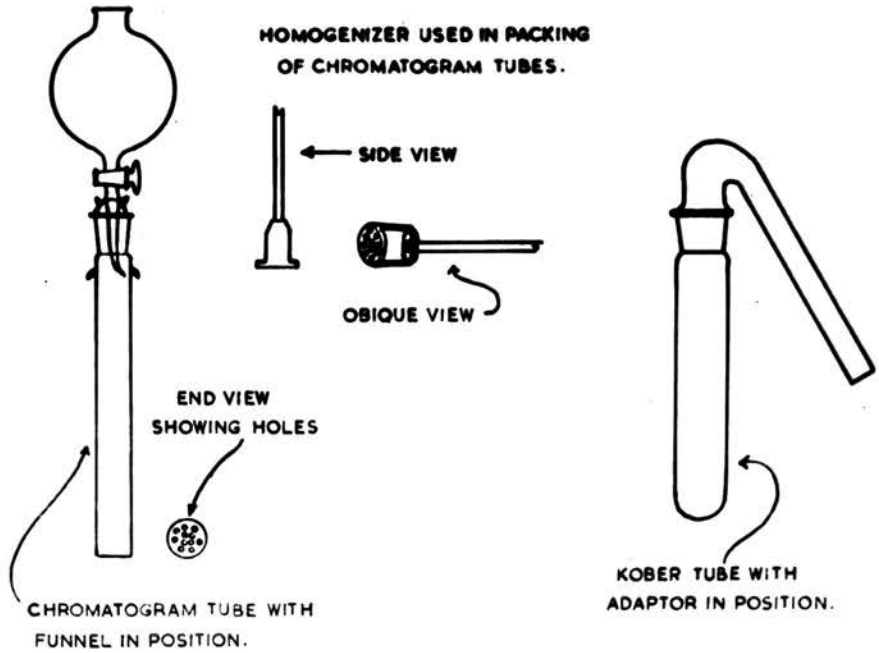
D. Standardization of Alumins

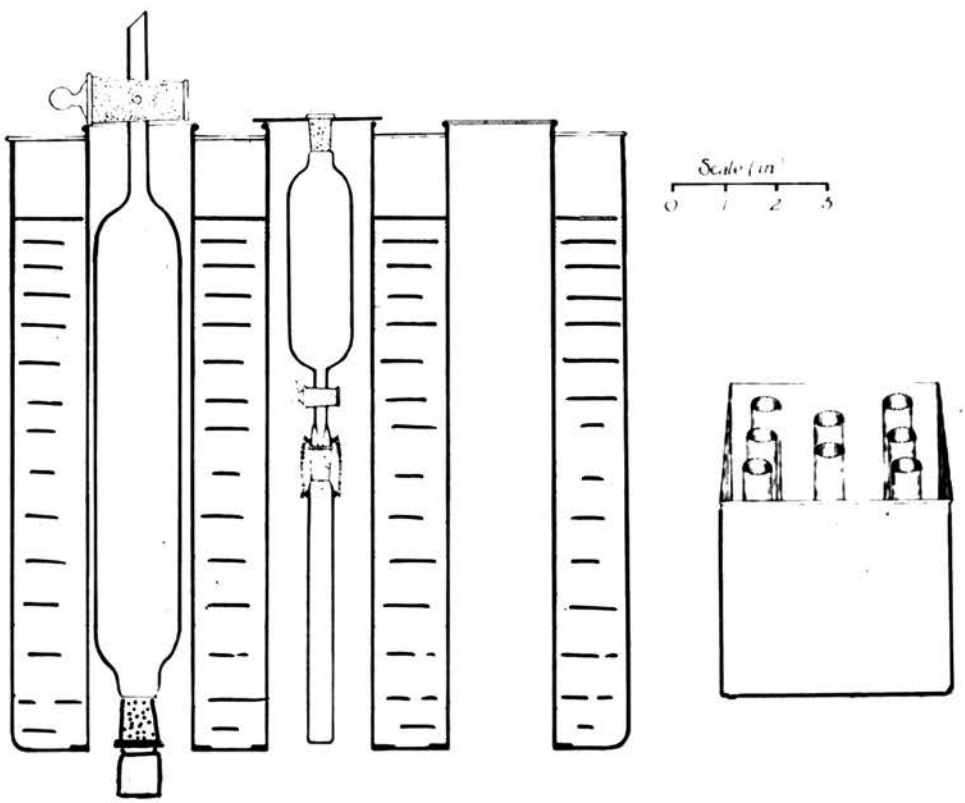
This was carried out according to the procedure of Brockmann and Schodder (1941).

E. Packing of partition chromatograms

Equal volumes of solvents, at the temperature at which the chromatogram was to be run, were shaken together for 1 min. and allowed to stand until clear. The appropriate amount of separated stationary phase was mixed with Celite (see text). An excess of the mobile phase was then added to the dampened Celite; a uniform slurry was obtained by thorough mixing and added in small portions (enough to give 1-2 cm. of height of column) to an empty chromatogram tube, supported with the open end below the surface of a few ml. of mobile phase in a test tube. The homogenizer (see Appendix III, p. 13) was moved rapidly up and down below the surface of the solvent through the slurry until particles of uniform size were obtained. These were then packed in small segments (about 2 mm.) by a slow firm movement of the plunger which trapped some of the particles and compressed them into a pad. The process/

process was repeated until the required height of the column was obtained. With a little practice the correct degree of pressure was found to obtain a percolation rate of 10 - 12 ml./hour. Before use, the Rp was measured by allowing 5 - 10 ml. of mobile phase to pass through the column. The top of the chromatogram was always covered with mobile phase except during the application of the solutes at which time the surface was allowed to go dry momentarily.





THERMOSTATICALLY CONTROLLED WATER BATH

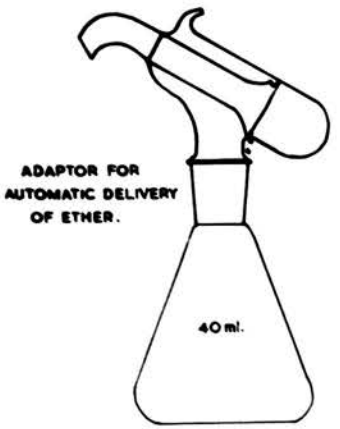
APPENDIX III

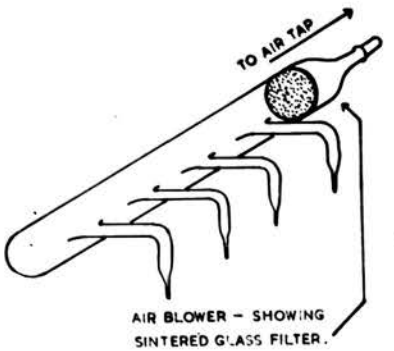
DESCRIPTION OF SPECIAL EQUIPMENT

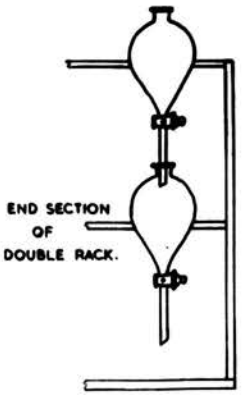
A. The homogenizer used for packing chromatograms, based on the suggestion of Martin (1949), is shown in the accompanying illustration. It consists of a brass disc, drilled with concentric holes, and fitted with a stirrup into which is screwed a stainless steel rod, 2 ft. in length. The diameter of holes for the oestrone-oestradiol column packer is SWG 19, while that for the oestriol column packer is SWG 22. Brass has been used for the disc in order to facilitate the boring of the holes.

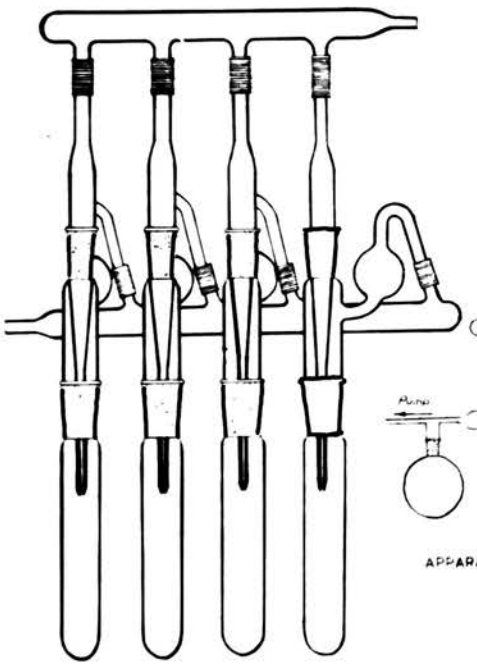
B. The funnel and chromatogram tube for the oestrone-oestradiol chromatogram are also shown. They form an air-tight system at the connecting B 19 joint so that the solvent level in the tube remains constant provided air locks in the capillary inlet are avoided.

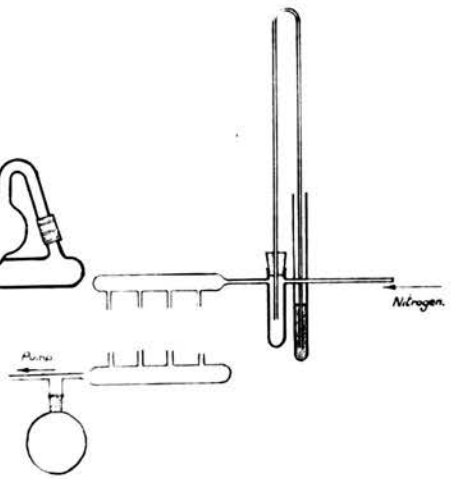
C. The thermostatically controlled bath is fitted with eight cylindrical chambers, open at both ends, and sealed into the bath by soldered flange joints. The water in the bath is maintained at a constant level by a device of conventional design, and is stirred by slow streams of air passing through a number of holes in/



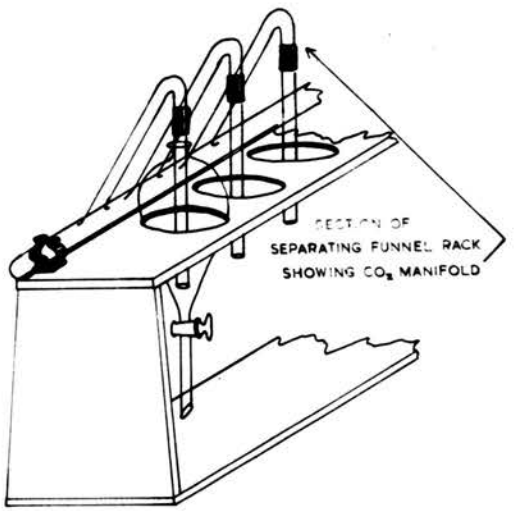








APPARATUS FOR REMOVAL OF ETHANOL.



in a glass tube lying on the bottom of the bath. The water is heated by a 50-watt immersion heater controlled, via a Sunvic relay unit (F102/4), by a mercury-toluene regulator. The cylindrical funnels are supported in the air chambers by soft zinc collars, and the chromatogram tubes are secured to the funnels at the interconnecting ground glass (B 19) joints by springs. The larger funnel shown in the figure is used for the equilibration of the solvent system. It is supported in an air chamber in the inverted position by its tap.

D. The apparatus for the removal of EtOH from Kober tubes is arranged to allow the evaporation of the solvent under reduced pressure in an atmosphere of N_2. The upper manifold is connected by polythene tubing to the jets within the tubes, and also to a source of dry N_2, the flow of which is regulated by a Hg. containing valve as shown in the smaller diagram. The lower manifold, which is attached by polythene tubing to the splash-head side arms of the tube, is connected to a water pump via a round bottomed flask in which the solvent collects. Before evaporation of the solvent was begun, the apparatus was evacuated and allowed to fill with nitrogen three times. During evaporation the Kober tubes were immersed in a boiling water bath and/

and the N_2 stream was left on.

E. The adaptor for the automatic delivery of a definite volume of ether is useful in the reextraction of oestriol.

The remaining apparatus is self-explanatory.

APPENDIX IV

SUPPLEMENTARY DATA ON THE COLOUR REACTION INVESTIGATION

Table 1. Effect of addition of m-cresol to hydroquinone reagent in overcoming depression by ether residues.

Method: 25 µg. of oestriol were extracted from 500 ml. of distilled water and 75 ml. A.R. HCl with ether (4 x 125 ml.). The combined ether extracts were washed with 8.5% $NaHCO_3$ (A.R.) and distilled water (2 x 25 ml.), and distilled to dryness. The residue was transferred to a Kober tube with EtOH (3 x 3 ml.) and the solvent puffed down in a stream of air. The colours of these 'extracts' were developed as shown below and compared with that developed by 25 µg. of pure oestriol - 'standard'.

Reagent 1 - 0.1 ml. of m-cresol was added to 4 ml. of 2% hydroquinone in 76% (v/v) H_2SO_4 immediately before use.

Reagent 2 - 4 ml. of 2% hydroquinone in 76% (v/v) H_2SO_4.

Colour reactions - see Appendix II, p. 7

$$D_{corr} = \frac{4D_{604} - 3D_{601} - D_{606}}{3.8}$$

Exp.	Oestriol used	Rgnt.	D_{604}	D_{601}	D_{606}	% Recovery D_{corr}
1	Standard	1	0.150	0.033	0	-
	Extract	1	0.168	0.060	0.025	94
	Extract	1	0.163	0.069	0.032	86
	Standard	2	0.160	0.047	0.002	-
	Extract	2	0.118	0.058	0.018	61
2	Standard	1	0.165	0.022	0	-
	Extract	1	0.192	0.081	0.038	90
	Standard	2	0.168	0.028	0.004	-
	Extract	2	0.125	0.059	0.025	57
3	Standard	1	0.165	0.028	0.003	-
	Extract	1	0.176	0.081	0.030	87
	Standard	2	0.177	0.032	0.004	-
	Extract	2	0.121	0.055	0.018	57
4	Standard	1	0.145	0.027	0.005	-
	Extract	1	0.140	0.065	0.029	76
	Extract	1	0.146	0.058	0.023	85
	Standard	2	0.152	0.050	0.002	-
	Extract	2	0.072	0.054	0.035	23
	Extract	2	0.098	0.052	0.020	48
5	Standard	1	0.155	0.022	0.002	-
	Extract	1	0.145	0.065	0.027	75
	Extract	1	0.157	0.080	0.035	75
	Standard	2	0.170	0.030	0	-
	Extract	2	0.115	0.052	0.023	52
	Extract	2	0.115	0.051	0.026	52
6	Standard	1	0.150	0.018	0	-
	Extract	1	0.145	0.074	0.035	69
	Standard	2	0.168	0.028	0.005	-
	Extract	2	0.119	0.058	0.027	54
7	Standard	1	0.171	0.028	0	-
	Extract	1	0.198	0.099	0.036	89
	Standard	2	0.187	0.035	0	-
	Extract	2	0.169	0.070	0.024	75

8/

Exp.	Oestriol used	Rgnt.	D_{604}	D_{601}	D_{606}	% Recovery D_{corr}
8	Standard	1	0.163	0.025	0	-
	Standard	1	0.169	0.028	0	-
	Extract	1	0.162	0.086	0.034	72
	Standard	2	0.164	0.029	0.004	-
	Standard	2	0.178	0.033	0.004	-
	Extract	2	0.119	0.055	0.024	54
9	Standard	1	0.168	0.035	0.004	-
	Extract	1	0.182	0.106	0.038	81
	Standard	2	0.178	0.030	0.004	-
	Extract	2	0.134	0.068	0.028	57

Table ii. Comparison between fresh (i.e. non-sulphonated) and aged (i.e. sulphonated) oestrone and oestradiol-17β hydroquinone sulphuric acid reagents in the presence of solvent residues.

Method - as in Table i, p. 16.

Reagent 1 - Hydroquinone sulphuric acid reagents for the respective oestrogens as in Appendix II, p. 7, 2 months old.

Reagent 2 - As for reagent 1, but freshly prepared with no heating.

Reagent	Oestrone used	D_{604}	D_{601}	D_{606}	% Recovery D_{corr}
1	Standard	0.230	0.060	0	-
	Standard	0.240	0.061	0.001	-
	Extract	0.227	0.075	0.011	92
	Extract	0.221	0.075	0.010	89
2	Standard	0.239	0.058	0	-
	Standard	0.245	0.061	0.002	-
	Extract	0.219	0.057	0.003	90
	Extract	0.229	0.074	0.010	90

Reagent/

Reagent	Oestradiol used	D_{404}	D_{401}	D_{406}	% Recovery D corr
1	Standard	0.165	0.047	0.161	
	Standard	0.172	0.034	0.172	
	Extract	0.168	0.048	0.002	98
	Extract	0.171	0.051	0.005	98
2	Standard	0.171	0.035	0.001	
	Standard	0.178	0.038	0.001	
	Extract	0.167	0.045	0.004	93

Table iii Effect of different degrees of intensity of illumination on the colour development of oestriol with hydroquinone sulphuric acid reagents.

Method - Dry residues of 25μg. of oestriol in test tubes (18 x 150 mm.) were heated in a bath of boiling water for 20 min. with 4 ml. of 2% hydroquinone in 76% (v/v) H_2SO_4, either freshly prepared or after ageing as shown. The tubes were shaken at 2 min. and 5 min. For Reaction 1, the solutions were diluted with 1 ml. of water, shaken and reheated for 5 min. The cooled solutions were diluted to 15 ml. with 30% (v+v) H_2SO_4, mixed with a footed stirring rod, and optical densities read with two filters at 10 min. and 60 min. For Reaction 2 the same procedure was carried out except that 2 ml. of water and 33% (v+v) H_2SO_4 were/

were used for the second and third stage diluents respectively. The tubes were exposed during the procedure to illumination of the type shown and in each case a control protected from light at all stages was done.

Reaction 1

Exp.	10 min. D_{SO_4}	10 min. D_{SO_4}	60 min. D_{SO_4}	60 min. D_{SO_4}
Freshly prepared reagent				
1. Exposed to dull daylight	0.202	0.051	0.205	0.045
Dark	0.185	0.077	0.190	0.065
2. Exposed to dull daylight	0.205	0.053	0.203	0.046
Dark	0.185	0.070	0.189	0.062
3. Exposed to hazy sunlight	0.175	0.046	0.161	0.045
Dark	0.180	0.068	0.187	0.056
Aged Reagent				
4. Exposed to dull daylight	0.183	0.062	0.196	0.047
Dark	0.165	0.060	0.173	0.055
5. Exposed to dull daylight	0.191	0.052	0.193	0.040
Dark	0.173	0.074	0.178	0.063
6. Exposed to hazy sunlight	0.178	0.052	0.166	0.050
Dark	0.168	0.169	0.175	0.058

Reaction 2/

Reaction 2

	10 min.		60 min.	
	D_{so4}	D_{so1}	D_{so4}	D_{so1}
Freshly prepared reagent				
7. Exposed to dull daylight	0.190	0.045	0.190	0.042
Dark	0.185	0.042	0.185	0.042
Aged Reagent				
8. Exposed to dull daylight	0.175	0.038	0.175	0.035
Dark	0.175	0.038	0.175	0.035

Table iv. Effect of illumination at different stages of the colour reaction (oestriol)

See text p. 40

Method - as in Table iii, p.19; a freshly prepared reagent was used.

Results

In sunlight at all stages

	Reaction 1 (1 ml. H_2O)		Reaction 2 (2 ml. H_2O)	
	D_{so4}	D_{so1}	D_{so4}	D_{so1}
10 min.	0.169	0.045	0.155	0.037
60 min.	0.160	0.044	0.145	0.038

In sunlight at end of second and third stages

10 min.	0.179	0.042	0.179	0.039
60 min.	0.174	0.042	0.169	0.039

In/

	Reaction 1 (1 ml. H_2O)		Reaction 2 (2 ml. H_2O)	
	D_{604}	D_{601}	D_{604}	D_{601}
In sunlight at end of third stage				
10 min.	0.195	0.048	0.190	0.043
60 min.	0.187	0.044	0.183	0.041
Not exposed to direct sunlight				
10 min.	0.195	0.048	0.190	0.043
60 min.	0.187	0.044	0.183	0.041

Table v. Effect of illumination on development of colour with oestrone, oestradiol-17β. (Hydroquinone sulphuric acid reaction).

See text p. 40.

Method - 25μg. oestrone and 4 ml. of 2% hydroquinone in 66% (v/v) H_2SO_4 (1 month old) in a test tube (18 mm. x 150 mm.) were heated for 20 min. in a boiling water bath, and the contents were mixed by shaking at 2 and 5 min. Water (0.5 ml.) was added to the cooled solution and, after mixing, the tube was reheated for 5 min. After cooling, 30% (v+v) H_2SO_4 was added to a final volume of 15 ml. and the contents mixed with a footed stirring rod. 25μg. of oestradiol-17β were treated in a similar manner with 2% hydroquinone in 60% (v/v) H_2SO_4 (1 month old) but no water was added in the second stage. During the procedure the tubes were/

were exposed to illumination of the type shown. Optical densities were measured at 10 min. and at 60 min. after final dilution.

Results

	10 min.		60 min.	
Oestrone	D_{604}	D_{601}	D_{604}	D_{601}
Exposed to hazy sunlight	0.248	0.056	0.240	0.056
	0.246	0.054	0.237	0.052
Dark	0.248	0.068	0.255	0.062
	0.244	0.065	0.248	0.060
Oestradiol-17β				
Exposed to hazy sunlight	0.179	0.039	0.172	0.038
	0.175	0.037	0.166	0.035
Dark	0.172	0.059	0.178	0.047
	0.170	0.057	0.178	0.049

Conclusions

It will be seen that even attenuated sunlight was sufficient to cause some instability of the pink colour produced and that in the absence of adequate illumination there was an incomplete conversion of yellow to pink as shown by the D_{601} readings.

Table vi. Comparison between pure reagents and reagent containing quinone, NO_3^-. (oestriol).

See Text p. 55, and Fig. 5

Reagent 1/

Reagent 1 - 2% hydroquinone in 76% (v/v) H_2SO_4 (Hopkins and Williams, A.R.).

Reagent 2 - 2% hydroquinone in 76% (v/v) H_2SO_4 (B.D.H., A.R.).

Reagent 3 - 2% hydroquinone in 76% (v/v) H_2SO_4 (Hopkins and Williams, A.R.) containing 1 mg.% $NaNO_3$, 2 mg.% quinone - see Appendix II, p. 8.

Reactions

Second stage concentrations were chosen in preliminary experiments to give the best compromise between Type II inhibition and fading for Reagents 1 and 3. The conditions for the pure reagents thus selected were 5.5 ml. of reagent and 100 mg. hydroquinone, 20 min. heat for first stage and 2 ml. of H_2O as diluent. For Reagent 3 the conditions were 5.8 ml. of reagent + 100 mg. hydroquinone, 20 min. heat and 1.7 ml. of H_2O as diluent. In each case 11 samples (50μg.) were so treated followed by reheating for the times shown below. The blanks were heated 20 min.

Time	Reagent 1 1 day old D_G	D_V	$\frac{D_G}{D_V}$	Reagent 1 6 days old D_G	D_V	$\frac{D_G}{D_V}$
0	0.334	0.441	.1	0.448	0.378	1.2
2	0.370	0.372	1.0	0.685	0.251	2.7
4	0.532	0.276	1.9	0.591	0.266	2.2
6	0.536	0.272	2.0	0.568	0.253	2.2
8	0.590	0.248	2.4	0.594	0.236	2.5
10	0.590	0.232	2.5	0.602	0.220	2.7
12	0.596	0.231	2.6	0.640	0.208	3.1
14	0.635	0.193	3.3	0.654	0.197	3.3
16	0.635	0.187	3.4	0.656	0.179	3.7
18	0.644	0.179	3.6	0.654	0.181	3.6
20	0.644	0.182	3.5	0.649	0.166	3.9

Time	Reagent 2 1 day old D_G	D_V	$\frac{D_G}{D_V}$	Reagent 3 1 day old D_G	D_V	$\frac{D_G}{D_V}$
0				0.643	0.298	2.2
2				0.690	0.269	2.6
4	0.722	0.244	3.0	0.700	0.241	3.2
6	0.737	0.190	3.9	0.715	0.221	3.2
8	0.721	0.179	4.0	0.725	0.198	3.7
10	0.716	0.173	4.1	0.720	0.197	3.7
12	0.716	0.170	4.2	0.735	0.197	3.8
14	0.698	0.161	4.3	0.751	0.190	4.0
16	0.700	0.170	4.1	0.725	0.177	4.1
18	0.682	0.170	4.0	0.746	0.176	4.2
20	0.672	0.159	4.2	0.736	0.176	4.2

Time = Time of heat, 2 nd stage (min.)

D_G = D_{SO4}

D_V = D_{SCl}

Table vii. Effect of ageing of reagent containing quinone. NO_3^- (oestriol).

See text, p. 56, and Fig. 6

Reagent - Reagent 3, Table vi

Reaction 1 - As for Reagent 3, Table vi.

Reaction 2 - Reaction 1 with additional hydroquinone (100 mg.) in second stage.

	Reagent 1			Reaction 1			Reaction 2		
	Reagent 1 1 day old		D_G	Reagent 1 2 wk. old		D_G	Reagent 1 2 wk. old		D_G
Time	D_G	D_V	$\overline{D_V}$	D_G	D_V	$\overline{D_V}$	D_G	D_V	$\overline{D_V}$
0	0.643	0.298	2.2	0.670	0.284	2.4			
2	0.690	0.269	2.6	0.674	0.256	2.6			
4	0.700	0.241	2.9	0.686	0.224	3.1	0.690	0.226	3.1
6	0.715	0.221	3.2	0.674	0.218	3.1	0.695	0.206	3.4
8	0.725	0.198	3.7	0.700	0.201	3.5	0.735	0.206	3.6
10	0.720	0.197	3.7	0.715	0.188	3.8	0.731	0.190	3.9
12	0.735	0.197	3.8	0.715	0.188	3.8	0.734	0.190	3.9
14	0.751	0.190	4.0	0.708	0.188	3.8	0.726	0.177	4.1
16	0.725	0.177	4.1	0.715	0.181	3.9	0.726	0.186	3.9
18	0.746	0.176	4.2	0.702	0.181	3.9	0.731	0.184	4.0
20	0.736	0.176	4.2	0.715	0.186	3.8	0.731	0.178	4.1

Table viii. Comparison between pure reagents and reagent containing quinone, NO_3^- (oestrone)

See text p. 57, and Fig. 7

Reagent 1 - 2% hydroquinone in 66% (v/v) H_2SO_4 (Hopkins and Williams A.R.).

Reagent 2 - 2% hydroquinone in 66% (v/v) H_2SO_4 (B.D.H. A.R.).

Reagent 3 - 2% hydroquinone in 66% (v/v) H_2SO_4 (Hopkins and Williams A.R.) containing 1 mg.% sodium nitrate, 2 mg.% quinone - see Appendix II p.8.

Reactions

Second stage concentrations were chosen in preliminary experiments to give the best compromise.

between Type II inhibition and fading for Reagents 1 and 3. The conditions for the pure reagents thus selected were 6 ml. of reagent + 100 mg. of hydroquinone, 20 min. heat for first stage and 1.5 ml. of H_2O as diluent. For Reagent 3 the conditions were 6.8 ml. of reagent + 100 mg. hydroquinone, 20 min. heat and 0.7 ml. of H_2O as the diluent. In each case 11 samples (50μ g.) were so treated, followed by reheating for the times shown below. The blanks were heated 20 min.

	Reagent 1 1 d. old			Reagent 1 10 d. old		
Time	D_G	D_V	$\frac{D_G}{D_V}$	D_G	D_V	$\frac{D_G}{D_V}$
0	0.710	0.343	2.1	0.742	0.346	2.1
2	0.834	0.288	2.9	0.780	0.310	2.5
4	0.798	0.269	3.0	0.938	0.202	4.7
6	0.820	0.240	3.4	0.884	0.218	4.0
8	0.855	0.208	4.1	0.894	0.207	4.3
10	0.875	0.195	4.5	0.899	0.188	4.8
12	0.880	0.182	4.8	0.894	0.182	4.9
14	0.900	0.178	5.1	0.884	0.180	4.9
16	0.870	0.165	5.3	0.888	0.176	5.0
18	0.885	0.170	5.2	0.879	0.175	5.0
20	0.875	0.165	5.3			
	Reagent 2 1 d. old			Reagent 3 1 d. old		
0						
2				0.959	0.175	5.5
4	0.945	0.186	5.1	0.949	0.170	5.6
6	0.961	0.186	5.2	0.949	0.173	5.5
8	0.955	0.180	5.3	0.949	0.165	5.8
10	0.960	0.179	5.4	0.938	0.165	5.7
12	0.926	0.174	5.3	0.910	0.154	5.9
14	0.921	0.172	5.4	0.926	0.160	5.8
16	0.886	0.170	5.2	0.948	0.160	5.9
18	0.902	0.174	5.2	0.910	0.160	5.7
20	0.890	0.177	5.0	0.926	0.168	5.5

Table ix. Effect of ageing of reagent containing quinone, NO_3^- (oestrone).

See text p. 58, and Fig. 8.

Reagent - Reagent 3, Table viii

Reaction 1 - As for Reagent 3, Table viii.

Reaction 2 - 6 ml. reagent + 100 mg. hydroquinone, 20 min. heat for 1st stage and 1.5 ml. H_2O as diluent.

Reaction 3 - Reaction 1 with additional hydroquinone (100 mg.) in second stage.

	Reaction 1 1 d. old			Reaction 1 10 d. old		
Time	D_G	D_V	$\frac{D_G}{D_V}$	D_G	D_V	$\frac{D_G}{D_V}$
0				0.923	0.250	3.7
2	0.959	0.175	5.5	0.896	0.235	3.8
4	0.949	0.170	5.6	0.923	0.224	4.1
6	0.949	0.173	5.5	0.923	0.216	4.3
8	0.949	0.165	5.8	0.953	0.208	4.6
10	0.938	0.165	5.7	0.942	0.206	4.6
12	0.910	0.154	5.9	0.942	0.203	4.6
14	0.926	0.160	5.8	0.942	0.199	4.7
16	0.948	0.160	5.9	0.942	0.196	4.8
18	0.910	0.160	5.7	0.942	0.201	4.7
20	0.926	0.168	5.5	0.942	0.202	4.7

	Reaction 2 10 d. old			Reaction 3 2 wk. old		
0	0.955	0.226	4.3			
2	0.960	0.216	4.4			
4	0.963	0.203	4.7	0.885	0.229	3.9
6	0.935	0.184	5.1	0.926	0.216	4.3
8	0.935	0.185	5.1	0.926	0.201	4.6
10	0.915	0.182	5.0	0.926	0.197	4.7
12	0.905	0.181	5.0	0.926	0.196	4.7
14	0.894	0.179	5.0	0.926	0.191	4.9
16	0.868	0.181	4.8	0.926	0.195	4.8
18	0.888	0.178	5.0	0.926	0.189	4.9
20	0.865	0.178	4.9	0.926	0.189	4.9

Table x. Comparison between pure reagents and reagent containing quinone, NO_3^- (oestradiol-17β)

See text p. 59, and Fig. 9

Reagent 1 - 2% hydroquinone in 60% (v/v) H_2SO_4 (Hopkins and Williams A.R.)

Reagent 2 - 2% hydroquinone in 60% (v/v) H_2SO_4 (B.D.H. A.R.)

Reagent 3 - 2% hydroquinone in 60% (v/v) H_2SO_4 (Hopkins and Williams A.R.), containing 1 mg.% $NaNO_3$ and 2 mg.% quinone - see Appendix II, p.8.

Reactions

Second stage concentrations were chosen in preliminary experiments to give the best compromise between Type II inhibition and fading for Reagents 1 and 3. The conditions for the pure reagents thus selected were 7 ml. of reagent + 100 mg. of hydroquinone, 20 min. heat for first stage and 0.5 ml. of water as diluent. For Reagent 3 the conditions were 7.5 ml. of reagent + 100 mg. of hydroquinone, 20 min. heat and no diluent. In each case 11 samples (50 μg.) were so treated followed by reheating for the times shown below. The blanks were heated for 20 min.

Time	Reagent 1 1 d. old D_G	D_V	$\frac{D_G}{D_V}$	Reagent 1 2 wk. old D_G	D_V	$\frac{D_G}{D_V}$
0	0.589	0.254	2.3	0.598	0.230	2.6
2	0.585	0.234	2.5	0.625	0.235	2.7
4	0.634	0.223	2.8	0.655	0.203	3.2
6	0.650	0.214	3.0	0.640	0.193	3.3
8	0.678	0.182	3.7	0.659	0.179	3.7
10	0.700	0.180	3.9	0.662	0.166	4.0
12	0.710	0.165	4.3	0.700	0.172	4.1
14	0.732	0.166	4.4	0.660	0.158	4.2
16	0.722	0.160	4.5	0.700	0.162	4.3
18	0.726	0.158	4.6	0.680	0.155	4.4
20	0.722	0.152	4.8	0.695	0.155	4.5
	Reagent 2 1 d. old			Reagent 3 1 d. old		
0				0.785	0.128	6.1
2				0.818	0.138	5.9
4	0.693	0.152	4.6	0.818	0.140	5.9
6	0.730	0.160	4.6	0.840	0.144	5.9
8	0.768	0.155	4.9	0.848	0.145	5.9
10	0.748	0.155	4.8	0.826	0.139	6.0
12	0.740	0.154	4.8	0.826	0.140	5.9
14	0.744	0.150	5.0	0.838	0.143	5.9
16	0.678	0.142	4.8	0.848	0.140	6.1
18	0.733	0.145	5.1	0.808	0.137	5.9
20	0.728	0.160	4.6	0.824	0.140	5.9

Table xi. Effect of ageing of reagent containing quinone, NO_3^- (oestradiol-17β)

See text p. 60, and Fig. 10

Reagent - Reagent 3, Table x

Reaction 1 - as for reagent 3, Table x.

Reaction 2 - Reaction 1 with additional hydroquinone (100 mg.) in second stage.

Time	Reaction 1 1 d. old D_G	D_V	$\frac{D_G}{D_V}$	Reaction 1 10 d. old D_G	D_V	$\frac{D_G}{D_V}$	Reaction 2 2 wk. old D_G	D_V	$\frac{D_G}{D_V}$
0	0.785	0.128	6.1	0.765	0.159	4.8			
2	0.818	0.138	5.9	0.771	0.159	4.8			
4	0.818	0.140	5.9	0.768	0.149	5.2	0.796	0.167	4.8
6	0.840	0.144	5.9	0.748	0.159	4.7	0 790	0.168	4.7
8	0.848	0.145	5.9	0.758	0.139	5.5	0.819	0.163	5.0
10	0.826	0.139	6.0	0.764	0.149	5.1	0.790	0.153	5.1
12	0.862	0.140	5.9	0.748	0.142	5.5	0.829	0.163	5.1
14	0.838	0.143	5.9	0.754	0.147	5.1	0.790	0.156	5.0
16	0.848	0.140	6.1	0.770	0.140	5.5	0.790	0.163	4.9
18	0.808	0.137	5.9	0.748	0.139	5.4	0.796	0.164	4.9
20	0.824	0.140	5.9	0.728	0.137	5.3	0.807	0.163	5.0

APPENDIX V

Alternative Methods of Purification of Oestriol by Partition Chromatography.

Type A.

This type was designed to retain acidic material and to permit oestriol and basic contaminants to move down the column. An attempt was made to have the mobile phase such that oestriol would flow more slowly than the basic contaminants and thus allow separation of these two factors. Small columns (1 x 10 cm.) packed in the usual manner were used. The solutes were applied to the columns in the mobile phase (3 x 1 ml.), letting each wash go into the top of the chromatogram before the next was applied. R_p was 10 ml./hr. and operating temperature 65° F. The solutes used were 150µg. of oestriol and the untreated oestriol fraction of male urine (1/5 of 24 hr. specimen).

1. Stationary Phase - N-NaOH and 20% NaCl.

   Mobile Phase - n-Butanol.

   A broad band of brown impurities (? acids) was retained on the top of the column; the oestriol was eluted completely by 7.5 ml. but was markedly contaminated (? bases). This is an ideal system for separating oestriol from hydroquinone since the latter is oxidized and retained on top of the column.

2. Stationary Phase - N-NaOH

   Mobile Phase - Ethylene dichloride

A broad/

A broad band of brown impurities was retained on the top of the column, but the oestriol was not eluted by 170 ml. A group of impurities (? indigoids) was eluted in the first few ml. This was the chromatogram which led to the use of the ethylene dichloride extract of the oestriol fraction from a strongly alkaline medium. Addition of 4% butanol to the $EtCl_2$ did not alter the elution pattern, but with 25% butanol, oestriol was eluted from 20-42 ml. with a peak at 26 ml.

3. Stationary Phase - NaOH/Borate buffer pH 10.

Mobile Phase - Ethylene dichloride.

Oestriol was eluted from 2-27 ml. with a peak at 7 ml. This did not permit separation from the basic contaminants.

Type B.

This type was designed to retain basic material and to permit oestriol and acidic contaminants to move down the column.

1. Stationary Phase - 30% H_2SO_4 (v + v)

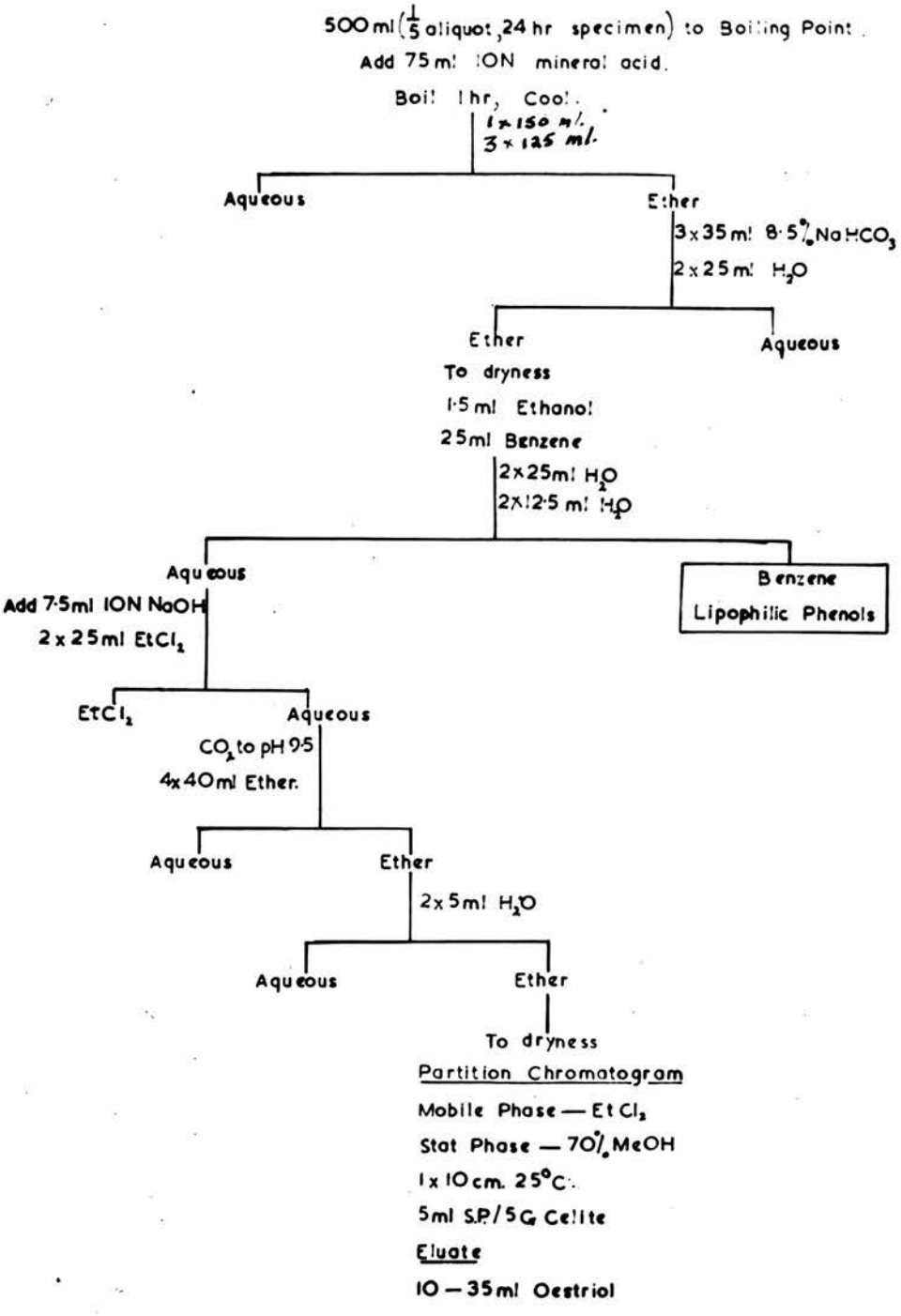
Mobile Phase - Benzene

A brown band was retained on top of the column. Oestriol was eluted by 100 ml. Adequate separation from basic contaminants was thus attained. The system was unsatisfactory for a routine method since oestriol did not dissolve in the mobile phase. It

was/

was necessary to allow an ether solution to evaporate in contact with dry Celite and to pack the dry powder on top of the column. This caused marked broadening of the band.

Figure 27. Procedure for Extraction and Purification of Oestriol.



APPENDIX VI

SUPPLEMENTARY DATA ON SOURCES OF ERROR

Table xii. Recovery of oestriol from 500 ml. of water and 75 ml. concentrated HCl (complete method)

Method - 25µg. of oestriol in 0.5 ml. EtOH were added to 500 ml. of distilled water in a 1 l. round bottomed flask. A.R. HCl (75 ml.) was added slowly with thorough mixing. The subsequent procedures are shown in Fig. 27 opposite.

The colours were developed using the hydroquinone colour reaction, stabilized by ethanol or m-cresol, and the correction formula (text p. 62) applied. Recoveries were calculated from standards run simultaneously.

A. Recoveries based on Colour Correction.

Recoveries at 25µg. level - 72, 73, 59, 60, 60, 57, 56, 63, 82, 63, 49, 45, 48, 70, 70, 63, 66, 43, 56, 76, 75, 72, 64, 47, 87, 77, 64%. (27 determinations) 64 ± 11.1

Recoveries at 50µg. level - 74, 71, 75, 72, 66, 74, 71, 60, 57, 66, 72, 72, 81, 40, 52, 65, 49, 64, 63, 69, 65% (21 determinations) 66 ± 9.6.

Table xiii. Effect of antioxidants in preventing low recoveries in ether extraction of oestriol from aqueous hydrochloric acid.

Method - 25µg. of oestriol in 0.5 ml. ethanol and antioxidant where shown, were added to 500 ml. of distilled water in a 1 l. round bottomed flask and treated as in Table xii.

Exp.	Antioxidant	D_{604}	D_{601}	D_{606}	D_{corr}	% Recovery
1	3.5 mg. hydroquinone	0.211	0.111	0.036	0.165	97
	7 mg. hydroquinone	0.222	0.143	0.043	0.163	96
	14 mg. hydroquinone	0.242	0.183	0.044	0.172	101
	70 mg. hydroquinone	0.252	0.168	0.050	0.168	99
	standard	0.170	0.029	0.002	0.170	-
		0.172	0.026	0.004	0.170	-
2	1.4 mg. hydroquinone	0.189	0.068	0.029	0.156	98
	"	0.189	0.084	0.032	0.150	94
	"	0.189	0.068	0.026	0.158	99
	standard	0.159	0.017	0.004	0.158	-
		0.160	0.015	0.	0.162	-
3.	1.4 mg. hydroquinone	0.189	0.102	0.035	0.143	95
	"	0.198	0.112	0.043	0.144	95
	standard	0.157	0.037	0.	0.152	-
		0.154	0.035	0.	0.151	-
4	1.4 mg. hydroquinone	0.222	0.115	0.038	0.174	95
	standard	0.185	0.032	0.003	0.184	-
5	7 mg. hydroquinone	0.218	0.091	0.036	0.175	100
	standard	0.179	0.027	0.005	0.175	-
6	7 mg. hydroquinone	0.184	0.074	0.024	0.153	95
	"	0.225	0.169	0.049	0.152	95
	standard	0.165	0.033	0.	0.163	-
		0.164	0.033	0.003	0.160	-
7						
7	7 mg. hydroquinone	0.185	0.069	0.026	0.154	96
	"	0.205	0.100	0.039	0.159	99
	standard	0.165	0.025	0.005	0.161	-
		0.165	0.025	0.005	0.161	-
8	100 mg. Vitamin C	0.188	0.159	0.045	0.121	68
	"	0.188	0.109	0.040	0.138	77
	"	0.187	0.154	0.052	0.116	65
	No antioxidant	0.168	0.064	0.022	0.143	80
		0.182	0.132	0.055	0.114	64
		0.175	0.084	0.031	0.138	77
	standard	0.186	0.035	0.007	0.181	-
		0.182	0.038	0.008	0.176	-
9	100 mg. Vitamin C	0.122	0.052	0.020	0.098	64
		0.116	0.058	0.028	0.084	55
		0.126	0.065	0.028	0.092	61
	standard	0.151	0.016	0.002	0.151	-
		0.150	0.016	0.001	0.150	-

10/

Exp.	Antioxidant	D_{so4}	D_{so1}	D_{soe}	D_{corr}	% Recovery
10	100 mg. Vitamin C	0.152	0.082	0.030	0.113	74
		0.122	0.055	0.015	0.101	66
		0.132	0.068	0.019	0.105	69
		0.143	0.062	0.022	0.115	75
	standard	0.152	0.015	0.	0.154	-
11	50 mg. amino-naphthol sulphonic acid	0.185	0.082	0.034	0.147	84
		0.187	0.085	0.035	0.145	85
		0.195	0.090	0.034	0.155	89
	No antioxidant	0.185	0.070	0.034	0.150	86
		0.180	0.085	0.032	0.142	81
		0.172	0.070	0.025	0.143	82
	standard	0.175	0.025	0.	0.175	-
		0.175	0.025	0.	0.175	-

Table xiv. Effect of purification of ether on recoveries of oestriol from dilute HCl.

Method - 25µg. of oestriol in 0.5 ml. EtOH were added to a solution of 500 ml. of distilled water and 75 ml. A.R. HCl in a 1 l. round bottomed flask. The solution was extracted with 4 x 125 ml. of ether of the type shown. The combined ethereal extracts were washed with 3 x 35 ml. 8.5% $NaHCO_3$, 2 x 25 ml. of water and distilled to dryness. The residue was transferred to Kober tubes with ethanol (3 x 3 ml.), and the solvent removed with the tube immersed in a bath of boiling water, either in a stream of air or in an air free stream of nitrogen. The colour reaction used is described in Appendix II, p. 7, and was stabilized with m-cresol. All recoveries shown are corrected for the non-pink component by the colour equation of Brown (1952c).

Results:/

Results:

1) Ether purified by washing with 3 x 1/5 vol. of acidic $FeSO_4$ (60 g. $FeSO_4$, 100 ml. H_2SO_4, 1 l. of H_2O diluted 1/5 before use.

   Examples of recoveries: 85, 81; 76, 85; 69, 69%.

2) Ether purified by refluxing with and distilling from NaOH.

   Examples of recoveries: 77, 85; 85%.

3) Ether purified by distillation from Na.

   Examples of recoveries: 75, 75; 84, 88, 81%.

4) Ether purified by standing 3 days with $KMnO_4$ (50g./l.) and NaOH (50g./l.) and distillation through KOH column.

   Recovery - 90%.

5) Ether purified by passing through Al_2O_3, Activity II (Brockmann).

   Recovery - 83% (Blank extraction gave 16%).

6) Ether purified by Ag_2O (See Appendix I, p.3).

   Examples of recoveries: 90, 84; 75, 67; 72%.

   (oestriol added to blank extract gave 100%).

N.B. Experiments conducted on different days are separated by semicolons.

SEPARATUM

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THE HYDROLYSIS OF THE CONJUGATED OESTROGENS IN HUMAN URINE

BY

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THE HYDROLYSIS OF THE CONJUGATED OESTROGENS IN HUMAN URINE

BY

G. F. MARRIAN & W. S. BAULD

INTRODUCTION

It is only fitting to begin this article by acknowledging the great debt owed by all workers in the field of the steroid hormones to *Bernhard Zondek*. In 1927 in collaboration with *Aschheim* (*Aschheim & Zondek*, 1927) he showed the urine of pregnant women to be a much richer source of the oestrogenic principle than any that had previously been examined, while three years later he reported the urine of pregnant mares to be an even richer source of this principle. These two discoveries were not only directly responsible for the rapid and dramatic developments in the chemistry of the oestrogens which occurred in the early 1930's, but indirectly, through their effect in this field, they also exerted later a profound influence over research in other related fields. Thus the brilliant achievements of German, Swiss and American chemists in elucidating the structures of progesterone, the androgens and the adrenocortical steroids certainly owed much to the available knowledge of the structure of the oestrogens; while it may justifiably be said that the methods of extraction and fractionation used today by all workers in the field of the urinary steroids are based on the procedures worked out twenty years ago by the small group of pioneers who followed up *Zondek*'s discoveries.

In this article an attempt has been made to review critically some of the available knowledge concerning the hydrolysis of the conjugated oestrogens in human urine. The reviewers have emphasized the historical aspects of the subject, since, in their opinion, many of those who today are concerned with other groups of urinary steroids have failed to profit by the mistakes made in the past by workers in the oestrogen field. In this connection it is interesting to compare the early work on the quantitative determination of the urinary oestrogens carried out during the period 1927—1935 with the work carried out during the past few years on the quantitative determination of the so-called urinary adrenocorticoids.

The presence of an oestrogenic principle in human urine was first demonstrated by *Loewe & Lange* in 1926, and by 1932 the importance of subjecting urine to some preliminary treatment with acid before extraction in order to obtain high yields of oestrogen was appreciated by most of the leading workers in the field. However, many of those who at that time were interested in the quantitative aspects of the urinary excretion of oestrogens were curiously reluctant to devote any time to the study of the optimum conditions for this acid pretreatment; and during the period 1932—1935 not a few papers appeared in the literature in which »quantitative« determinations of urinary oestrogens were carried out on extracts made from unhydrolysed urine or from urine that had been treated with acid in a purely arbitrary manner. When the first systematic studies on the hydrolysis of the conjugated urinary oestrogens were reported in 1934—1935 it was at once apparent that the numerous data already in the literature on the excretion of oestrogens during the menstrual cycle and during pregnancy were, from a quantitative standpoint, quite valueless. In retrospect it seems clear that progress would have been greatly accelerated had those interested in the quantitative aspects of oestrogen excretion given top priority to the investigation of the optimum conditions for pre-treatment of the urine with acid.

It is disconcerting to see how history has repeated itself

in the case of the urinary adrenocorticoids. The interval between the first demonstration of biological activity in urine and the discovery of the effect of acidification of the urine upon the yield of extractable activity was actually longer in the case of the adrenocorticoids (1931 to 1946) than it was in the case of the oestrogens. The majority of those interested in the quantitative aspects of urinary adrenocorticoid excretion have shown the same strange reluctance to investigate the optimum conditions for the hydrolysis of the conjugates as was shown by the early workers in the oestrogen field; and too often they have been content to carry out »quantitative« determinations on extracts prepared from unacidified urine or from urine treated with acid in an arbitrary manner. Only during the past year has the problem of the hydrolysis of the conjugated urinary corticoids been studied systematically; and although this problem has yet to be solved satisfactorily, it is already clear from preliminary findings in several laboratories that the numerous data in the literature on the excretion of these steroids can have little or no quantitative significance.

There can be little doubt that among those who are concerned with the quantitative determination of urinary steroids the view is widely held that results of clinical value and of physiological interest can be obtained using sub-optimal conditions for the preliminary hydrolysis of the urine, provided that these conditions are carefully standardized. In some circumstances this may be true, but in the opinion of the present writers this view is a dangerous one which should be discouraged whenever possible. It has been the cause of no little confusion in the past in connection with the determination of the urinary oestrogens, and there is danger that it may lead to even more confusion in the future in connection with the much more complicated problem of the determination of the urinary adrenocorticoids.

HISTORICAL

The early investigations which led to the discovery of the presence in urine of the conjugated oestrogens make an interesting but somewhat confused story. This confusion was largely due to the fact that at first a clear distinction was not made between the effect of acidification in causing hydrolysis of the conjugated oestrogens in urine and the effect of acidification in suppressing the ionization of free oestrogen in alkaline solution.

In their early work on the oestrogens and chorionic gonadotrophin in the urine of pregnant women *Zondek & Aschheim* (1928) acidified the urine with acetic acid before extraction with ether or benzene. There is no suggestion in the published work that this procedure was adopted in order to increase the yield of extractable hormone, and indeed it is doubtful whether any significant hydrolysis of the oestrogen conjugates could have occurred under these conditions.

Undoubtedly considerable credit for the discovery of the presence of the conjugated oestrogens should be given to *Glimm & Wadehn* who in 1929 showed that a considerable fraction of the oestrogenic activity in the urine of pregnant women is in a form in which it cannot be extracted by ether. Furthermore they tentatively considered the possibility that the unextractable oestrogen might be excreted in combination with glucuronic or sulphuric acids. They went on to show that on heating urine with alkali the ether-soluble oestrogen present was partly converted into an ether-insoluble form (doubtless due to salt formation), and that on subsequent treatment with acid increased yields of ether-extractable oestrogen could be obtained. The true significance of these findings was not appreciated by *Glimm & Wadehn,* since they apparently believed that the effect of acid in increasing the yield of extractable oestrogen was due to a reversal of the effect produced by alkali. It seems clear that they did not understand that the effect of acid was due, not only to a reversal of this effect, but also to hydrolysis of conjugated oestrogens.

By 1930 the acidification of urine prior to extraction was

being carried out as a routine in two laboratories, but it is clear that in neither case was this practice initiated with any idea of effecting the hydrolysis of conjugated oestrogens.

Funk in 1929 had briefly reported on the acidic property of the oestrogenic hormone, and in the same year *Marrian* had confirmed and extended this finding in a series of experiments in which the distribution was studied of ether-soluble oestrogen from the urine of pregnant women between ether and neutral, acidic and alkaline aqueous media. With the mistaken idea of eliminating loss of extractable oestrogen by salt formation in alkaline urines, *Marrian* (1930 a, b) later adopted the practice of acidifying urine (pH not recorded) immediately before extraction with ether, and reported that by doing so he obtained greatly increased yields of active material.

In the same year *Doisy, Veler & Thayer* (1930) also reported increased yields of oestrogen from the urine of pregnant women when the urine was acidified to pH 4 and allowed to stand for some time prior to extraction. At that time these workers were employing olive oil as a solvent for the extraction of oestrogen, and the practice of acidification of the urine was adopted in the first instance in order to minimize emulsion formation. However, it was soon appreciated by these workers (*Veler, Thayer & Doisy,* 1930) that the increased yields of oestrogen could not be explained entirely on this basis.

In the light of modern knowledge concerning the conditions necessary to attain complete hydrolysis of the conjugated oestrogens in the urine of pregnant women it may seem strange that such marked increase in yield of extractable oestrogen should have been obtained by *Marrian* and by *Doisy* and his co-workers by acidification at room temperature. It must be remembered, however, that the increased yields reported by these workers, although appearing highly satisfactory at the time, were almost certainly minute compared with those which could have been obtained by modern methods of hydrolysis.

The stimulus to investigate more drastic methods of acid

pre-treatment of the urine of pregnant women was provided by the work of *Zondek* (1930) on oestrogens in the urine of pregnant mares. He showed that the oestrogen in this source is in a form in which it is non-extractable by chloroform, and in the following year he showed (1931) that this non-extractable form could be converted into an extractable form by boiling the urine for a few minutes with acid. This finding was shortly afterwards confirmed by *Lipschütz & Poch* (1932). At about the same time *Collip, Browne & Thomson* (1932) briefly mentioned that the urine of pregnant women contains an ether-insoluble oestrogen resembling that which they had found in extracts of human placenta.

The first clear-cut figures showing the value of boiling the urine of pregnant women with acid before extraction appear to have been those of *Marrian* in 1933, and the first attempt to determine the optimal conditions of hydrolysis was made soon afterwards by *Borchardt, Dingemanse & Laqueur* (1934). During the next two years more detailed studies of the same problem were reported by *Cohen & Marrian* (1935), who employed a colorimetric method of determining oestrogens based on the highly specific colour reaction discovered in 1931 by *Kober,* and by *Smith & Smith* (1935). The method recommended by the former workers involved acidification of the urine to pH 1.0 with HCl, the addition of a further 3.3 ml. of concentrated HCl per 100 ml. urine and heating in an autoclave for 2 hours. The *Smiths'* method involved boiling at atmospheric pressure for 10 minutes after the addition of 15 volumes per cent of concentrated HCl. These two methods may be considered to be the prototypes of the majority of those in use at the present time.

Destruction of the oestrogens during acid hydrolysis.

Cohen & Marrian (1934, 1935) observed considerable losses of ether-extractable oestrogen on heating urine in the presence of acid for long periods. They also observed similar losses on prolonged heating of acidified aqueous solutions of pure oestriol and oestrone. Their results suggested that this

destruction of oestrogen might be oxidative, since they found that it could be increased by frequent introduction of oxygen. Recent researches by others, and in particular by *Falk & Heard* (see *Heard & Saffran,* 1949), *van Bruggen* (1948), and *Rosenmund* (1948) have provided ample confirmation of the fact that destruction of oestrogens does occur on heating with acid, and have made it almost certain that this destruction is indeed largely oxidative in character.

Falk & Heard were able to isolate considerably greater yields of crystalline oestriol from the urine of pregnant women or from aqueous solutions of oestriol glucuronide after hydrolysis with acid under anaerobic conditions than after hydrolysis in a vessel open to the air. In their experiments anaerobic conditions were maintained by an atmosphere of nitrogen, or by adding zinc to the hydrolysis mixture, or by carrying out the hydrolysis in an autoclave.

Van Bruggen (1948) has shown that extensive inactivation of oestriol, oestrone and oestradiol-17 β occurs when these are heated in aqueous acid solution, but that this inactivation can be prevented by carrying out the process in an atmosphere of nitrogen and in the presence of 1-amino-2-naphthol-4-sulphonic acid. In contrast to the findings of *Falk & Heard,* however, he has reported that these protective measures are ineffective in increasing the yield of extractable oestrogen from the urine of pregnant women.

The experiments of *Rosenmund* (1948) have shown that the inactivation which occurs in solutions of oestriol, oestrone and oestradiol-17 β on heating in acid solution can be greatly increased by the addition of ferric chloride and completely prevented by the addition of ascorbic acid or pyrogallol. He has furthermore shown that urine contains a principle, which exerts a protective action, like that of ascorbic acid and pyrogallol, in inhibiting the inactivation of added oestrogens, and that this inhibitor is not present in all urines in amounts sufficient to prevent inactivation completely. This most important latter finding would seem to explain adequately the discrepancy between the results of *Falk & Heard,* and of *van*

Bruggen. It also provides an explanation of the unpublished observation of *Stevenson & Marrian* that whereas the oestrogen in some urine specimens from pregnant women is apparently stable to boiling with acid for long periods, in others marked destruction occurs under the same conditions.

Acid hydrolysis of the conjugated oestrogens in the presence of zinc.

In 1937 *Smith & Smith* reported that the addition of zinc dust to urine before hydrolysis with HCl resulted in a very marked increase in the amount of ether-extractable oestrogenic activity compared to that which could be obtained from the same urine after their usual hydrolytic treatment. In a long series of subsequent papers they have developed the theory that this increase (for which they have used the expression T_{Zn}/T_o) is due largely to the new formation of oestrogenic substances by the reduction of oestrogenically inactive oestrogen oxidation products which they have claimed are present in urine. No satisfactory chemical evidence for the presence in urine of such oestrogen oxidation products has yet been provided; nevertheless the Smiths believe that these hypothetical substances may have an important physiological role in the control of the gonadotrophic activity of the anterior hypophysis (*Smith,* 1944) and in the maintenance of progesterone secretion by the corpus luteum and placenta (*Smith & Smith,* 1940; *Smith, Smith & Schiller,* 1941). They have also suggested a close relationship between excess formation of oestrogen oxidation products and the development of toxaemias of pregnancy (*Smith & Smith,* 1938; 1941 a, b; 1948). In view of the wide interest among obstetricians and gynaecologists in these theories, the evidence upon which they are based must be discussed critically and at some length.

The*Smiths* have recognized that the observed T_{Zn}/T_o values may be due in part to the reduction of oestrone to the more potent oestradiol-17 β and indeed they have shown that whereas the oestrogenic activities of oestriol and oestradiol are unaffected by heating with HCl and zinc, that of oestrone

is increased 3—5 fold (*Smith & Smith,* 1941 c). Nevertheless they have claimed that their determinations on urine carried out at different stages of the menstrual cycle and pregnancy exclude the possibility that the whole of the observed T_{Zn}/T_o values could be due to reduction of oestrone. Thus they have claimed to have shown that during menstruation (*Smith & Smith,* 1938; 1941 c) and during labour (*Smith, Smith & Schiller,* 1941) very high urinary T_{Zn}/T_o values (5—8.5) are associated with the complete absence of oestrone from the urine. They also have claimed that at the 38th week of pregnancy, when the urinary excretion of oestrone is at a maximum, the urinary T_{Zn}/T_o values are lower (*ca.* 2.0) than at any other time.

It must be pointed out at this stage of the discussion that whereas the ordinary hydrolysis procedure of the Smiths involves boiling with acid for 10 minutes their »zinc hydrolysis« involves boiling for 3 hours. It might therefore be thought that more complete hydrolysis of the urinary conjugated oestrogens during the prolonged boiling would contribute largely to the observed T_{Zn}/T_o values. The *Smiths* have indeed reported (1941 c) that »zinc hydrolysis« of urine following an ordinary hydrolysis and extraction yields additional oestrogenic activity. However, they have claimed to have shown that during the menstrual cycle the additional activity obtained by zinc hydrolysis following ordinary hydrolysis and extraction is lowest at the time of menstruation when the urinary T_{Zn}/T_o ratios are maximal, and on these grounds deny that more complete hydrolysis of the conjugated oestrogens can account for the observed ratios.

The *Smiths'* original observation that the addition of zinc dust to urine before hydrolysis with HCl increases the yield of extractable oestrogen has been confirmed by other workers using chemical methods of determination (*Stevenson & Marrian* — see *Marrian,* 1948; *Falk & Heard* — see *Heard & Saffran,* 1949; *Stimmel,* 1949), and there can be no doubts about its essential validity. However, the correctness of the *Smiths'* interpretation of this original observation, and the validity of

their later experimental findings advanced in support of this interpretation, cannot be unreservedly accepted.

Firstly, it has been established by *Stevenson & Marrian* (1947) and *van Bruggen* (1948) that the 10 minutes boiling with 15 volumes per cent of concentrated HCI, which the *Smiths* have employed throughout in their ordinary method of hydrolysis, is insufficient to hydrolyse completely the conjugated oestrogens in urine. It follows that the *Smiths'* T_{Zn}/T_o ratios must all be in error, and it would seem that the significance of variations in these values at different stages of the menstrual cycle and pregnancy may be less than was originally supposed. In any case it is certain that more complete hydrolysis of the conjugated oestrogens during the prolonged zinc-HCl treatment must have made a larger contribution towards the observed T_{Zn}/T_o values than the *Smiths* believed.

It should also be pointed out that the inadequate time of boiling used by the Smiths in their hydrolysis without zinc may itself have been to some extent the cause of the observed variations in the $T_{Zn}T_o$ ratios to which so much physiological significance is attached. The *Smiths'* hydrolysis conditions (without zinc) are certainly sufficiently vigorous to hydrolyse completely oestrone sulphate (*Heard & Saffran,* 1949) and also, in all probability, the sulphate esters of the other two urinary oestrogens. On the other hand these conditions are certainly not adequate for the complete hydrolysis of oestriol glucuronide in urine, and it is likely that they would not be for the other two oestrogen glucuronides also. Accordingly, if there was a day to day variation in the relative proportions of the oestrogens excreted as sulphates and as glucuronides, there would inevitably be some corresponding variation in the T_{Zn}/T_o values as determined by the *Smiths'* method. That such variations in oestrogen sulphate and oestrogen glucuronide excretion may occur is indicated by the recent work of *Cohen & Bates* (1949).

Secondly, it is certain that the protection against oxidative destruction of the oestrogens provided by nascent hydrogen in the zinc hydrolysis procedure (see preceding section) must

have been responsible to a considerable extent for the T_{Zn}/T_o ratios greater than unity obtained by the *Smiths*. Their procedure for hydrolysis without zinc must have resulted not only in incomplete hydrolysis of the conjugated oestrogens, but also in some destruction of the free oestrogens liberated during hydrolysis. On the other hand, the prolonged zinc-HCl treatment must have given complete hydrolysis of the conjugated oestrogens with little or no destruction.

Since the *Smiths* themselves admit that the oestrogenic activity of oestrone can be enhanced 3 to 5 fold by the zinc-HCl treatment (1941 c), and since in their experiments the various factors mentioned above must have been contributing to the production of high and variable urinary T_{Zn}/T_o ratios, it seems hardly necessary to postulate the presence in urine of any reducible oestrogen oxidation product in order to explain their results.

This problem has been recently reinvestigated by *Stimmel* (1949) using the *Smiths'* methods of hydrolysis with and without zinc, and a colorimetric method, based on the *Kober* reaction, for the determination of oestrogens. In confirmation of the results of the *Smiths* he has found that increased yields of extractable oestrogens are obtained using the zinc-HCl hydrolysis procedure, and he has advanced evidence which he believes excludes the possibility that these increased yields are entirely due to incomplete hydrolysis and destruction of oestrogen in the procedure without added zinc. *Stimmel* has also shown that 16-ketooestrone (*Huffmann & Lott*, 1948) can be partially converted into a *Kober* chromogen by zinc-HCl treatment, and he has cautiously suggested that the increased yields of oestrogen obtainable from urine after zinc-HCl hydrolysis might be partly accounted for by the presence in urine of this oestrogen derivative.

The possibility that 16-ketooestrone may be a urinary metabolic product of the oestrogens is an attractive one, and indeed was first suggested by one of the present writers in 1938 in the course of some speculations on oestrogen metabolism (*Marrian*, 1938—39; 1939). However, it does not seem to the

present writers that Stimmel's results conclusively prove that urine contains any oestrogen derivative which, like 16-keto-oestrone, can be reduced to a *Kober* chromogen by the zinc-HCl treatment. It has been shown by *Stevenson & Marrian* (see *Marrian,* 1948) that the yield of ether-extractable Kober chromogen obtainable from the urine of pregnant women can be doubled merely by lengthening the time of boiling with acid (15 vols per cent of concentrated HCl) from 10 to 60 minutes. In view of this finding it would seem that Stimmel has greatly underestimated the possible effect of incomplete hydrolysis in his experiments.

The problem of zinc-HCl hydrolysis was investigated some years ago by *Stevenson & Marrian* (see *Marrian,* 1948) who compared the yields of ether-extractable *Kober* chromogen obtained after 60 minutes boiling with 15 volumes per cent of concentrated HCl with those obtained after boiling *for the same time* with the same concentration of acid and added zinc. It was found that the addition of zinc increased the yields of chromogen by not more than 10—30 per cent. These results were not published in detail, nor was any comment made concerning their possible significance. It should therefore be stated now that it was believed that these small increases could be quite adequately explained by inhibition of oxidative destruction of oestrogen by the nascent hydrogen and by more complete extraction due to the presence of a high concentration of zinc chloride in the hydrolysis mixture.

Enzymatic hydrolysis of the conjugated oestrogens in urine.

In 1933 *Marrian* found that a most effective method of obtaining high yields of ether-extractable oestrogen from the urine of pregnant women was to allow the urine to stand without preservative for several days until a considerable growth of bacteria had developed. This finding was confirmed by *Cohen & Marrian* (1934), and shortly afterwards *Patterson* (1937) reported that incubation of urine inoculated with B.

coli gave yields of free oestriol comparable to those obtained on acid hydrolysis.

The isolation of oestriol glucuronide from the urine of pregnant women (*Cohen & Marrian,* 1936, *Cohen, Marrian & Odell,* 1936) suggested that the bacterial agent responsible for the hydrolysis of the conjugated oestrogens might be a glucuronidase. In following up this line of reasoning it was shown by *Odell, Skill & Marrian* (1937) that mouse intestine contains an enzyme capable of hydrolysing pure oestriol glucuronide, while *Fishman* (1939) showed that partially purified preparations of ox-spleen β-glucuronidase would do the same. More recently *Buehler, Katzman, Doisy & Doisy* (1949) have shown that glucuronidase preparations obtained from *E. coli* cultured in media containing menthol glucuronide is an effective means of hydrolysing the conjugated oestrogens in the urine of pregnant women. These authors reported yields of total oestrogen (determined biologically and colorimetrically) by enzymatic hydrolysis which in nearly every case were somewhat higher than those obtained by acid hydrolysis. It is doubtful, however, whether these results were in fact as good as they appeared, since the conditions of acid hydrolysis employed (10 minutes boiling with 15 vols. per cent of concentrated HCl) could not have been optimal.

For the quantitative determinations of oestrogens in urine enzymatic hydrolysis seems to offer certain advantages over acid hydrolysis: — destruction during hydrolysis would be avoided, while the resulting oestrogen fractions, being much less contaminated with unwanted impurities than those obtained after acid hydrolysis, would be much more suitable for determination by colorimetric methods. However, until the problems involved in the preparation and preservation of adequate supplies of standardized preparations of both glucuronidase and sulphatase have been solved, it is doubtful whether enzymatic methods of hydrolysis will be practicable for routine quantitative work.

Enzymatic methods of hydrolysis have recently been employed effectively for the study of certain special problems re-

lated to the conjugated urinary oestrogens. *Cohen & Bates* (1949), using an extract of *Aspergillus oryzae* (Mylase P) as a source of phenol sulphatase, have obtained evidence which suggests that oestriol and oestradiol, as well as oestrone, may be present in the urine of pregnant women as sulphates. These results indicate that there may be considerable day to day variations in the mode of conjugation of each of the three oestrogens. It should, however, be pointed out that *Cohen & Bates* were not entirely justified in assuming that any conjugated oestrogen not hydrolysed by phenol sulphatase is necessarily glucuronide.

Grant & Marrian (1950) recently have made use of ox-spleen β-glucuronidase for the hydrolysis of oestriol glucuronide in their work in which the uronic acid moiety of the latter compound was identified as D-glucuronic acid.

DISCUSSION

During the interval of eighteen years which has elapsed since definite proof was first advanced for the necessity for hydrolysis of human urine prior to extraction of oestrogens, no complete agreement seems to have been reached amongst the workers concerned as to the most satisfactory method of hydrolysis to adopt in the quantitative determination of urinary oestrogens.

Although a few workers believe that the original *Cohen-Marrian* (1935) autoclaving method, or some modification thereof, gives the most satisfactory results (cf. *Callow et al.*, 1939; *Engel et al.*, 1950), there is no satisfactory evidence that such methods are significantly superior to a suitably modified *Smith & Smith* (1935) procedure. In the opinion of the present writers autoclaving methods are unsuited for accurate routine work because of the difficulty of obtaining accurate timing of the hydrolysis and because of the inconvenience involved.

The available evidence indicates the following modifica-

tions should be made in the original *Smith & Smith* (1935) procedure: —

I. The time of boiling should be increased from 10 to 60 minutes, clear evidence having been obtained by *Stevenson & Marrian* (1947), *Marrian* (1948), *van Bruggen* (1948), *Stimmel* (1949), and *Engel* (1950) that 10 minutes' boiling with 15 volumes per cent of concentrated HCl is inadequate to obtain maximal hydrolysis of the conjugated oestrogens in human urine. It should be noted that *Jayle, Crépy & Judas* (1949) have also adopted the procedure of boiling for 60 minutes with 15 volumes per cent of concentrated HCl.

II. As suggested by *Stevenson & Marrian* (1947) the HCl should be added to the urine after it has been brought to the boil. By doing so accurate timing of the hydrolysis is facilitated and destruction due to heating with acid in the presence of oxygen is decreased.

III. As suggested by *van Bruggen* (1948) and by *Rosenmund* (1948) a mild reducing agent should be added to the urine before hydrolysis in order to afford protection against oxidative destruction. Although *Rosenmund* (1948) has shown that urine contains a natural protective agent, it is not always always present in a concentration sufficient to prevent oxidative destruction.

Dingemanse et al. (1939) have suggested that »layering« the surface of urine by an immiscible solvent during hydrolysis may be advantageous. There is no conclusive evidence, however, that this procedure is of any value when other precautions against oxidative destruction are observed.

It is noteworthy that *van Bruggen* (1948) obtained higher yields of oestrogen from pregnancy urine by refluxing a butanol extract of the urine with acid (2 hours) than he was able to obtain by any other method of hydrolysis. It seems possible that this increased yield might be

due to the more efficient hydrolysis consequent upon the higher temperature and/or to the removal of substances catalysing oxidative destruction which may have been present in the urine. It is clear that this procedure of van Bruggen's merits further attention.

Other »open flask« methods of acid hydrolysis differing from the *Smith & Smith* procedure in the concentration of acid used have been described by *Venning, Evelyn, Harkness & Browne* (1937), *Cherry & Bernstein* (1939), *Bachman & Pettit* (1941) and *Finkelstein* (1948). There is no evidence that these procedures are superior to a suitably modified *Smith & Smith* procedure.

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