STATISTICAL ANALYSIS OF DATA FROM COUNTERCURRENT DISTRIBUTION. III. THE BILIARY AND URINARY EXCRETION PATTERNS OF ESTRIOL IN A HUMAN SUBJECT

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ABSTRACT

The application of the previously reported (1,2), new mathematical approach for the analysis in countercurrent distribution data, to the determination of the percentage and number of compounds in the whole CCD train rather than to individual peaks is found to be very successful. The utility of the method is typically examplified by the analysis of biliary and urinary excretion patterns of estriol-6, 7-H^3 in a human subject. The fractionation of these biological media was also performed by CCD, but without prior treatment of any type. This assured both minimal artifact formation and no loss of radioactivity. The combination of mathematical approach and CCD therefore provides a truer picture of the excretion pattern than has been hitherto possible.

Since estrogens were first discovered, progress in the methodology of isolation and identification of steroids has been remarkable, especially with the advent and application to this field of techniques such as paper-, thin layer-, gas-, and partition chromatography, and countercurrent distribution (CCD). Recently, emphasis in the investigation of these hormones has shifted from the metabolism of the steroids per se to the metabolism of their conjugates. The latter are more polar molecules, more difficult to separate chromatographi-

cally than the corresponding aglycones and are being isolated with a widening variety of conjugating groups.

For reasons outlined below, CCD is unique among these techniques. Solutes, or portions thereof, are not readily "lost" as occurs by irreversible adsorption on paper chromatograms or chromatography columns. CCD affords very mild treatment of compounds, minimizing deleterious effects. If the solute behaves ideally and the binomial destribution is followed, CCD data are amenable to very precise mathematical analyses, which have not yet been developed for any other type of separation method. The purpose of such analyses is to ascertain the purity of the compound in question by comparison of a curve calculated from the parameters of the partition coefficient and the amount of solute with that observed from the data. Recently, a new mathematical approach was perfected and tested with the aid of which the percentage of each authentic standard present in a CCD train could be accurately determined after a smaller number of transfers (1,2) than were required for resolution. The method improves one of the limitations of CCD, viz., low resolving power. The utility of an analytical procedure cannot be ascertained, however, until it has been applied to practical situations. To this end, and to remain within the context of investigation in this laboratory, the biliary and urinary excretion of a normal subject injected with tritium labelled estriol was investigated, and the pattern of metabolites and conjugates in both media was compared.

Fractionation of raw bile and urine was performed by distribution in 10% ammonia: n-butanol[1]. A method is presented which overcomes the emulsions formed by bile in the presence of n-butanol. In spite of the tediousness of the procedure, it was considered to be worth the extra effort. Any other method of fractionation would decrease the accuracy of the analysis, since losses would be greater than the accuracy of the analytical method, at least under ideal conditions.

Although this is the first reported analysis of estriol excretion and conjugation utilizing the statistical analysis of CCD data, Emerman et al. (3) have recently reported results following the infusion of doubly labelled estriol-3-sulfate into T-tube patients. Since the sulfate moiety was removed after introduction of the conjugate into the body, there was, therefore, little metabolic difference between an infusion of estriol or its sulfate. Results obtained by Emerman et al. (3) are consequently, closely related to data presented below, and biliary and urinary excretion patterns would be expected to be similar. The latter authors fractionated bile using a sequence of precipitation with alcohol, chromatography on inactivated neutral alumina, paper chromatography, acetylations and saponifications.

In the present work, emphasis was placed mainly on CCD for fractionation and on enzymatic hydrolysis for identification. In the case of the latter, it was necessary to synthesize estriol-

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1 10 ml. concentrated ammonia (sp. gr. 0.90) made to 100 ml. with distilled water.

3-sulfate for use as a standard. Although this compound has been frequently mentioned in the literature, no rigorous chemical identification has been reported. Elemental analysis and spectrophotometric data are presented here which show that this molecule is hydrated in a manner similar to the estradiol (4) and estrone (5) derivatives. Also presented is evidence that the partition coefficient of this compound exhibits wide variation with concentration.

METHODS AND RESULTS

ANALYSES: Ultraviolet and infrared spectra were determined in Cary Model 14 and Beckman IR 9 spectrophotometers, respectively. Elemental analysis was performed by Spang Microanalytical Laboratories. The equipment and method of determination of radioactivity were identical to those previously reported (1,2). An IBM 7040 computer was used for curve analysis using a modified program to precalculate dpm from cpm. In all distributions reported below, there were at least 500 cpm in the aliquot taken for determination of radioactivity in the peak tube of the smallest peak in the CCD train.

COLLECTION OF BILE AND URINE: A 55 year old normal female subject was injected intravenously four days after cholesystectomy with a single injection of 50 μc of estriol-6,7-H^3 (0.14 mc/ mole) in 2 ml. of 95% ethanol diluted to 30 ml. of with normal sterile saline solution. The plastic pouch attached to the T-tube was emptied of its bile contents and aliquots taken for counting at the times indicated in Fig. 1. It can be seen that negligible amounts of radioactivity were excreted in the first hour and that the highest concentration of tritium occurred at 1½ hours after injection. Urine was also collected and the percentage of radioactivity excreted conformed to that previously published (6).

FRACTIONATION OF BILE AND URINE: The bile collected from 1½-4 hours was combined (a total of 48.1 ml. containing 21.3 X 10^6 dpm) and the volume was reduced in a rotary evaporator (temperature did not exceed 40°C.) to less than 18 ml. This concentrate was made 10% with respect to ammonia, the volume being increased to 20 ml. The initial steps of a countercurrent distribution were performed outside the CCD train in the following manner. The alkaline solution was divided into two equal portions, which were made lower phase only. Pre-equilibrated n-butanol (10ml.) was added and the two phases in tubes 0 and 1 were mixed with a rocking motion simulating that of the CCD instrument. The two phases were allowed to separate, and emulsions were broken by centrifugation. Each upper phase was transferred to the tube with the next higher number, and fresh upper phase was added to tube 0.

This was repeated in regular distribution manner until a total of 21 manual transfers had been accomplished. It was noted that severe emulsions occurred in the first two tubes for about 10 transfers, after which emulsions abated. When the time required for phase separation without centrifugation had decreased to approximately one hour, the solutions were inserted into a previously prepared CCD train and the distribution continued automatically until 99 transfers had been accomplished. The total time for the above fractionation was approximately two weeks.

For fractionation of urine, 10 ml. (containing 7.87×10^6 dpm) from the 0-2 hour collection was used directly as the lower phase of tube 0 in the CCD train. Settling time was 1 hour at the beginning, since no severe emulsions were encountered.

To avoid confusion, peaks from bile and urine were prefixed B and U, respectively. Major peaks were then numbered whereas sub-fractions were further designated by a letter. Since the objective of the present work was to test the CCD method, exhaustive investigation of compounds below the 5% level was not attempted. Previous experience with the computer method has shown that the estimate of a compound present in a peak should be accepted only if an adequate number of transfers has been made. If n is not adequate then a resolvable peak may be falsely estimated to contain a single component. To ascertain if such a condition exists, fractions from initial distributions were re-distributed for larger numbers of transfers.

BILE FRACTIONS: The distribution (Distribution #1, n=99) of human bile after a single intravenous injection of estriol is shown in Fig. 2. It is apparent that with only two peaks, the pattern of the biliary excretion is relatively simple. The major peak (80%, K=0.087) contains a minor, less polar component (6%, K=0.182). The second peak is present in 10.5% and had a K=0.614. It can be seen from the above figure that the first peak was well fitted, while the fit for the second one was very poor, indicating deviation from the mathematical model, viz., the binomial distribution.

The solutes in tubes 0-19, containing the components estimated with K values of 0.087 and 0.182, designated B-I, were redistributed (Distribution #2, Fig. 3). Because of the good fit between observed and predicted curves, it is apparent that the estimate in Distribution #1 of a compound with K=0.087 (80.1%) was valid, and that this compound (B-I-b, tubes 12-35) is represented as K=0.075 (91.9%) in Fig. 3 (Distribution #2). B-I-b was assumed to be almost pure and constituted 79.4% of the radioactivity present in the original bile[1]. However, the component with K=0.182 (6.2% in Fig. 2)

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[1]Based on the following calculation: From Distribution #1, the percentages of the compounds with K=0.087 (80.1%) and K=0.182 (6.2%) were combined (86.3%). On redistribution, the major component (Fig.3, Distribution #2) was 91.9% of 86.3%, or 79.4% of the radioactivity in the original bile.

was not single, and on further distribution (Fig. 3) it was resolved into two compounds: K=0.113 (3.8%) and K=0.227 (0.6%). These compounds were disregarded as was B-I-a (the solutes in tubes 0-11, Distribution #2).

To prove that B-I-b is estriol-3-sulfo-16-glucosiduronate, aliquots were incubated with bacterial B-glucuronidase[1] and Mylase P[2], respectively. To the dry material from the former was added carrier estriol-3-sulfate, and the mixture was distributed (Fig. 4, Distribution #3). With the amount of carrier sulfate used, the K value of this compound is not constant with concentration and, therefore, the computer method was not used to estimate the percentage of the material hydrolyzed. Rather, the total radioactivity used for this experiment was compared with that in the peak. These figures were 214,000 dpm and 156,000 dpm, respectively; therefore, the percentage hydrolyzed was 72.8. The statistical method of Baggett and Engel (7) was used to evaluate the slope of this line. The analysis indicated that the observed slope is closer to zero than 20-30% of those results which could be obtained from an experiment in which two compounds, known to be identical, are being compared[3]. It cannot be concluded, therefore with statistical significance that B-I-b and estriol-3-sulfate are different. However, it appears from Fig. 4 that the curve for radioactivity is wider than that of absorbance on the right hand side. Since the carrier used was an analytical sample, it is possible that the radioactive compound contained a less polar contaminant. It can be considered, however, that the major portion of the radioactive peak has been identified as estriol-3-sulfate.

[1]B-Glucuronidase incubation in 15 ml. of 0.05M acetate buffer, pH 7.0, at 37°C. with 1500 units of Bacterial Type II, Sigma Chemical Co. After 20 hours, an equal volume of ethanol was added and solvents evaporated.

[2]Mylase P incubation, similar to the above except for use of 2mg./ml. Mylase P, purchased from Mann Research Laboratory, and 0.05M acetate buffer, pH 6.0.

[3]The isotope effect reported when paper chromotography was used by Gold and Crigler (8) to prove identity, has also been observed in the present week and undoubtedly contributes to lowering the values in the above and following identifications. In related unpublished work, a mixture of very pure H^3-6,7-estriol-3-glucuronide and estriol-3-C^{14}-glucuronide, when distributed in the system: ethyl acetate: n-butanol: 0.2% ammonia had a β value of 1.010 with the tritium label being more polar. This β value gave a peak tube separation of 1.08 tubes after 499 transfers. For the 99 transfer distributions, the magnitude of this isotope effect should be quite small.

No further identification was necessary since the above work is acceptable evidence that the compound in peak B-I-b does contain both sulfate and glucosiduronate moieties. The sites of conjugation (3 and 16, respectively) are inferred from the work of Emerman, et al. (3) who showed that the major excretory product in human bile after injection of estriol-3-sulfate is estriol-3-sulfo-16-glucosiduronate. The K value of this compound in the present work is very close (0.075) to that found when a C^{14}-labelled standard[1] was distributed in the same system (10% ammonia: n-butanol).

After incubation of the sulfoglucuronide (B-I-b) with Mylase P, the dried material, after mixing with estriol-C^{14}-16-glucosiduronate, was dissolved in the lower phase of tube 0 in the CCD train using the system: ethyl acetate: 0.2% ammonia; 1:3:4. Analysis of the curve for the tritium component gave the following results (n=99):

Distribution #4	K	22.2	%	83.1
		0.09		15.4
		17.20		1.5

The three compounds have K values compatible with estriol-16-glucosiduronate, estriol-3-sulfo-16-glucosiduronate and estriol, respectively. Statistical analysis (7) indicates that the observed slope is closer to zero than 10-20% of those results which could be obtained from an experiment in which two compounds, known to be identical, are being compared.

On redistribution of the second bile peak, B-II, (Distribution #5, Fig. 5) the patterned discrepancy between the observed points and the best predicted curve, first observed for this peak in Fig. 2 (Distribution #1), was again evident in Distribution #5 (Fig. 5). The observed curve has a sharper rise than the predicted one and has a tailing edge. This "tail" was fitted with another compound. Since it is apparent that the observed curve does not exhibit the shape associated with the binomial distribution, the computations can only be used to obtain a very gross indication of the number and quantity of compounds present.

This peculiar skewing of the peak described above gave a clue that it could be composed of estriol sulfate, since the partition coefficient of estrogen sulfates is shown (see preparation and properties of estriol-3-sulfate) to vary with concentration. The computed K values for the peak tube although valid in this instance may not be duplicated in other distributions where different concentrations of sulfate are present.

It should be noted that this distribution represents 10% of the radioactivity present in the original bile (Fig. 2). Therefore, the first compound above (Distribution #5) represents 6.6% of the bile radioactivity and the second 1.2% and so on.

[1]We are indebted to Dr. Mortimer Levitz for this gift of C^{14}-16-estriol-3-sulfo-16-glucuronide.

To the dried residues of the CCD tubes containing the second peak (Distribution #5, the first two compounds[1], peak B-II-b) were added (total percentage approximately 8% of the biliary output of radioactivity) 10 mg. of carrier estriol-3-sulfate and the mixture was distributed in the system: ethyl acetate: n-butanol: 0.2% ammonia; 1:1:2 for 99 transfers. The statistical analysis of the specific activity line for tubes 38 to 75 indicates that the observed slope is closer to zero than 50-70% of those results which could be obtained from an experiment in which two identical compounds are being compared. It cannot be concluded, therefore, with statistical significance that B-II-b and estriol-3-sulfate are different.

Aliquots of peak B-II-b were separately treated using Mylase P and B-glucuronidase in the usual manner and the dried incubation media were distributed individually (n=99) in the system: ethyl acetate: n-butanol:0.2% ammonia; 1:1:2. Results of the computer analysis are as follows:

Distribution #6 (Mylase P)	K	12.7	%	100.0
Distribution #7 (B-Glucuronidase)	K	1.00	%	90.6
		0.02		5.2
		15.8		4.2

It is apparent that while Mylase P effected 100% hydrolysis in Distribution #6 (K of 12.7 corresponds to estriol), -glucuronidase in Distribution #7 had little effect (K of 1.00 corresponds to estriol-3-sulfate, K of 15.8 corresponds to estriol).

The above evidence is compatible with the fact that peak B-II-b is composed of estriol-3-sulfate. It can be concluded, therefore, that the radioactivity excreted in the bile of the subject following injection of estriol-6,7-H^3 consisted of 80% estriol-3-sulfo-16-glucoronide and 8% estriol-3-sulfate together with other minor components.

URINE FRACTIONS: The distribution (n=99, 10% ammonia:n-butanol[1]) of an aliquot from the 0-2 hour urine collection of the T-tube subject following injection of estriol-6,7-H^3 is shown in Fig. 6 to be composed of two main peaks: U-I and U-II (Tubes 0-16 and 17-46, respectively). U-II was redistributed (n=99) in the system: ethyl acetate:n-butanol:0.2% ammonia; 1:3:4 (Fig. 7). Previous experience in this laboratory has shown estriol-16- and 3-glucosiduronates to have K values of about 2 and 0.3, respectively, in this system. In dilute ammonia (Distribution #9), the 3-phenol is not ionized (9), and the 16-glucuronide has a relatively high

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[1]Because of the tailing of the sulfate peak, it is doubtful if the second compound exists. However, addition of computed percentages for these two compounds is quite valid and gives the amount of radioactivity in the peak as a percentage of that in the CCD train.

K value. Only estriol-3-sulfate is known to have a similar partition coefficient under these conditions. There is ample evidence (10) that the major urinary product of estriol in man is its 16-glucosiduronate. However, in view of recently reported (1) estriol-17-glucosiduronate, it was of interest to examine the 16-glucuronide peak for purity. After 299 transfers in the same system (Distribution #9), analysis of the curve is given in Fig. 8 (Distribution #10) and shows that the peak is 98.6% pure.

When U-I (Distribution #9) was redistributed (Fig. 9) two peaks (K=0.054, tubes 9-24 and K=0.017, tubes 0-8) were designated U-I-b and U-I-a, respectively. It should be noted that U-I-a and U-I-b represent 2.7 and 9.0% of the urinary radioactivity, respectively. No further work was done on peak U-I-a except to ascertain that it is more polar than either estriol-3, 16-diglucosiduronate and estriol-3-sulfo-16-glucosiduronate. An aliquot from peak U-I-b was treated with bacterial B-glucuronidase in the usual manner. After distribution (n=99) in the system: ethyl acetate:n-butanol:0.2% ammonia; 1:1:2 results of the computer analysis were as follows:

Distribution #12	K	2.28	%	93.7
		0.02		6.3

The partition coefficient of the first peak (at tube 69) corresponded to estriol-3-sulfate.

When another aliquot from peak U-I-b was hydrolyzed with Mylase P and the dried incubation medium distributed (n=29) in the system:ethyl acetate:butanol:0.2% ammonia; 1:3:4 results of the analysis were as follows:

Distribution #13	K	2.22	%	52.5
		0.12		47.5

The partition coefficient of the first compound corresponds to that of estriol-16-glucosiduronate in this system.

From the above results and those of Emerman et al. (3) it was concluded that the above compound is estriol-3-sulfo-16-glucosiduronate.

In the present work, therefore, the radioactivity excreted in the urine of the T-tube patient after injection of estriol-6, 7-H^3 contained 64.8% estriol-16-glucosiduronate and 9.0% estriol-3-sulfo-16-glucosiduronate which were adequately identified. A compound in the estriol-3-glucosiduronate region was present in 0.8% and was not identified further.

ESTRIOL-3-SULFATE: This conjugate was prepared from estriol-16, 17, diacetate which was in turn synthesized by reductive hydrolysis of the triacetate using sodium borohydride according to the method of Tsuneda, et al. (12). Radioactive diacetate was obtained by acetylation of estriol-6,7-H^3 with a drop each of pyridine and acetic anhydride, evaporation of the solvents, addition of 1 mg. carrier triacetate and reduction with sodium borohydride. The products of the reaction were separated by paper

chromatography in the system: benzene:methanol:water; 10.0:5.5:4.5. After scanning, the area corresponding to the co-chromatographed estriol-16,17-diacetate was eluted in methanol and the solvent evaporated. Estriol-16,17-diacetate (prisms) melted at 175-176.5°C. (lit.[12] 171-3°C.) $\lambda_{max.}^{MeOH}$ 281 mμ (ϵ=2120).

Estriol-3-sulfate was prepared similarly to the estradiol derivatives (4). Upon evaporation of the major portion of the butanol extract and allowing the solution to stand overnight in the cold, crystals were deposited, collected and dried in vacuo at 110°C. When a sample was heated on the Kofler block, it turned red at 252°C., softened at 260°C., re-solidified, turned deep red, and did not melt up to 310°C. $\lambda_{max.}^{MeOH}$ 276 mμ (= 750) and 269 mμ (ϵ= 780). $\lambda_{max.}^{KBr}$ 3425, 2938, 1250, and 1055 cm.$^{-1}$. Analysis: calculated for $C_{18}H_{23}O_6SNa$ 1.5 H_2O; C 51.78; H 6.28; O 28.74; S 7.68; Na 5.51. Found C 51.89; H 6.02; O 28.96; S 7.65; Na 5.48.

The radioactive sample was prepared as follows: labelled estriol-16,17-diacetate was diluted with 25 mg. carrier and the mixture reacted to form the sulfate. Purification was by countercurrent distribution in 10% ammonia in water and n-butanol.

To examine the effect of concentration on the partition coefficient, 10 ml. of each phase of pre-equilibrated 10% ammonia in water and n-butanol were transferred to test tubes. Estriol-3-sulfate was added and the tubes were rocked at constant temperature (30°C.). The phases were then allowed to separate until clear, and aliquote were removed for counting. Table I gives the variation of K with concentration of this compound and shows there is a two-fold increase in K as the concentration is decreased from 3 mg./ml. to 0.5 mg./ml. Table 1 accurately indicates the trend but the K values cannot be duplicated when countercurrent distribution is performed in an instrument. This phenomenon has been observed in the present work and by other investigators[1]; repeatable results are expected with the use of the instrument since the partition coefficient obtained in the latter case is an average of several determinations (one in each tube across the peak). The peak tube of estriol-3-sulfate in the system:ethyl acetate:n-butanol:0.2% ammonia; 1:3·4 has varied from 70 (with trace radioactive material from biological origin) to 50 or less when 10 mg. carrier was added to the same material. The skewness of the peak also changes markedly with concentration. A binomial curve, which can be fitted with a theoretical, can be obtained if the peak tube is at 70 in the above system. Distortion increases, however, with concentration until at a peak tube of 50, the left hand edge is sharp while the right hand side tails markedly[2].

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[1]Private communication from Dr. W. Roy Slaunwhite, Jr.

[2]A mathematical approach to this problem is being incorporated into the computer program used in the present work (1,2).

DISCUSSION

Work in this laboratory on estrogen conjugates in bile and urine has pointed to the need for new and powerful tools to separate and quantitatively measure the degree of purity of these polar compounds. For separation, the method must have high resolution and yet afford mild treatment to labile compounds. There also should be no loss of steroids or their conjugates under any conditions. For ascertaining purity, the method should produce, directly or indirectly, data which can be analyzed to give a reliable indication of the purity of the compound. Identification of steroid conjugates presents special problems. The compounds are closely related in polarity, and to obtain them in high purity involves tedious separations. Carriers cannot be obtained in quantity, if at all, and are difficult to crystallize and and sometimes are sufficiently labile so they do not withstand manipulations. It is believed that crystallization to constant specific activity is not applicable to these compounds. For identification, therefore, the method should compare the unknown with a standard, with enough sensitivity to give a "not identical" verdict when the compounds are not the same, yet not be so sensitive as to reject identity if either the compound to be tested or standard have a minor contaminant, or if there is an isotope effect.

The work presented in this communication proves that the combination of countercurrent distribution with new statistical approaches can provide the tool that fulfills the above requirements. Concurrently, the burden of proof of identity, as exam-

plified by peak U-I-b, rests on enzymatic hydrolysis and identification of the resulting mono-conjugates by admixture with the proper standard, countercurrent distribution and determination (by application of the method of Baggett and Engel (7)) that the slope of log specific activity vs. tube number is not significantly different from zero. The conclusion that B-I-b is estriol-3-sulfo-16-glucuronide is strengthened by the fact that this compound, from which is obtained the 3-sulfate (Fig. 4) and the 16-glucosiduronate had already been rigorously identified by Emerman, et al. (3) as the major constituent of human bile after administration of estriol-3-sulfate.

In the present work, estriol was selected in order to minimize complications of analysis due to metabolite formation, and hence, no attempt was made to identify the aglycone as such. In this connection, Emerman, et al. (3) have stated that following complete hydrolysis of biliary and urinary conjugates and final paper chromatography of these hydrolysates, recovery of the steroid (exclusive of the efficiency of the hydrolysis procedures; which were between 88 and 100%) was between 87 and 96%. The remaining radioactivity was ascribed to impurities which were not further investigated. It has been the experience of many workers that in paper chromatography there is almost always a certain amount of radioactivity 'stuck at the origin', which could be breakdown products or the result of strong adsorption to the paper. While Emerman, et al. (3) could recover only 87 to 96% of the amount chromatographed on paper, this is not indicative of, nor did the above authors prove, that the remaining 4 to 13%

represent an aglycone other than estriol. However, the question of whether CCD and the statistical modification used in the present work can separate the mono-conjugates of estriol and a metabolite thereof, e.g. 6-hydroxy estriol has not been investigated. Predictably, this is a difficult resolution for any technique, and would best be performed on the aglycones after hydrolysis rather than on the conjugates per se.

After injection of estriol, Fig. 1 shows that there was a latent period of 1½ hours before significant excretion of biliary radioactivity commenced. This time lag probably is a reflection of the efficiency of the liver in extracting the steroid from the blood, the time consumed in the conjugation reaction itself, as well as time of excretion from the individual cells into the bile.

To compare the results presented above with those from the work of Emerman, et al. (3), it is necessary to describe the methodology in both cases. In the present work, the only pretreatment of the bile was to reduce its volume in a rotary evaporator under mild conditions. The fractionation procedure was then commenced, and after the given number of transfers, the amount of radioactivity present in each peak was calculated as a percentage of the total in the CCD train. Emerman, et al. (3) infused intravenously estriol-3-sulfate labelled with H^3 and S^{35} into a T-tube patient and examined the bile and urine for the excretion pattern. Bile was made 70% with respect to methanol and left in the cold. The precipitate was centrifuged, and the supernatant after being concentrated was made 95% with respect

to ethanol. The precipitate was washed repeatedly with 95% ethanol. However, in one of the samples, 7% of the tritium could not be recovered from this precipitate.

The ethanolic extract was chromatographed on de-activated (9% water) "neutral alumina" and four fractions were obtained by elution with 89-90% and 70% and 25% ethenol in water, respectively, and also with 0.2% ammonia. All quantities were related by these authors to the total H^3 radioactivity eluted from the column, and not to the amount originally present in the bile. After this preliminary separation, purification was achieved by partition chromatography on celite and paper.

In the present studies, biliary excretion of estriol presented a simple pattern (Fig. 2): a major polar peak (B-I) comprising 86% of the radioactivity, a smaller (B-II), less polar peak with 11% of the activity and a non-polar minor (1.3%) peak. The first bile peak (B-I) consisted of almost pure estriol-3-sulfo-16-glucosiduronate. The latter compound comprised 79.4% of the biliary radioactivity. The second peak, B-II (Fig. 2) was composed of almost pure estriol-3-sulfate, which totalled 8% of the biliary radioactivity.

Emerman, et al. (3) obtained the following: 77% estriol-3-sulfo-16-glucosiduronate (very little labelled sulfur), 4% estriol-3-sulfate (the S^{35}/H^3 ratio as was infused) and 4% estriol-16-glucosiduronate, with traces in the estriol-3-glucosiduronate area.

The amount of double conjugate in the present results compares favorably with those obtained by Emerman et al. (3) above. Also compatible is the presence of estriol-3-sulfate. The fact

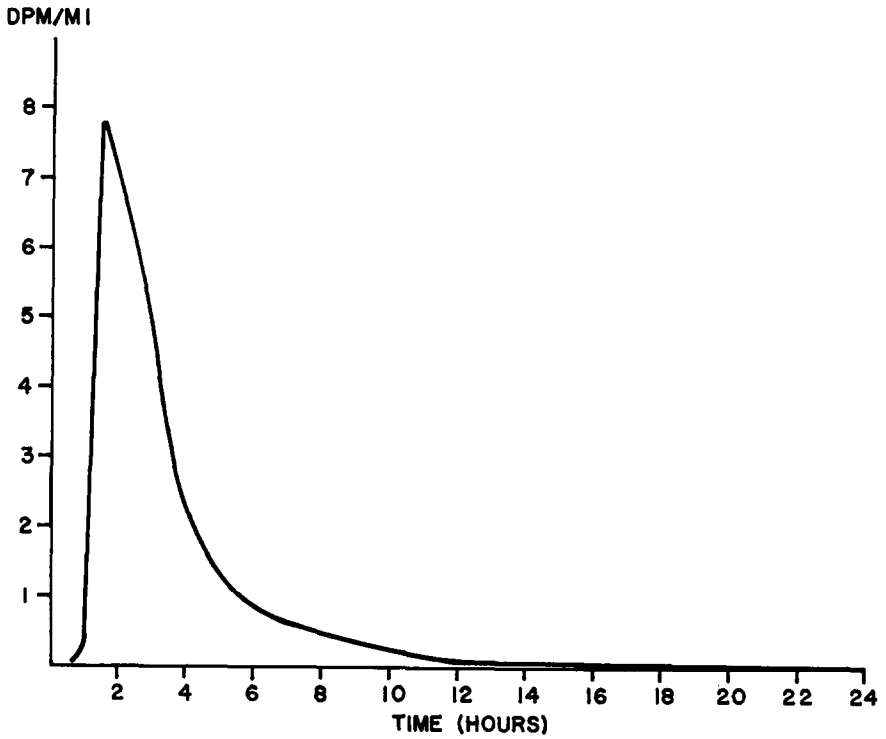
that in the latterwork, estriol-3-sulfate was recovered unchanged does not preclude its specific activity being low because of the presence of endogenous compound. In the present work the latter conjugate was found to comprise 8% of the biliary radioactivity. Absent, however, in the present data are both estriol-3-and estriol-16-glucosiduronate. These two compounds have an identical K of 0.65 in the system 10% ammonia:n-butanol. The possibility that they are included in the second biliary peak B-II (Fig. 2) is quickly checked by reference to Fig. 5, in which the system used was ethyl acetate:n-butanol:0.2% ammonia; 1:3:4. In this system estriol-3-glucuronide exhibits a K of 0.69, while the 16-glucuronide has a K of approximately 2.0. These two compounds were not observed in Fig. 3. The 4% estriol-16-glucosiduronate found by Emerman, et al. (3) could therefore be an artifact created by the hydrolysis of some of the estriol-3-sulfo-16-glucoronide when chromatographed on the alumina.

Results obtained for urine in both researches were entirely compatible: estriol-16-glucosiduronate in the present work was found to comprise 65% of the radioactivity in the first two hours urine collection, while estriol-3-sulfo-16-glucosiduronate comprised 9% of the tritium radioactivity. Emerman, et. al.(3) reported 52% and 7% of the above two conjugates, respectively.

TABLE 1

Variation of the Partition Coefficient with Concentration of Estriol-3-Sulfate

Concentration	K
3 mg/ml	1.22
2 mg/ml	1.59
1 mg/ml	1.80
0.5mg/ml	2.46



LEGENDS

Fig. 1: Excretion of radioactivity per ml. of bile as a function of time following injection of estriol-6,7-H^3 into a T-tube patient.

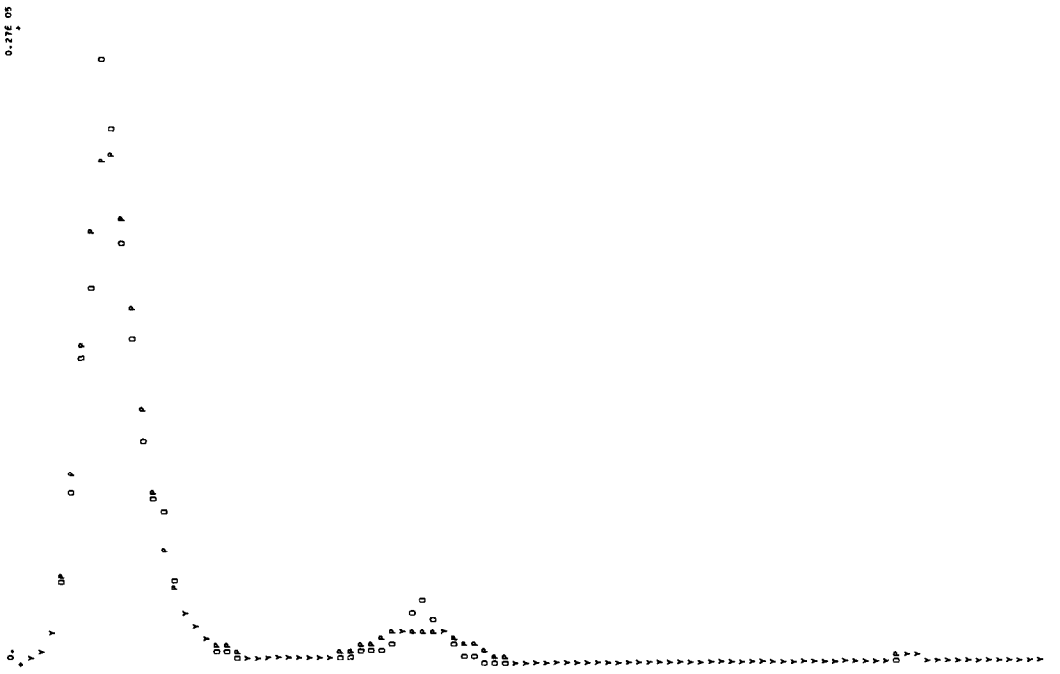


Fig. 2: Photograph of the computer output following analysis of a countercurrent distribution of bile fractionation. o = observed data, p = predicted data, y = a point at which o and p coincided. The system used was 10% ammonia:n-butanol. n = 99 transfers.

Distribution #1

K:	%:	
0.087	(30.1	
0.182	6.1)	B-I
0.614	(10.5)	B-II
0.007	0.4	
5.856	1.3	
0.333	1.3	

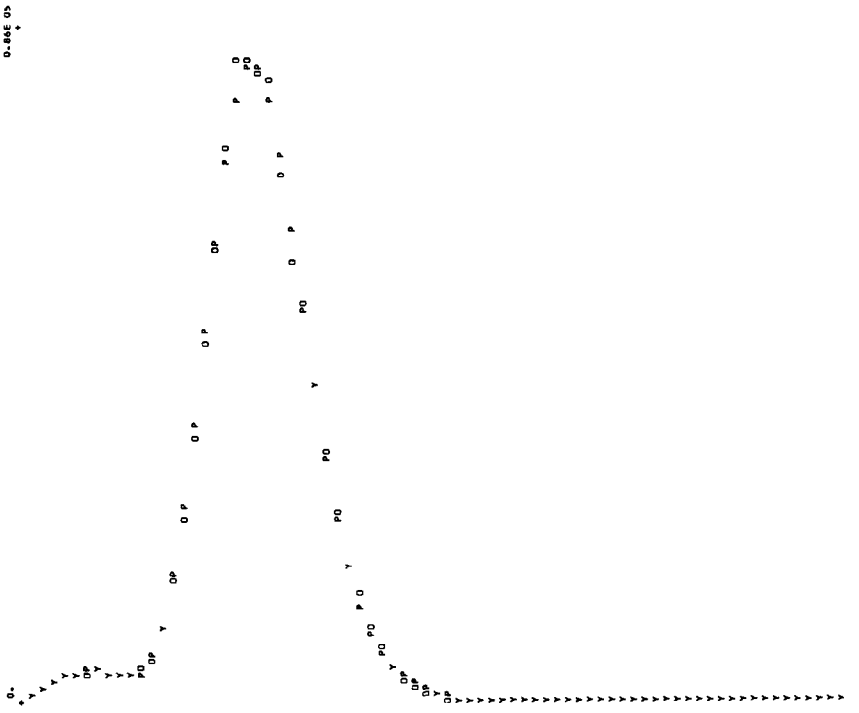


Fig. 3: Analysis of countercurrent distribution of B-I. n = 299 transfers in the system 10% ammonia:n-butanol.

Distribution #2

K:	0.021	%:	(2.8
	0.006		0.8) B-I-a
	0.075		(91.9
	0.112		3.7) B-I-b

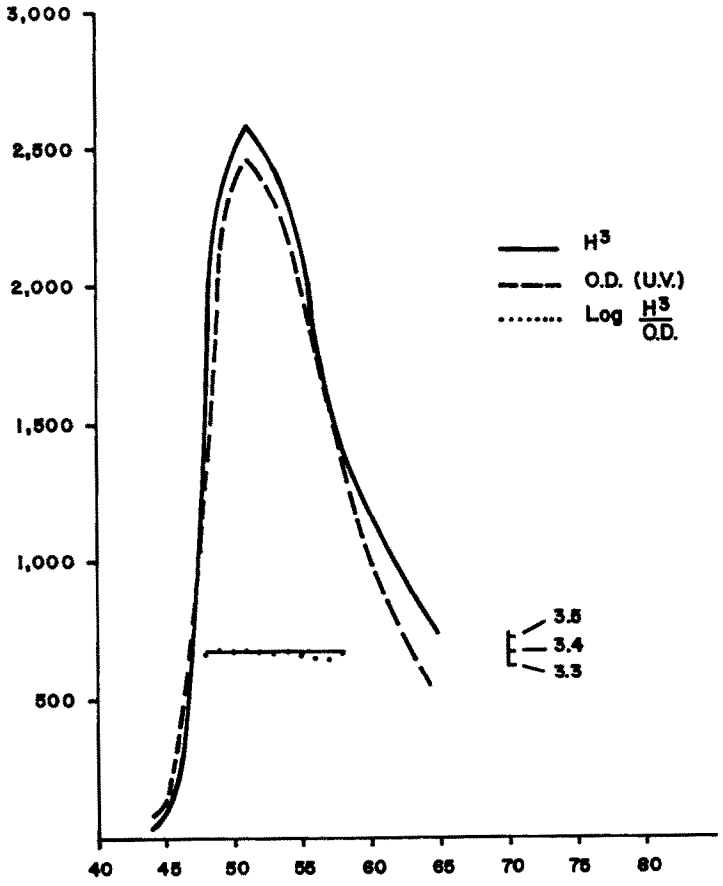


Fig. 4: The distribution of estriol-6,7-H^3-3-sulfate from hydrolysis of peak B-II-b with bacterial B-glucuronidase. Carrier estriol -3-sulfate (10 mg.) had been added. The optical density curve is skewed because of the change of partition coefficient with concentration. The distortion of the radioactive curve exceeds that of the carrier because of the presence of impurity on the right hand side.

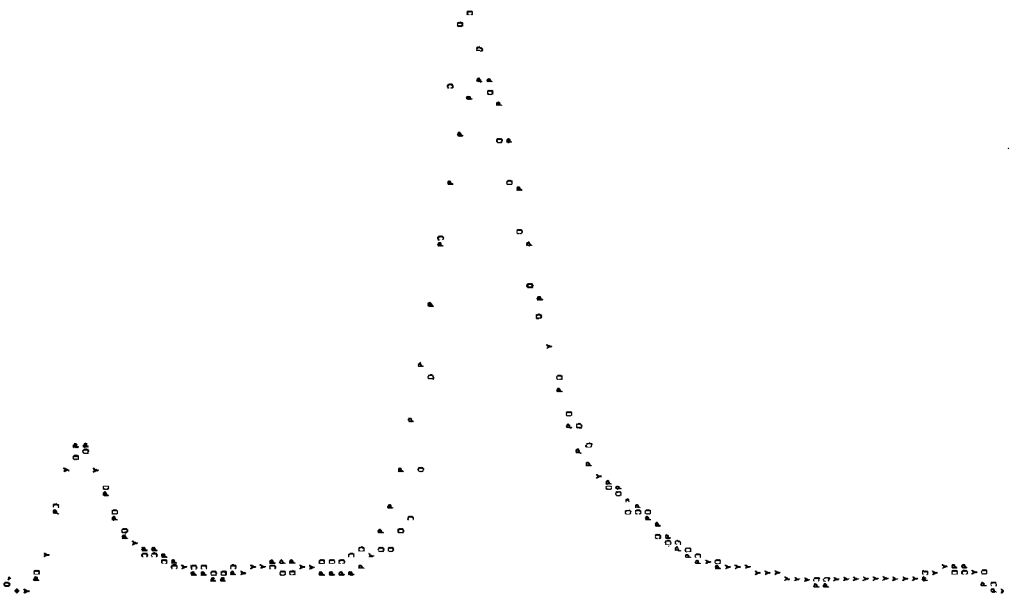


Fig. 5: Analysis of B-II, n = 99 transfers in the system ethyl acetate:n-butanol:0.2% ammonia; 1:3:4.

Distribution #5

K:	% :	
0.880	60.4)	
0.062	11.6)	B-II-b
1.525	8.9	
0.152	3.6	
18.075	1.6	
0.358	3.7	
2.788	2.6	
6.198	1.5	

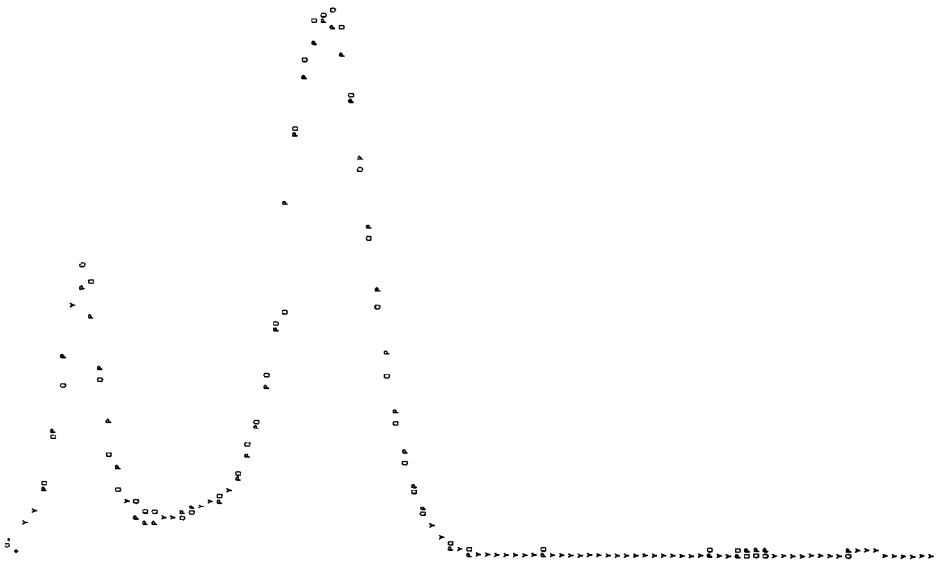


Fig. 6: Analysis of an aliquot from the 0-2 hour urine of T-tube patient after injection of estriol-6,7-H^3. n = 99 transfers in the system 10% ammonia:n-butanol.

Distribution #8
(Fig. VI)

K:	%:	
0.008	(0.8	
0.067	18.3	
0.117	2.1)	U-I
0.267	(4.5	
0.510	71.8)	U-II
1.297	0.8	
2.420	0.6	
4.270	0.6	
11.902	0.6	

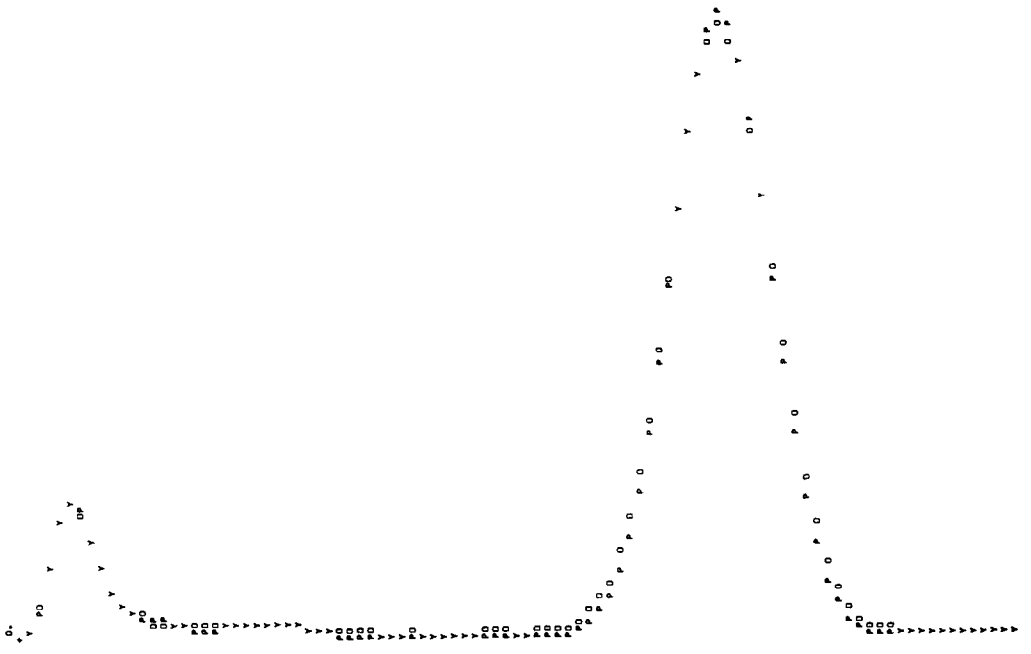


Fig. 7 : Analysis of U-II, n = 99 transfers in the system: ethyl acetate:n-butanol:0.2% ammonia; 1:3:4.

Distribution #9

K:	0.050	%:	(8.6
	0.229		1.1) U-II-a
	2.033		(90.3) U-II-b

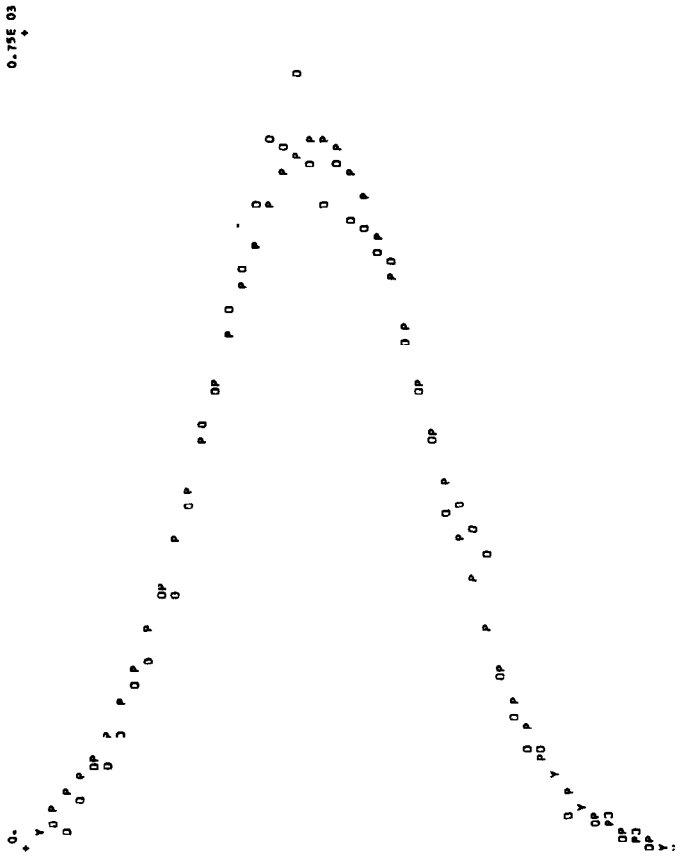


Fig. 8: Analysis of U-KK-b, n = 299 transfers in the system: ethyl acetate:n-butanol:0.2% ammonia; 1:3:4.

Distribution #10

K:	2.001	%:	98.6
	2.487		1.4

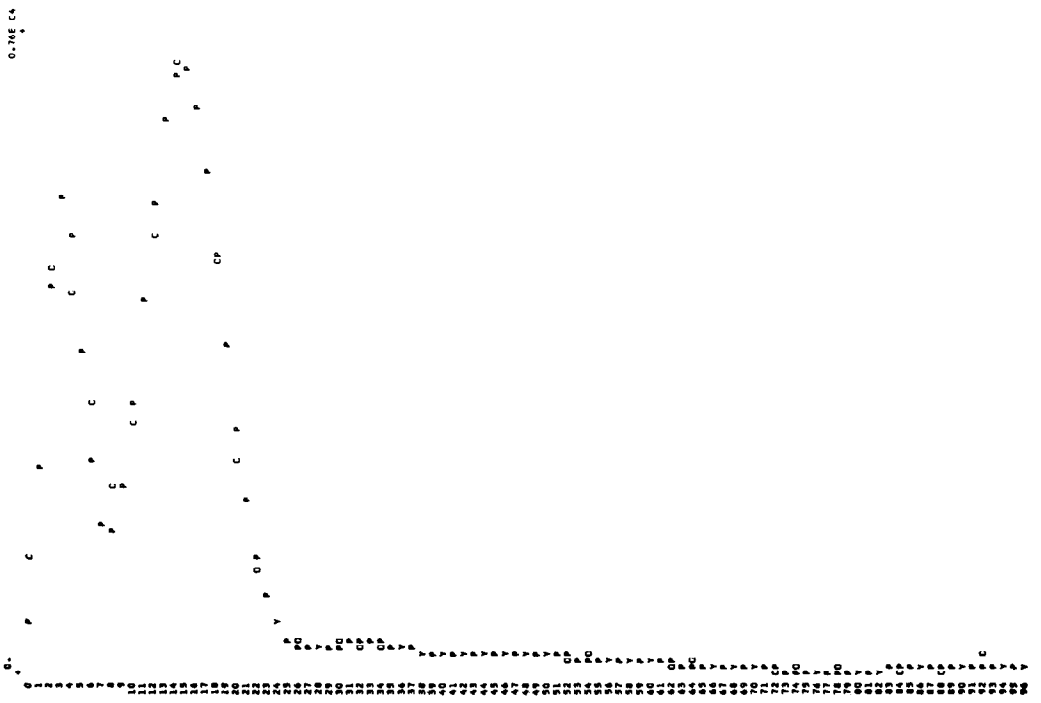


Fig. 9: Analysis of U-I, n = 299 transfers in the system: ethyl acetate:n-butanol:0.2% ammonia; 1:3:4.

Distribution #11

K:		%:	
	0.006	(1.5	
	0.016	14.8)	U-I-a
	0.053	(49.5)	U-I-b
	0.120	7.5	
	0.193	7.4	
	0.288	6.6	
	0.426	6.0	

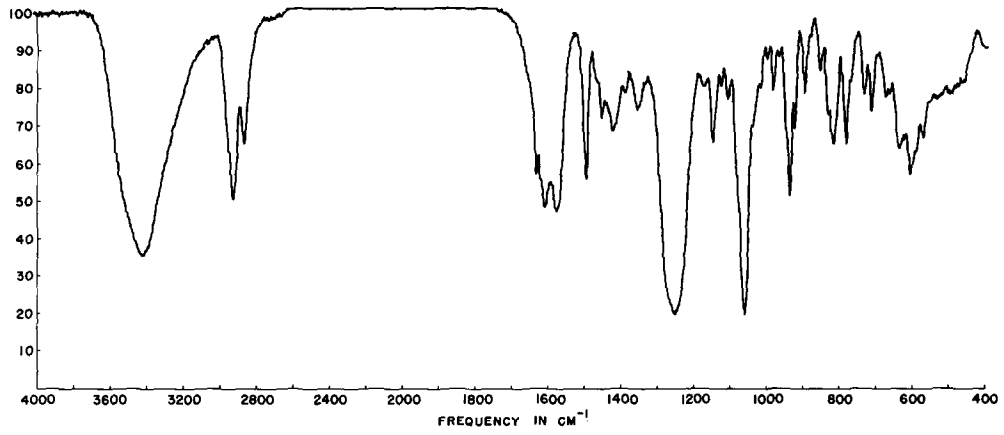


Fig. 10: Infra-red spectrum of estriol-3-sulfate. Dilution 1 mg. of sulfate:400 mg. KBr.

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