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EXCRETION OF 15α-HYDROXYESTRIOL AND ESTRIOL IN MATERNAL URINE DURING NORMAL PREGNANCY

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SUMMARY

The daily urinary excretion of 15α-hydroxyestriol (estetrol) and estriol was determined by a gas–liquid-chromatography method in 130 urine samples collected from 43 healthy pregnant women. The earliest sample was from the 9th week of pregnancy. The results of the estriol determination by this GLC method agree with those published before, the mean excretion of estriol being about 30 mg in 24 h towards the end of pregnancy. The mean amount of estetrol excreted in normal pregnancy urine increased from 0·28 mg in the 20th week to 2·09 mg in the 40th week of pregnancy. Some differences were observed in the rates of increase of estriol and estetrol excretion. Possible reasons for these differences are discussed. There was no significant difference in the variability of consecutive 24-h excretion of estetrol and estriol. If the collection time is shortened, the variability of estetrol excretion increases like that of estriol. The amount of estetrol excreted in maternal urine before partus is correlated with the weights of the fetus and placenta, like that of estriol.

INTRODUCTION

RELIABLE information on the development and condition of the fetus during pregnancy is of great importance for the clinician. Therefore several methods have been developed for the determination of steroid hormones and their metabolites originating from the fetoplacental unit. Of these, the one most widely used by clinicians is the determination of estriol in maternal urine. However, this method is influenced by several factors, which decreases its value for clinical purposes. Urine must be collected for at least 24 h or, when shorter collection periods are used, the environment must be very carefully stabilized but even so the variation between consecutive collections is about 20%[1]. The variation between individuals is also very great; at the same period of normal pregnancies the difference may be as much as 300%. Therefore the value of a single determination of estriol in maternal urine is limited. In addition, a part of the precursors of estriol in late pregnancy is derived from the neutral steroids synthesized in maternal adrenals[2] and a part, albeit a relatively small one, of the urinary estriol in normal late pregnancy is derived from the metabolism of estrogens in the mother[3, 4].

In 1965, Schwers *et al.*[5] in perfusion experiments with estrone, estrone sulfate and estradiol demonstrated that from these precursors the fetus is able to synthesize an estrogen that is more polar than estriol. Hagen *et al.*[6] isolated a very polar estrogen from the urine of malformed newborn children after an estradiol load, and Adlercreutz and Luukkainen[7] found an estetrol in pregnancy urine. In 1967, Zucconi *et al.*[8] identified this estrogen as 15α-hydroxyestriol (estetrol).

According to Schwers *et al.*[9], Gurpide *et al.*[10] and Mancuso *et al.*[11], the 15α-hydroxylation of estetrol is obviously carried out in the fetal liver. YoungLai and Solomon[12], in their studies on estetrol biosynthesis, have claimed that the 15α-hydroxylated C-19 steroids are important intermediate compounds. This is in agreement with the results of Schwers *et al.* who found that the biosynthesis of estetrol in the fetal compartment seems only partially to take place vis estriol[13]. In the same study, YoungLai and Solomon[12] estimated that more estetrol than 15α-hydroxyestradiol is synthesized from a given amount of labeled precursors injected into the maternal circulation or into the fetus during intrauterine transfusion. So it seems, on the basis of these reports, that the biosynthesis of estetrol differs from that of estriol and that this compound is probably a more specific product of the fetal organism than estriol. Therefore the amount of this estrogen excreted in maternal urine might prove to be a good indicator of the well-being of the fetus during pregnancy.

Previous studies in this laboratory[14, 15] have shown that the amounts of estetrol excreted into maternal urine are relatively high in late pregnancy. Though a nonpregnant woman is able to synthesize estetrol from estrone sulfate[16, 17], the maternal contribution to the total urinary excretion seems to be almost negligible. However, the amounts of estetrol excreted are relatively large and its determination in pregnancy urine might have some clinical value; no quantitative data on the excretion of estetrol in pregnancy urine are available as yet. The aim of the present study was to determine the urinary excretion of estetrol and estriol and to make comparisons between the amounts of these two steroids excreted into maternal urine during pregnancy.

EXPERIMENTAL

One hundred and thirty 24-h urine samples were collected from 43 healthy pregnant women. The pregnancies of these women revealed no pathologic symptoms or complications by history or examination. About one half of the material was obtained by repeated collections of urine from four volunteers, starting from the third month, and from four others, starting from the sixth month, to the end of pregnancy. The urine was collected until the 36th week of pregnancy every fourth week and after that every second week until delivery. Of these eight women three were primiparas and five were multiparas and the age of the subjects varied from 24 to 30 years. Other urine samples originated from one, two or in some cases more frequent collections from healthy pregnant women. The parity in this group was in proportion to that of the group followed through most of pregnancy and the age varied from 18 to 38 years. No environmental standardization was used during collections and most of the samples were collected at home by volunteers and only a few in hospital just before delivery. The urine samples were processed immediately; if this was not possible they were kept at −20°C until analyzed. In order to determine the correlations between the daily estrogen excretion and fetal and placental weight, urine samples were collected from 20 healthy, pregnant women during the last week before delivery. In all these cases the Apgar score was 9 or 10 at 1 min and the infants were healthy at subsequent examinations. The infants and placentas were weighed immediately after birth. The variability of urinary estetrol and estriol excretion was investigated from 34 pairs of consecutive 24-h urine samples and from 21 pairs of consecutive 8-hr urine samples, which were collected from healthy pregnant women between the 22nd and 41st week of pregnancy.

The method of Heikkilä and Adlercreutz[18] based on gas chromatographic determination of estetrol and estriol in urine was used. The outline of this method is shown in Fig. 1.

RESULTS

Estetrol values of one hundred and thirty 24-h urine samples collected from normal pregnant women are plotted against the week of pregnancy in Fig. 2 and the corresponding estriol values in Fig. 3. From these figures it can be seen that the estetrol value increases as a function of time. This increase seems

(1) Gel filtration of 50 ml filtered pregnancy urine.
(2) Hydrolysis of conjugates with *Helix pomatia* extract.
(3) Extraction with 3 × 1/1 vol. of ethyl acetate.
(4) Chromatography on silica gel column.
(5) Chromatography on partially deactivated acid alumina.
(6) GLC quantification of the TMSi derivatives of the steroids.

Fig. 1. Outline of the method used for the determination of estetrol and estriol in maternal urine.

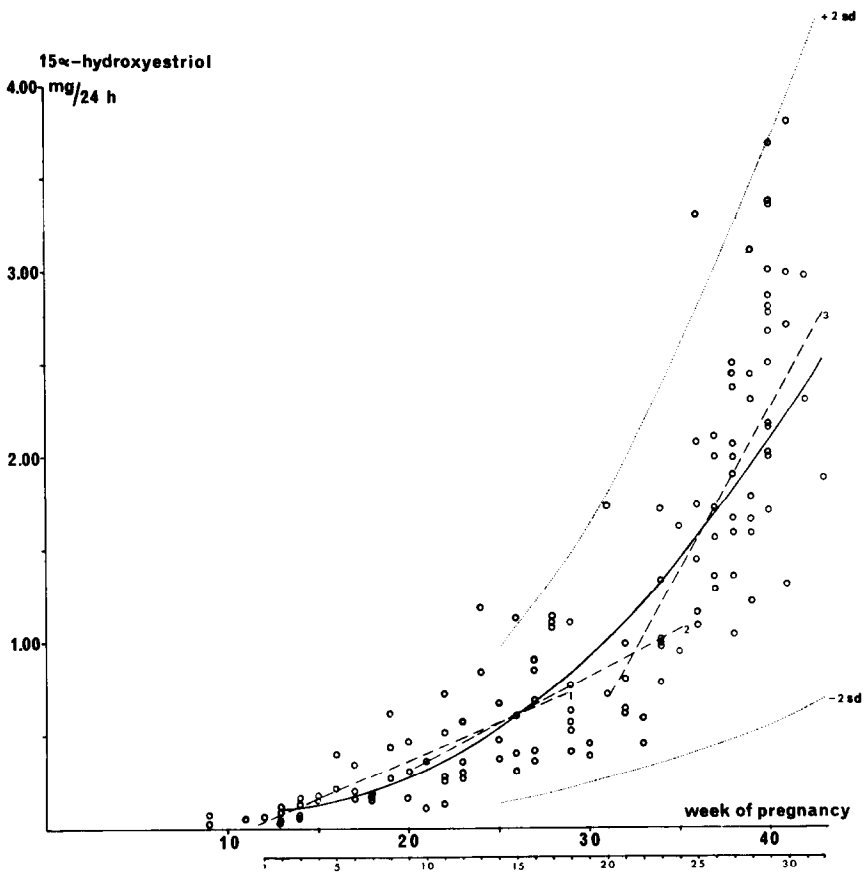

Fig. 2. The excretion of 15α-hydroxyestriol during normal pregnancy. 130 determinations from 43 subjects. The continuous curve is a parabola that depicts the mean excretion of 15α-hydroxyestriol and the dotted curves represent the variation (±2 S.D.) in this material. The broken lines are regression lines calculated from the results from 12–27[1], 21–34[2] and 31–43[3] weeks of pregnancy.

not to be linear and the increase of estetrol seems to differ from that of estriol. In order to obtain a better idea of the difference in the rate of increase of the two estrogens during normal pregnancy the results were analyzed statistically and graphically.

In order to depict the mean excretion of estetrol in maternal urine during normal pregnancy an attempt has been made to find the best curve which represents the estetrol values determined. The mean values of the results in each week

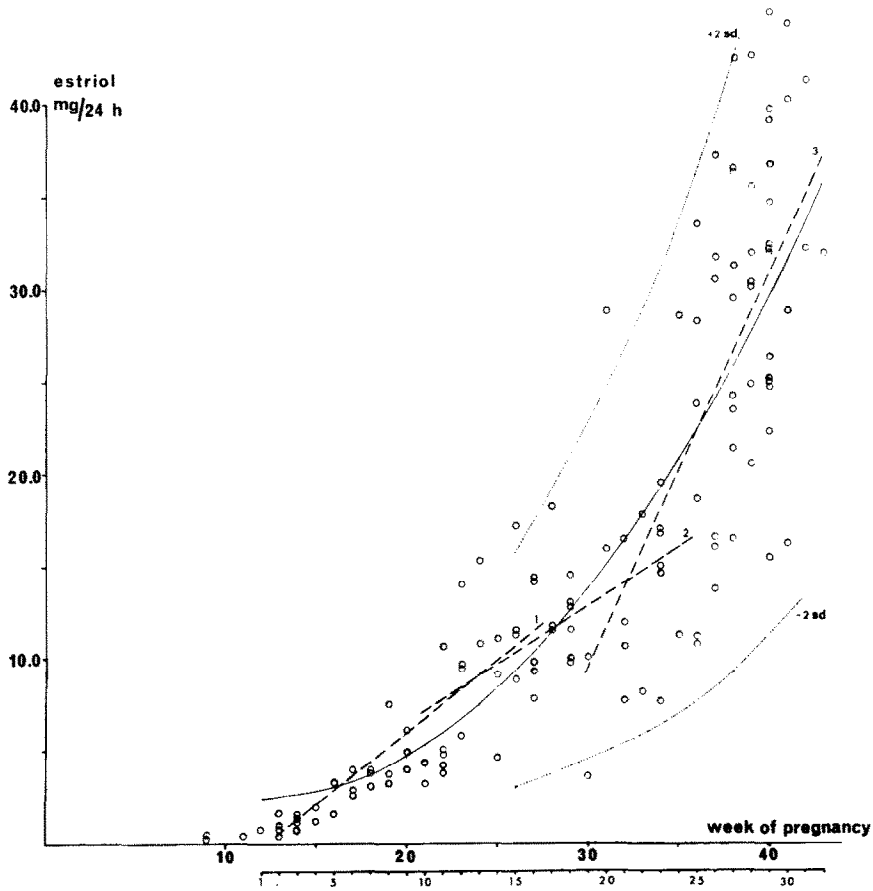

Fig. 3. The excretion of estriol during normal pregnancy. 130 determinations from 43 subjects. The continuous curve is a parabola that depicts the mean excretion of estriol and the dotted curves represent the variation (±2 S.D.) in this material. The broken lines are regression lines calculated from the results from 12–27[1], 21–34[2] and 31–43[3] weeks of pregnancy.

of pregnancy (except the results in the 9th–12th weeks, which are, because of the small number of determinations during these weeks, combined into a single mean value) were used for fitting an equation for a quadratic curve by the method of least squares. The equation calculated in this way for all the estetrol values is $y = 99{\cdot}43 - 2{\cdot}55x + 2{\cdot}45x^2$, where y = amount of estrogen in mg and x = week of pregnancy expressed in time units as seen in the figures below the abscissa. The correspondingly calculated equation for estriol is $y = 2503 - 67{\cdot}2x + 34{\cdot}4x^2$. The parabolas corresponding to these equations are drawn in Figs. 2 and 3. These parabolas describe the increase in the daily urinary excretion of estetrol and estriol throughout pregnancy in this material.

According to these results, the mean urinary excretion of estetrol of normal pregnant women in 24 h increased from 0·28 mg in the 20th week to 0·94 mg in the 30th week and to 2·09 mg in the 40th week of pregnancy. The corresponding mean values for estriol excretion were: 4·69 mg in the 20th week, 13·65 mg in the 30th week and 29·49 mg in the 40th week of pregnancy.

The variation limits for normal data are calculated from the estetrol and estriol results by using the calculated parabolas as follows: in each week the corresponding parabola value is taken as 100 and the determined estrogen values are expressed as per cents of this, and from these the coefficients of variation were calculated separately. The lines corresponding to ±2 S.D. are drawn in Figs. 2 and 3.

When the results were analyzed statistically, it was observed that the increase in the excretion of each of the estrogens as a function of time was significant ($t_{estetrol} = 10{\cdot}82^{***}$ and $t_{estriol} = 14{\cdot}97^{***}$).† When the coefficients of the parabolas were compared (values were made commensurable by multiplying the estetrol values by ten)‡ it was found that the two curves were significantly different ($t_x = 16{\cdot}2^{***}$ and $t_{x2} = 3{\cdot}9^{***}$). This agrees with the conclusion drawn from the graphical analysis that there are clear differences in shape between the parabolas. Thus the rate of increase of estetrol excretion differs from that of estriol excretion in normal pregnancies. When the curves were compared by the x^2 test, it was demonstrated that the parabola for estriol excretion corresponds a little, but not significantly, better to the empirical values than that for estetrol. The change in the ratio of estriol and estetrol excreted during pregnancy can also be seen from the results presented in Table 1.

From Figs. 2 and 3 it can be seen that the parabolas do not describe smaller variations in the rate of increase of the hormone excretion in this material, because, for instance, in the 23rd–28th weeks of pregnancy most of the values for estriol are above and in the 29th–34th weeks most of the values are below the parabola in this material. In order to check whether there really are any gestational variations in the rate of increase of hormone excretion, the material was analyzed in smaller sliding groups by linear regression analysis§. This could be simplified to three linear curves, which partly overlap each other. The lines are calculated from hormone values determined in the weeks 12–27, 21–34 and 31–43. The corresponding equations and their r-values are presented in Table 2. These regression lines, like the parabolas, clearly indicate that the increase of estriol excretion is more rapid than that of estetrol in the first half of pregnancy: the regression coefficient of estriol is nearly twice as great as that of estetrol. A very interesting point is the change in the rate of estriol increase between the 23rd and 32nd weeks of pregnancy: the regression coefficient is reduced as compared to the equation of the first line. No such diminution can be detected in the rate of increase of estetrol excretion. The regression coefficient is slightly greater for the second line than for the first one. During the last two months the regression lines of estriol and estetrol are almost parallel.

When the results of estetrol and estriol determination in individual urine samples were scrutinized, it was observed that a low estetrol value is accompanied by low estriol and vice versa. Therefore the results of estetrol and estriol determination in urine samples collected after the 32nd week of pregnancy were analy-

†*** = statistically significant at the significance level $P < 0{\cdot}001$.

‡The word "commensurable" is not a very proper one to be used in the connection with the measure taken here. The estetrol values were multiplied by ten in order to facilitate comparison of the two hormones and especially their trends in graphical presentation. This measure has not, of course, affected the shape of the curves.

§Partial linear regression lines were used in order to show the relationship between or make it possible to compare different periods of pregnancy in terms of the general directions or the average increase in excretion. Such comparisons are much easier with linear than with non-linear regressions.

Table 1. The mean excretion of estriol and estetrol and the amounts of estetrol as percentages of the estriol values in seven normal pregnant subjects followed through the latter half of pregnancy

Week of pregnancy	Estriol (mg/24 h)	Estetrol (mg/24 h)	Estetrol as % of estriol
24	8·4	0·45	5·3
28	12·9	0·70	5·4
33	16·1	0·91	5·6
38	26·9	1·71	6·3
40	30·7	2·50	8·5

Table 2. Equations and corresponding correlation coefficients for regression lines calculated from the results of estriol and estetrol determination. The regression lines are drawn in Figs. 2 and 3. (x = week of pregnancy expressed as values presented in Figs. 2 and 3 below abscissa, y = amount of estrogen (mg))

Line	Weeks of pregnancy	Equations for regression lines	Correlation coefficients
		Estriol	
1	12–27	$y = -1{\cdot}32 + 0{\cdot}80x$	0·86
2	21–34	$y = 0{\cdot}61 + 0{\cdot}64x$	0·52
3	31–43	$y = -31{\cdot}41 + 2{\cdot}15x$	0·63
		Estetrol	
1	12–27	$y = -0{\cdot}016 + 0{\cdot}041x$	0·72
2	21–34	$y = -0{\cdot}123 + 0{\cdot}049x$	0·53
3	31–43	$y = -3{\cdot}085 + 0{\cdot}187x$	0·70

zed by linear regression analysis. A significant correlation was found between the weekly averages of estetrol and estriol determined in individual urine samples ($t = 14{\cdot}5^{***}$). Thus there is a significant covariation in the amounts of these estrogens excreted in maternal urine. The amount of estetrol seems to be about $7 \pm 2\%$ of that of estriol.

Weights at birth of 9 female and 11 male infants, the weights of the corresponding placentas and the amounts of estriol and estetrol excreted in 24 h in maternal urine before delivery are shown in Table 3. In Figs. 4 and 5 the estrogen values are plotted against the fetal weights and it can be seen that there is a positive correlation between fetal weight and the amount of estrogen excreted. The mathematical expressions for the least square regression line for these values are: for estetrol $y = -0{\cdot}61 + 0{\cdot}86x$ (where x = the weight of the fetus in kg and y = the amount of estrogen in mg) and for estriol $y = -15{\cdot}6 + 12{\cdot}4x$. The corresponding correlation coefficients are: for estetrol $r = 0{\cdot}71$ and for estriol $r = 0{\cdot}75$. Statistically, the correlations are significant ($t_{\text{estetrol}} = 4{\cdot}277^{***}$ and $t_{\text{estriol}} = 4{\cdot}916^{***}$). Between the weights of fetus and placenta there is also a positive correlation ($r = 0{\cdot}73$). In order to compare the regression lines for estetrol and

Table 3. The birth weights and weights of placentas of twenty newborn infants and the results of estriol and estetrol determinations during the last week of the corresponding pregnancy

Sex	Weight at birth (kg)	Estriol (mg/24 h)	Estetrol (mg/24 h)	Weight of placenta (g)
	3·290	31·9	1·88	440
	3·840	32·1	3·37	525
	3·700	39·0	3·68	590
	3·330	30·0	2·30	660
Female	3·330	22·5	2·30	650
	2·950	11·2	1·73	430
	4·410	45·6	2·37	610
	3·170	16·0	1·28	630
	2·500	15·4	1·70	375
	3·520	27·7	2·26	600
	3·970	31·8	2·85	750
	4·390	26·7	3·12	700
	3·100	29·4	1·58	570
	4·050	30·8	2·72	825
Male	3·140	20·3	2·58	450
	4·250	43·5	2·78	760
	3·000	28·2	2·07	520
	2·500	15·0	1·10	485
	3·220	24·6	2·50	500
	3·460	22·3	2·86	510

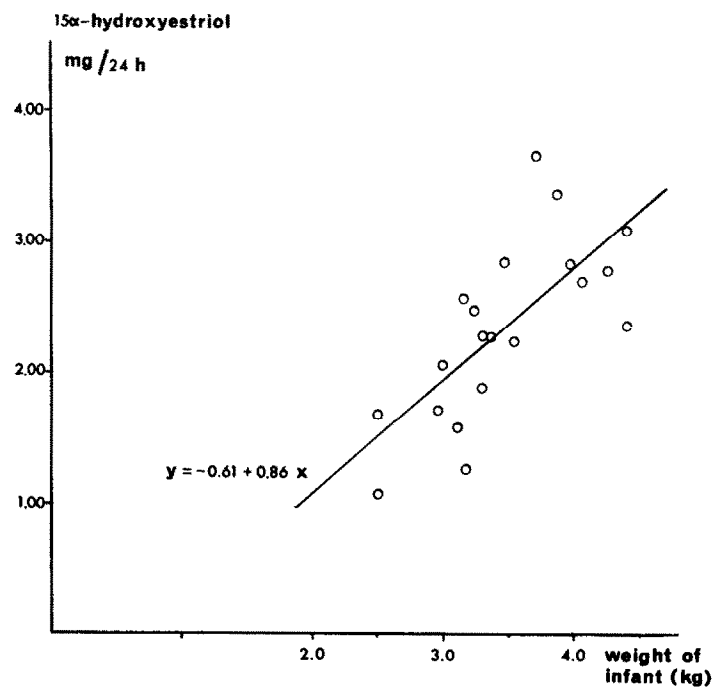

Fig. 4. Correlation between 15α-hydroxyestriol excretion and the weight of the newborn infant.

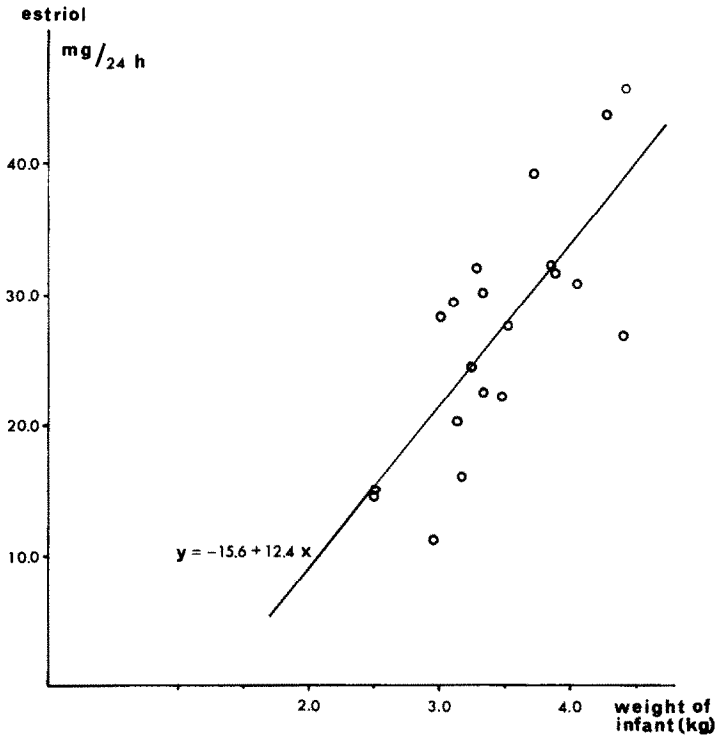

Fig. 5. Correlation between estriol excretion and the weight of the newborn infant.

estriol, the results are rendered commensurable (results of estetrol multiplied by ten). From these values it is evident that the regression coefficients differ significantly from zero ($t_{\text{estetrol}} = 4{\cdot}51^{***}$ and $t_{\text{estriol}} = 5{\cdot}64^{***}$) but no statistically significant difference can be observed between the regression lines for estetrol and estriol ($t = 1{\cdot}31$, significance level 80%).

In Table 4 are presented the coefficients of variation for the excretion of estetrol and estriol in urine in consecutive collections. The coefficients of variation are calculated from the equation of Snedecor[19]. The coefficients of variation for 24-h collections are calculated from 34 pairs and for 8-h collections from 21 pairs of consecutive urine samples. It was found that the coefficient of variation of the 8-h collection group is almost double that of the 24-h collection group for both estriol and estetrol. The difference between the variations of estetrol and estriol is not significant in either the 24-h or the 8-h collection group.

Table 4. Coefficients of variation of estetrol and estriol excretion in consecutive urine samples

	Coefficient of variation (%)	
	24-h collections	8-h collections
Estetrol	18·64	34·56
Estriol	19·40	36·49

DISCUSSION

The amount of the urinary estriol determined by gas chromatography method agrees very well with the values published before[20–23]. The mean value of the estriol excreted in 24 h exceeds 5 mg in the 21st week, 15 mg in the 31st week and is nearly 30 mg at the end of pregnancy. The 95% probability limits for variation do not differ significantly from those published before, though the data presented in this investigation are relatively meager. The amount of estetrol excreted in normal pregnancy urine in 24 h is fairly large, as has been stated in preliminary reports[14, 15, 18]; the mean parabola reaches nearly 2·1 mg at the end of pregnancy and the highest values exceed 3·5 mg in 24 h. The amount of estetrol excreted in maternal urine is thus comparable to that of 16α-hydroxyestrone and is greater than the amount of any other known estrogen, except estriol, during normal pregnancy. It is to be emphasized that the means presented are parabolic estimates and possibly would differ from those calculated from statistically sufficiently large and representative weekly samples, if such samples could have been used in the study. However, since the present sample is randomly dispersed over the last 30 weeks of pregnancy, it is possible that the real population means for different weeks of pregnancy would be close to the estimates given here.

As regards relative dispersion, the results of estetrol and estriol determination do not differ significantly from each other, as can be seen from the lines drawn in Figs. 2 and 3 to represent the variation limits (±2 S.D. = 95% probability). The differences between individuals are the major cause of the dispersion, because the methodological differences between estriol and estetrol are not great[18]. The individual variation in the excretion of estetrol seems, according to the results of this investigation, not to be smaller than that in the excretion of estriol.

When the slopes of the parabola and the regression lines of estetrol excretion are compared with those of estriol (when they have been made commensurable) it can be observed that the rate of increase of estriol is greater than that of estetrol in the first 27 weeks of pregnancy. Thereafter, the slope of estriol is a little straighter, but increases even more rapidly than that of estetrol. After the 32nd week of pregnancy the regression lines are nearly parallel. The slope that was found in the estriol excretion curve between the 24th and 32nd weeks has been reported previously by Klopper and Billewicz[20]. The present data were too limited for a mathematical analysis such as was made in the investigation of Klopper and Billewicz, but statistical treatment of the results of the present investigation confirms their results. It should be observed that the same phenomenon in the slope of the estriol excretion curve has now been demonstrated with the aid of a colorimetric method used by Klopper and Billewicz and with the GLC method used in the present investigation. Such a slope does not appear in the estetrol excretion curve and therefore the rate-limiting enzyme in the biosynthesis of estetrol is not the same as that for estriol during this time or else the relatively small amounts of estetrol synthesized during this stage of pregnancy are too little affected for the phenomenon to be detected.

Another clear phenomenon is the slower increase of estetrol excretion during the first 32 weeks of pregnancy. The more rapid increase in the excretion of estriol than in that of estetrol in the first two trimesters of pregnancy might have been due to a difference in precursors. However, the investigations of YoungLai and Solomon[12] indicate that most probably the same C-19 steroids (chiefly dehydroisoandrosterone sulfate) act as precursors for both estrogens. Therefore the

rate-limiting reaction in estetrol biosynthesis in these trimesters obviously may be the 15α-hydroxylation which would be carried out both before and after aromatization. Further, in the beginning of pregnancy a relatively large proportion of the estriol precursors are derived from the maternal compartment, while in late pregnancy these precursors mainly originate from the fetal adrenals[24]. Estetrol formation, on the other hand, seems to be of fetal origin throughout pregnancy. Therefore the excretion of estetrol in maternal urine may perhaps give a clearer reflection than estriol of the changes in fetal development and well-being in the first two trimesters of pregnancy.

The observed covariation in the amounts of estetrol and estriol in individual urine samples is easy to understand, because these steroid hormones are so nearly related and the difference in their biosynthesis is not great. This covariation will be of great importance and help in future analyses of pathologic pregnancies. If only the biosynthesis of a single estrogen is affected, the results determined from a urine sample will not be in the same proportion as in this normal material.

Comparison of the amounts of estriol excreted and the fetal weight shows a linear correlation and this correlation is also statistically highly significant. The results of this report thus agree well with those published previously[20–23]. The positive linear correlation between the estetrol in the maternal urine and the weight of the fetus is also highly significant. Because there is a fairly strong positive correlation between the weight of the fetus and that of the placenta, there is naturally also a correlation between the excreted amounts of these two estrogens and the placental weight. These correlations are very easy to understand, because the aromatization of the steroid molecule is carried out in the placenta and the 15α- and 16α-hydroxylations are carried out almost quantitatively in the liver of the fetus. Since the regression lines of estriol and estetrol plotted as functions of fetal weight do not differ significantly from each other and the coefficients of correlation are of the same order of magnitude, estetrol is no better than estriol as an indicator of the weight of the fetus in utero or of the function of the placenta in normal pregnancies.

The variation in the excretion of estriol in consecutive 24-h collections seems to be of the same order as in previous reports[1, 25], e.g. Klopper gave 17·6 as the coefficient of variation in urine samples collected in relatively stabilized conditions from hospitalized patients. In this material the coefficient of variation in 24-h collections is 19·4, but the material is collected by ambulatory volunteers, which increases the error in collection. When the collection time is 8 h, the coefficient of variation is nearly twice as great for both estrogens. So estetrol is not more suitable for shorter periods of collection than estriol is. There is no significant difference between estriol and estetrol in 24-h collections. It therefore appears that, as regards estetrol, the amount excreted in the urine of the same person on consecutive days varies almost exactly like that of estriol, and so in this respect estetrol is no better than estriol for clinical use in normal pregnancies.

Analysis of the amounts of estriol and estetrol in different pathological pregnancies will definitively show whether estetrol has any advantages over estriol for the clinician.

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