Journal of Steroid Biochemistry, 1972, Vol. 3, pp. 31–38. Pergamon Press. Printed in Great Britain

THE EXCRETION OF OESTROGEN CONJUGATES IN LATE PREGNANCY URINE

J. AHMED and A. E. KELLIE
The Courtauld Institute of Biochemistry, The Middlesex Hospital Medical School, London, W1P 5PR, England

(*Received* 2 *August* 1971)

SUMMARY

A scheme of analysis is described which permits the separation and determination of nine oestrogen conjugates in late pregnancy urine without prior hydrolysis. The oestrogen conjugates, oestrone-3-glucuronoside, 16α-hydroxyoestrone-3-glucuronoside, oestriol-3-glucuronoside, oestradiol-17β-glucuronoside, 16α-hydroxyoestrone-16α-glucuronoside, oestriol-16α-glucuronoside, epi-oestriol-16β-glucuronoside, 16α-hydroxyoestrone-3-sulphate-16α-glucuronoside, oestriol-3-sulphate-16α-glucuronoside have been measured in the urine of five pregnant women to record the scatter of normal values. They have also been measured in the urine of one woman at intervals during the last 12 weeks of pregnancy.

THE URINARY excretion of oestrogen conjugates increases substantially in late pregnancy. Unlike the C_{19} and C_{21} steroid hormones which are converted to inactive metabolites before conjugation and excretion, the principal oestrogens, oestrone, oestradiol and oestriol, are excreted in substantial amounts without modification in the conjugated form. Several different types of oestrogen conjugate have been recognised according to the type and position of conjugation, namely: (1) ring-A glucuronosides [1], (2) ring-D glucuronosides [2, 3], (3) sulphates [4], (4) the bis-conjugates, sulphate-glucuronosides [5, 6], (5) the bisconjugates, sulphate-N-acetylglucosaminides [7]. There is little excretion of free oestrogens, and the sulphate conjugates, which are important in the C_{19} steroid series, form a negligible proportion of the total oestrogen excretion [4, 8].

The rise in the excretion of oestrogens during pregnancy has been known for a considerable time [9] but measurement of the oestrogens has been achieved only after hydrolysis of all conjugated forms, a procedure which destroys the identity of individual conjugates. Previous identification of oestrogen conjugates has been on a qualitative or semi-quantitative basis and no simultaneous determination of several oestrogen conjugates has been reported. In the present work an attempt has been made to separate and measure individual oestrogen conjugates in late pregnancy urine. Methods based on gel filtration, partition and ion-exchange chromatography [10] have been used to separate and measure oestrone-3-glucuronoside, 16α-hydroxyoestrone-3(?)-glucuronoside, oestriol-3-glucuronoside, oestradiol-17β-glucuronoside, 16α-hydroxyoestrone-16α-glucuronoside, oestradiol-17β-glucuronoside, 16α-hydroxyoestrone-16α-glucuronoside, oestriol-16α-glucuronoside, 16-epi-oestriol-16β-glucuronoside, oestriol-3-sulphate-16α-glucuronoside and 16α-hydroxyoestrone-3-sulphate-16α-glucuronoside. These oestrogen conjugates have been measured in the urine of a group of pregnant women (36–40 weeks) to record the scatter of normal values and in the urine of an individual woman at intervals during the last 12 weeks of pregnancy.

METHODS

Chromatographic separation of oestrogen conjugates

The separation of the oestrogen conjugates of late pregnancy urine (150 ml) was achieved by chromatographic analysis on a variety of media as described by Smith and Kellie[10] and illustrated in Fig. 1. Practical details and evidence of

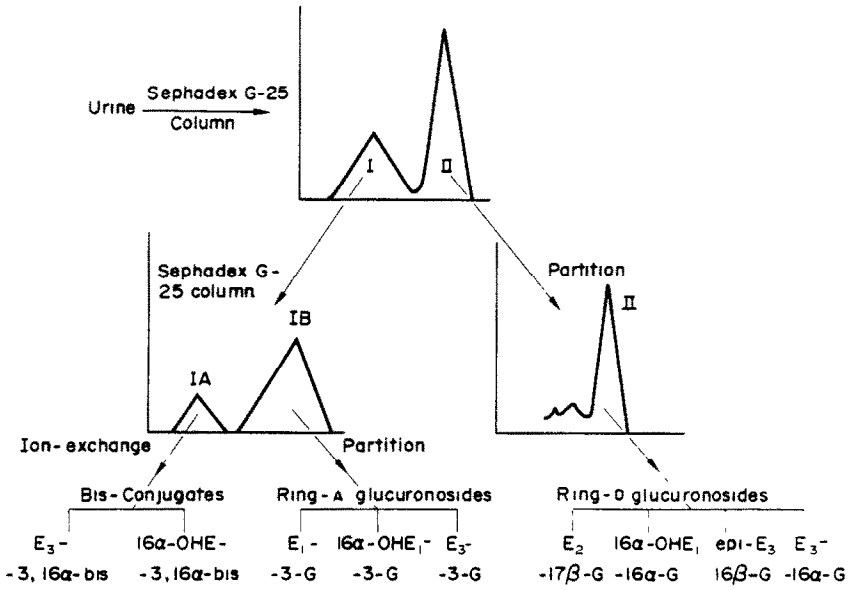

Fig. 1. Scheme of separation of oestrogen conjugates in late pregnancy urine on a variety of chromatographic media. E_1 = Oestrone, E_2 = Oestradiol, E_3 = Oestriol, 16α-OHE_1 = 16α-Hydroxyoestrone, S = Sulphate conjugate, G = Glucuronoside.

identity of individual oestrogen conjugates will be found in this source. The principal steps were:

Gel filtration of urine[1]: formation of peaks I and II. This initial separation was carried out on a column of Sephadex G-25 (superfine grade, 50 cm × 3 cm dia.) and led to the separation of peaks I and II. The latter fraction contained mainly ring-D oestrogen monoglucuronosides.

Sub-division of peak I oestrogen conjugates into sub-fractions IA and IB. Conjugates eluted in peak I in the initial separation were re-applied to the Sephadex column in a minimum volume of 1·5 M-acetate buffer pH 4·0 in the presence of 0·05 M-*p*-hydroxyphenylacetic acid and yielded two sub-fractions IA and IB. The former sub-fraction contains oestrogen bis-conjugates and the latter oestrogen ring-A monoglucuronosides.

Analysis of sub-fraction IA (bis conjugates). This separation was carried out on an ion-exchange column of Ecteola-cellulose powder (Whatman) eluting with a gradient of sodium sulphate solution and led to the separation and measurement of oestriol-3-sulphate-16α-glucuronoside and 16α-hydroxyoestrone-3-sulphate-16α-glucuronoside.

Analysis of sub-fraction IB (ring-A monoglucuronosides). Components of this sub-fraction were separated by partition chromatography on Celite in the system 2-methylpropan-2-ol-ethylene dichloride–acetic acid–water (3:7:3:7 by Vol.)

and led to the measurement of oestrone-3-glucuronoside, 16α-hydroxyoestrone-3(?16α)-glucuronoside and oestriol-3-glucuronoside. Separation of the 3- and 16α-glucuronosides of 16α-hydroxyoestrone is incomplete on the initial Sephadex gel column and traces of the latter conjugate may have been measured in this fraction.

Sub-division of peak II oestrogen conjugates (ring-D monoglucuronosides) The separation of oestrogen ring-D conjugates eluted in peak II from the initial Sephadex column separation was also achieved by partition chromatography on Celite in the system 2-methylpropan-2-ol–ethylene dichloride–acetic acid–water (15:85:30:70 by Vol.). The following oestrogen conjugates were separated and measured: oestradiol-17β-glucuronoside, 16α-hydroxyoestrone-16α-glucuronoside, oestriol-16α-glucuronoside and 16-epi-oestriol-16β-glucuronoside. The oestradiol-17β-glucuronoside was a minor component and could only be determined in some urines. Oestriol-16α-glucuronoside was by far the largest single conjugate component of this fraction and of late pregnancy urine.

Measurement of oestrogen conjugates

In each chromatographic separation a small portion of serial fractions was removed and the oestrogen content was determined by the Kober colorimetric method[11]. No preliminary hydrolysis of the oestrogen conjugates was necessary and the colours given were indistinguishable from those of the corresponding free oestrogen standards. It has been established[12] that the oestrogen glucuronosides may be treated in this way and presumably the hydrolysis occurs during the preliminary heating with the Kober reagent. The oestriol Kober reagent (76%H_2SO_4) was used throughout.

RESULTS

Analysis of oestrogen conjugates in late pregnancy urines (5)

The scheme of analysis described above has been applied to late pregnancy urine from five different subjects and the concentrations of individual oestrogen conjugates, expressed as mg/24 h, are given in Table 1. The overall recovery of oestrogen conjugates by summation ranged from 62–79% of the total oestrogen excretion as determined by Kober reaction in peaks I and II. Sulphate conjugates which are eluted in peak II have not been separated and measured. The figures in parentheses (Table 1) are expressed as a percentage of the total excretion.

In the urines examined, the major portion of the excreted conjugates (55·7–70·0 70·0%) were found in peak II; the ring-D glucuronosides, and the major component of this fraction, oestriol-16α-glucuronoside, represented 36·7–43·2% of the daily output. The other components recognised in peak II, all of which were ring-D glucuronosides, were by comparison minor conjugates, no single compound exceeding 2% of the daily excretion.

Less than half (29·8–44·2%) of the urinary conjugates were excreted in Peak I which embraces the bis-conjugates and ring-A glucuronosides. Within the sub-fraction IA (the bis-conjugates) oestriol-3-sulphate-16α-glucuronoside was the greater component but in no case did it exceed 8% of the total urinary excretion. The two bis-conjugates identified in this sub-fraction, although minor components, were found in all the urines examined.

The three conjugates identified in sub-fraction IB are ring-A glucuronosides conjugated at the phenolic hydroxyl group at C-3. Oestrone and oestriol-3-glucuronosides were positively identified in this fraction and 16α-hydroxy-

Table 1. Excretion of oestrogen conjugates in late pregnancy urines (mg/24 h)

Patient Ref.	Weeks of pregnancy	Urine vol. ml	Bis-conjugates 16α-OHE$_1$-3-S-16α-G mg	Bis-conjugates E$_3$-3-S '16α-G mg	Ring-A glucuronosides E$_1$-3-G mg	Ring-A glucuronosides 16α-OHE$_1$-3-G mg	Ring-A glucuronosides E$_3$-3-G mg	Ring-D glucuronosides E$_2$-17β-G mg	Ring-D glucuronosides 16-epiE$_3$-16α-G mg	Ring-D glucuronosides 16α-OHE$_1$-16α-G mg	Ring-D glucuronosides E$_3$-16α-G mg	Total mg
ES	36	1470	0·35(1·1)	1·72(5·4)	1·64(5·2)	0·77(2·5)	2·20(7·0)	0·27	0·29	0·29	12·2(38·5)	19·72
EL	36	750	1·26(2·8)	3·53(5·3)	1·04(2·3)	2·34(5·3)	7·19(16·2)	—	0·12	0·81	18·5(41·6)	34·79
AJS	37	1080	0·64(1·4)	2·55(5·8)	1·77(4·0)	1·79(4·1)	5·31(12·1)	0·50	0·60	0·41	19·0(43·2)	32·57
MBM	39	2114	0·46(1·5)	1·88(5·3)	0·53(1·6)	1·00(2·8)	2·42(6·8)	←———	Fraction lost	———→		35·60
WO	40	1480	0·50(0·9)	1·63(8·0)	0·13(4·0)	3·21(6·1)	7·20(13·6)	—	0·14	0·37	19·4(36·7)	35·58

Figures in parentheses represent per cent of total excretion.
E$_1$ = Oestrone, E$_2$ = Oestradiol, E$_3$ = Oestriol, 16α-OHE$_1$ = 16α-Hydroxyoestrone, S = Sulphate conjugate, G = Glucuronoside.
"–" = Not detected by Kober.

Table 2. Changes in the excretion of oestrogen conjugates during late pregnancy (mg/24 h)

Week of pregnancy	Urine vol. (ml)	Bis-conjugates 16α-OHE$_1$-3-S-16α-G (mg)	Bis-conjugates E$_3$-3-S-16α-G (mg)	Ring-A glucuronosides E$_1$-3-G (mg)	Ring-A glucuronosides 16α-OHE$_1$-3-G (mg)	Ring-A glucuronosides E$_3$-3-G (mg)	Ring-D glucuronosides 16-epiE$_3$-16α-G (mg)	Ring-D glucuronosides 16α-OHE$_1$-16α-G (mg)	Ring-D glucuronosides E$_3$-16α-G (mg)	Total (mg)
28	1700			1·18(6·0)	1·27(6·4)	2·90(14·7)	0·12	0·30	6·70(33·9)	12·47
32	2180	0·21(0·8)	0·46(1·8)	1·76(6·9)	1·65(6·5)	3·75(14·7)	0·14	0·28	8·90(34·9)	17·15
34	1600	0·33(1·2)	0·55(2·0)	1·33(4·8)	1·66(6·0)	3·17(11·4)	0·20	0·44	13·0(47·0)	20·68
37	2310	0·24(0·6)	0·72(1·8)	1·05(2·7)	2·47(3·3)	3·65(9·4)	0·16	0·36	15·8(40·3)	24·45
40	1480	0·50(0·9)	1·63(3·1)	2·13(4·0)	3·21(6·1)	7·20(13·6)	0·14	0·37	19·4(36·7)	34·58

Figures in parentheses represent per cent of total excretion.
E$_1$ = Oestrone, E$_2$ = Oestradiol, E$_3$ = Oestriol, 16α-OHE$_1$ = 16α-Hydroxyoestrone, S = Sulphate conjugate, G = Glucuronoside.

oestrone-3-glucuronoside when added to urine was eluted in this subfraction between the named glucuronosides. Synthetic 16α-hydroxyoestrone-16α-glucuronoside is eluted mainly in peak II but there is some overlap into sub-fraction IB and this compound may have contributed to the component measured as 16α-hydroxyoestrone-3-glucuronoside. Together, ring-A monoglucuronosides represent a significant part of the total urinary oestrogen conjugates (20·2–31·8%) and the major component of this, as of other fractions, was the conjugate of oestriol (6·8–16·2%).

Technically the oestrogen conjugates in peak II are the most difficult to separate because of the great preponderance of oestriol-16α-glucuronoside. The other components, oestradiol-17β-glucuronoside, 16α-hydroxyoestrone-16α-glucuronoside and epi-oestriol-16β-glucuronoside, are minor metabolites. Although oestradiol-17β-glucuronoside is probably present in all late pregnancy urines, it was only possible to determine the amount in two of the urines examined.

The pattern of excretion of oestrogen conjugates with advancing pregnancy

The object of this study was to observe any progressive change in the pattern of excretion of oestrogens during the latter stages of pregnancy. Urine samples representing complete daily excretion were received and analysed at 28, 32, 34, 37 and 40 weeks gestation; the last sample was obtained within a few days of a normal delivery (male child: 4 kg at birth). The amounts of the individual oestrogen conjugates excreted at these times are given in Table 2; as previously, the values given in parentheses are expressed as a percentage of the total oestrogen conjugate excretion.

DISCUSSION

The pattern of excretion of oestrogen conjugates in the urines from five subjects examined was similar, yet even in this limited number there was a wide range of variation of individual conjugate forms. All of the pregnancies went normally to full term and it is not known whether the pattern of excretion is grossly disturbed in abnormal circumstances. The complete analysis of individual urine samples is tedious and time-consuming and the method cannot be recommended as a practical routine procedure; nevertheless, there is a recognisable pattern of normal excretion and this may justify a further study of excretion in abnormal pregnancy, e.g. in toxaemia of pregnancy, diabetic pregnancy, etc.

It is clear from the analysis of consecutive urines from a pregnant subject (Fig. 2) that there is no dramatic change in the pattern of excretion of oestrogen conjugates with advancing pregnancy. The ratio peak II/peak I conjugates shows a slow but progressive rise to the 37th week indicating selective conjugation as ring-D conjugates, and as might be expected the same tendency is apparent in the ratio oestriol-16α-glucuronoside/oestriol-3-glucuronoside (Table 3). This follows from the fact that these oestriol conjugates are the major components of peak II and sub-fraction IB respectively. Tikkanen and Adlercreutz[13] have achieved the separation of different types of oestriol conjugates in pregnancy urine. The quantitative relationship of the main oestriol conjugates, recently established by Tikkanen[14], showed that oestriol-16α-glucuronoside was the conjugate present in the largest proportion followed by oestriol-3-glucuronoside in late pregnancy urines. Goebelsman *et al.*[15] maintain that the latter ratio rises with advancing pregnancy although this is disputed by Hähnel[4]. In the present study the ratio

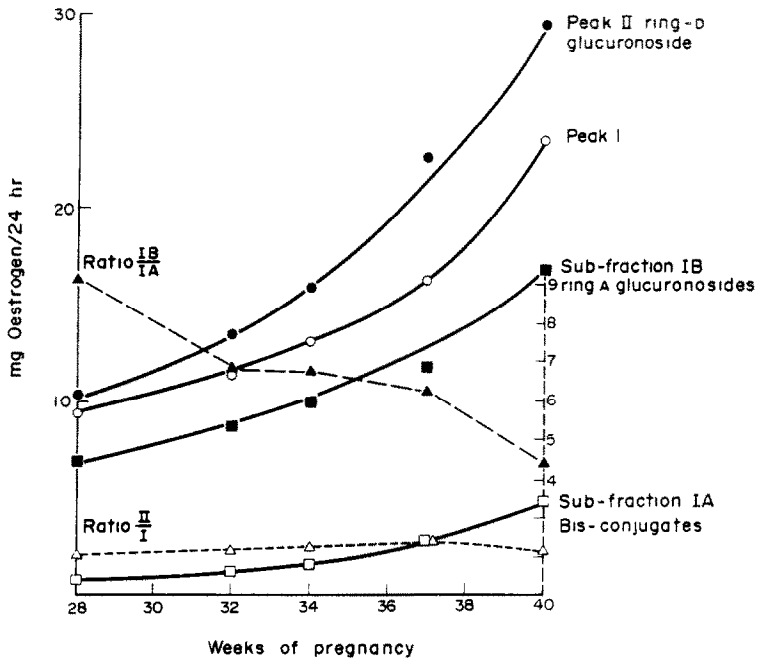

Fig. 2. Excretion of oestrogen conjugates with advancing pregnancy. Peak II – total Ring-D glucuronosoides; Peak IA – total bisconjugates; Peak IB – total Ring-A glucurosides; Peak I – sum of Peaks IA and IB. The origin of these fractions is shown in Fig. 1.

Table 3. Relative changes in forms of conjugation with advancing pregnancy

Weeks of pregnancy	Ratio Pk II/Pk I	Ratio IA/IB	Ratios of oestrogen conjugates C-16/C-3 gluc.	C-3 gluc./bis-conjugates
28	1·11	8·2	2·31	
32	1·14	6·0	2·37	8·2
34	1·31	5·8	4·10	5·8
37	1·43	5·4	4·33	5·1
40	1·25	3·5	2·70	4·3
Comment	Rise to 37th wk.	Continuous fall	Rise to 37th wk.	Continuous fall

Peak I C-3 plus bis-conjugates (IA–IB); *Peak II* Ring-D conjugates; *Peak IA* bis-conjugates; *Peak IB* Ring-A conjugates.

increased until the 37th week but had fallen significantly by the 40th week. The reason for the latter change is uncertain but may have been associated with the preparation for delivery which occurred within a short interval of the receipt of the last urine sample.

A progressive fall was also observed in the ratio IB/IA which indicated that in spite of a steady increase in the excretion of C-3 glucuronosides throughout the period of study, the bis-conjugates (3-sulphate-16α-glucuronosides) represented an increasing proportion of peak I conjugates. This tendency is also apparent in the changing ratio oestriol-3-glucuronoside/oestriol-3-sulphate-16α-glucurono-

side (Table 3). Other ratios which have been calculated did not reveal any consistent change throughout the period of study.

Oestrone was recognised in the urine samples only as the C-3 glucuronoside although trace amounts of oestrone-3-sulphate are excreted[8, 16]. At full term the glucuronoside represented 13·6% (7·2 mg) of the total oestrogen excretion and this must be considered as the major urinary conjugate of oestrone. Oestradiol, which has the highest biological activity, and which is a major component of late pregnancy plasma[17], could only be identified as oestradiol-17β-glucuronoside in some of the urines. Oestriol, and 16α-hydroxyoestrone, are compounds formed by the placenta from precursors of foetal origin and they are the major products of the foeto-placental unit. Both compounds were excreted in three conjugated forms, as the C-3- and C-16α-glucuronosides and as the bis-conjugates. Oestriol is excreted mainly as the 16α-glucuronoside whereas the principal conjugate of the ring-D α-ketol is the C-3 glucuronoside.

In the process of identification of the separated conjugates and their parent steroids by paper and thin layer chromatography[10] no evidence of any other important oestrogen metabolites such as 2-hydroxyoestrogens and 2-methoxyoestrogens[18] was obtained. Moreover, in the Kober determinations of the purified conjugates no interference was observed. It can be concluded that the C2 substituted oestrogens, which give a red–violet colour with the Kober reagent[19] were absent and they may have been eluted earlier in the initial separation by gel filtration on Sephadex G-25.

Although the oestrogens of pregnancy are formed mainly by the foeto-placental unit they do not appear to be released in the conjugate form. In spite of the important role of steroid sulphates in the biogenesis of oestrogens, within the unit only trace amounts are excreted and there is some experimental evidence that oestrogen sulphates produced within the foeto-placental unit are hydrolysed before entering the maternal circulation[20]. In contrast, there is little evidence of oestrogen-glucuronoside formation within the unit[21], yet over 95% of human excretory oestrogens are excreted in this form.

It seems probable that the oestrogens produced in the foeto-placental unit are conjugated elsewhere. Oestriol, the major product, can be conjugated as the 16α-glucuronoside in the human liver and as the 3α- and 16α-glucuronosides in the human small intestine[22]. The conjugation of 16α-hydroxyoestrone with glucuronic acid probably occurs at the same sites. The formation of the bis-conjugates of these oestrogens involves passage through the liver[23]. The results suggest that there is no great change in the pattern of oestrogen excretion in late normal pregnancy and the organs principally involved, namely the liver and intestines, are able to deal with the vast increase in endogenous production.

ACKNOWLEDGEMENTS

The authors acknowledge the interest and encouragement of Professor R. H. S. Thompson, D.M., D.Sc., F.R.C.P., the Director of the Courtauld Institute of Biochemistry. One of us, J. Ahmed, M.B., B.S., M.Sc., acknowledges financial support from the Government of East Pakistan and is indebted to the British Council for a Student Award Grant.

REFERENCES

1. C. G. Beling: *Acta. endocr. (Kbh)* **43** (1963) Suppl. 79.
2. M. Neeman and Y. Hashimoto: *Tet. Lett.* **5** (1961) 183.
3. M. Neeman and Y. Hashimoto: *J. Am. chem. Soc.* **84** (1962) 2972.

4. R. Hähnel: *J. Endocr.* **38** (1967) 555.
5. R. F. Straw, P. A. Katzman and E. A. Doisy: *Endocrinology* **57** (1955) 87.
6. J. C. Touchstone, J. W. Greene, R. C. McElroy and T. Murawec: *Biochemistry* **2** (1963) 653.
7. H. Jirku and M. Levitz: *J. clin. Endocr.* **29** (1969) 615.
8. J. McKenna, E. Menini and J. K. Norymberski: *Biochem. J.* **79** (1961) 11P.
9. J. B. Brown: *J. Obstet. Gynaec. Br. Commonw.* **66** (1954) 795.
10. E. R. Smith and A. E. Kellie: *Biochem. J.* **104** (1967) 83.
11. W. Nocke: *Biochem. J.* **78** (1961) 593.
12. H. A. F. Blair and A. E. Kellie: *Biochem. J.* **107** (1968) 655.
13. M. J. Tikkanen and H. Adlercreutz: *Acta Chem. Scand.* **24** (1970) 3755.
14. M. J. Tikkanen: *Acta endocr. (Kbh) Suppl.* **155** (1971) 123.
15. U. Goebelsmann, I. Cooke, N. Wiqvist and E. Diczfalusy: *Acta endocr. (Kbh)* **52** (1966) 30.
16. R. Hähnel and M. G. A. Rahman: *Biochem. J.* **105** (1967) 1047.
17. J. Ahmed: Ph.D. Thesis, Univ. of London. *Free and Conjugated Oestrogens in Human Late Pregnancy* (1971).
18. H. Breuer: *Vitam. Horm.* **20** (1962) 285.
19. H. Breuer: *Hoppe-Seyler's Z. physiol. Chem.* **319** (1960) 136.
20. J. Sjövall, K. Sjövall and R. Vihko: *Steroids* **11** (1968) 703.
21. E. Diczfalusy, C. Franksson and B. Martinsen: *Acta endocr. (Kbh)* **38** (1961) 59.
22. H. Breuer and R. Knuppen. *Methods in Enzymology*, (Edited by R. B. Clayton). Academic Press, London (1969) p. 691.
23. M. Levitz and J. Katz: *J. clin. Endocr.* **28** (1968) 862.