Journal of Steroid Biochemistry, 1975, Vol. 6, pp. 1477–1481. Pergamon Press. Printed in Great Britain

ENTEROHEPATIC CYCLING OF ^{3}H-ESTRONE IN THE BULL: IDENTIFICATION OF ESTRONE-3-GLUCURONIDE*

BENJAMIN S. LEUNG,† J. RICHARD PEARSON and ROBERT P. MARTIN
The Department of Biochemistry, Colorado State University, Fort Collins, Colorado 80521, U.S.A.

(*Received* 18 *March* 1975)

SUMMARY

Following surgical preparation of a bull with indwelling catheters in the gallbladder and duodenum, the animal was injected with radio-estrone. Bile was collected. Subsequently, this bile containing radioactive metabolites, all of which appeared to be conjugated with glucuronic acid, was infused into the duodenum. In another similar experiment, pure radio-esterone was added to otherwise non-radioactive bile and infused into the duodenum. Urine and newly formed bile was collected during and following the two infusions. In each case the bile collected post-absorption was radioactive, and it contained only conjugated metabolites. These were distributed into three peaks of radioactivity which appeared at about 30, 180 and 330 min after infusion. Analysis of the bile collected post-absorption revealed that there were two major metabolites, estrone and 17α-estradiol. Biliary metabolites were purified and separated by column and paper chromatography. Using color reactions, chromatography, enzymatic hydrolysis, formation of derivatives, crystallization to constant S.A. and U.V. and I.R. spectra, estrone conjugates was identified as estrone-3-glucuronide. 17α-estradiol was in the form of a mono-glucuronide and a diglucuronide similar to biliary metabolites of 17β-estradiol. The ratio of radioactivity excreted in bile to that excreted in urine was 2:5 after the infusion of the conjugated metabolites of estrone and 1·2:1 after the infusion of pure estrone. It is clear that in this animal enterohepatic cycling of estrogens took place, but the physiological role and the biochemical components to the process remain to be elucidated.

INTRODUCTION

Rakoff *et al.*[1] found that after i.v. injection of 17β-estradiol in dogs, excretion of estrogen in bile decreased after an initial peak and then rose again after 72 h. They explained this phenomenon on the basis of an enterohepatic cycling of estrogen. The same authors also showed that after instillation of 17β-estradiol into an isolated loop of jejunum, free estrogen was present in blood draining that segment of intestine [2]. Martin *et al.*[3] have found that following injection of [^{14}C]-testosterone in dogs, most of the radioactivity appeared in the bile. When the collected bile was infused into the duodenum several days later, radioactivity appeared in portal vein blood and in newly formed bile. Since the bovine utilizes mainly the biliary route for excretion of the metabolites of testosterone [4] and of 17β-estradiol [5], a study of the enterohepatic cycling of estrogens in the bull was undertaken. Biliary metabolites of 17β-estradiol was found to consist of three major fractions identified as 17α-estradiol-3-β-D-glucuronide, estrone-3-β-D-glucuronide and estradiol-3-17α-β-D-diglucuronide [6]. We present here our findings following an injection of ^{3}H-estrone and subsequent infusion of the radio-metabolites in the duodenum.

MATERIALS AND METHODS

Solvents were of reagent grade and redistilled before use for extraction and chromatography. [16-^{14}C]-Estrone was purchased from New England Nuclear Corporation, Boston; [6^{3}H]-estrone and [6-^{14}C-D-glucuronic acid potassium salt was obtained from the Radio-Chemical Center, Amersham, England. Each radioactive compound was ascertained by chromatography to be of acceptable quality. Amberlite, XAD-2, was obtained from Rohm & Haas, Philadelphia, Penn; alumina (Woelm neutral, activity I) from Alupharm Chemicals, New Orleans, LA and Sephadex G-10 from Pharmacia Fine Chem., Inc., Piscataway, NJ.

Catheters were surgically placed in the fundus of the gall bladder and in the lumen of the duodenum 2 cm. below the sphincter of Oddi [7] in an Angus × Guernsey bull (4 years; 725 kg). These catheters were kept patent by daily flushing with 10% sodium bicarbonate solution. Twelve days after surgery, [16-^{14}C]-estrone (43·4 μCi, 1·13 mg), dissolved in 6 ml ethanol–propylene glycol–water (1:1:1 by vol.), was injected into the left jugular vein. Bile was collected continuously in 20 ml portions with polyethane vials

* Estrone-3-Glucuronide: 17-oxoestra-1,3,5(10)-triene-3-yl-β-D-glucopyranosiduronic acid.

† Present address: University of Oregon Health Sciences Center, Department of Surgery, Portland, Oregon 97201, U.S.A.

during and after the injection. After 50 and 100 μl aliquots were taken for counting, the rest of the collection was frozen at -15°C and used five days later for the second infusion as described below. Urine was collected as voided; 250 μl sample of each collection was counted. Radioactivity was determined as described previously [5].

The first duodenal infusion. Freshly collected bile was found to be devoid of radioactivity three days after the original injection of [^{14}C]-estrone. Subsequently, a 1 ml ethanolic solution of [6-^{3}H]-estrone (1·45 mCi; 1·5 mg), mixed with 20 ml of non-radioactive bile, was infused through the duodenal catheter. Bile and urine were collected during and after the 2-min period of infusion.

The second duodenal infusion. A portion (46 ml) of the bile collected after the original injection of [^{14}C]-estrone was diluted with 15 ml of water. Two days after the first duodenal infusion, when radioactivity was no longer detected in freshly collected bile, this mixture which contained 1·2 μCi of [^{14}C]-estrone metabolites was infused into the duodenum. Bile and urine were collected.

After each infusion the amount of radioactivity excreted in each sample of bile and urine was determined. The urine was not extracted. The bile was extracted at pH 9 with ether, and hydrolysis of the residue conjugated metabolites with β-glucuronidase was conducted as previously reported [5]. The free estrogens were identified by their paper chromatographic mobilities relative to authentic standards, color reactions, crystallization to constant S.A. with authentic standards, and crystallization of a derivative to constant S.A. [5].

Purification and separation of conjugated metabolites. A procedure described by Bradlow[8] for urinary estrogen conjugates was applied for extraction in good yield of steroid conjugates in the bile. Amberlite, XAD-2, was packed in a 2·5 cm. × 100 cm. glass column and was washed in succession with 2 l. of dionized water, 1 l. of methanol and 2 l. of deionized water before use. A portion of bile (150 ml) was diluted with an equal vol. of water and applied to the column. Free steroids and certain substances were removed by washing the column with 200 ml water, and the steroid conjugates were eluted with methanol.

The crude fraction from the Amberlite column was purified on a dry column of 300–500 g alumina, deactivated with 9–10% water. Gradient elution with increasing water content in ethanol removed colored material and viscous contaminants from the bile. Estrogen conjugates were eluted with 0·5 M ammonium hydroxide solution and the ammoniacal eluate was then passed through an Amberlite XAD-2 column. The methanol eluate containing the estrogen conjugates was concentrated to a small volume by flash evaporator.

Separation of estrone conjugates from other metabolites was achieved on 6-in. wide Whatman No. 3 papers after running for 20 h in system 1. Estrogen conjugates were eluted from the chromatogram by refluxing cut strips of the paper in a soxhlet extractor with methanol water (9:1 v/v) for about 6 h. The estrone conjugate once separated from the metabolites was further purified by thin-layer chromatography, once in solvent system *b* and twice in solvent system *a*. Crystallization and identification were then attempted.

Identification of the carbohydrate moiety. After hydrolysis of the estrogen conjugates with dilute hydrochloric acid in a sealed tube [9], the carbohydrate moiety was identified by color reactions and by paper chromatography in solvent system 2. Attempts to quantify glucuronic acid from a solution of unhydrolyzed estrone-conjugate by the carbazole reaction [10] were unsuccessful due to the interference of the estrogen peak at about 500 nm. Thus, the following procedure was adopted.

A known amount ($1·06 \times 10^{6}$ d.p.m./24 μg) of authentic-[6-^{14}C]-D-glucuronic acid-potassium salt was added as a tracer to a known amount of estrogen conjugate (0·3–1·0 mg, based on radioactivity of injected or infused estrone). The mixture was incubated with 5000 units of β-glucuronidase (Ketodase) in an acetate buffer (pH 4·5) for 76 h at 37°C. Under similar conditions, control samples gave 96% hydrolysis. After incubation, all of the hydrolysate was transferred to a 1·5 × 85 cm. column of Sephadex G-10 and eluted with 60–70 ml of deionized water at about 3 ml per 20 min. The radioactive eluate was dried down and chromatographed on No. 3 Whatman paper in solvent system 2. The radioactive glucuronic acid area was eluted and made up to a known volume in water. After development of color in the carbazole reaction [10], the samples and standards were quantitated at 530 nm in a Beckman DK-2A spectrophotometer. From the radioactivity of estrone-conjugate and results of the carbazole reaction, the stoichiometric relation between estrone and glucuronic acid can be determined.

RESULTS

Figure 1 represents the excretion of radioactivity in bile after i.v. injection of [^{14}C]-estrone. The

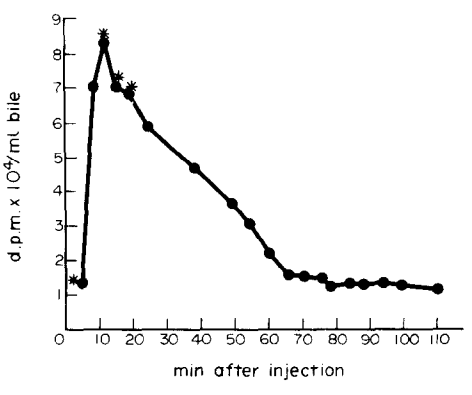

Fig. 1. Levels of radioactivity in bile of a bull after i.v. injection of 43·4 μCi of [16-^{14}C]-estrone. The asterisks indicate bile samples which were used for duodenal infusion.

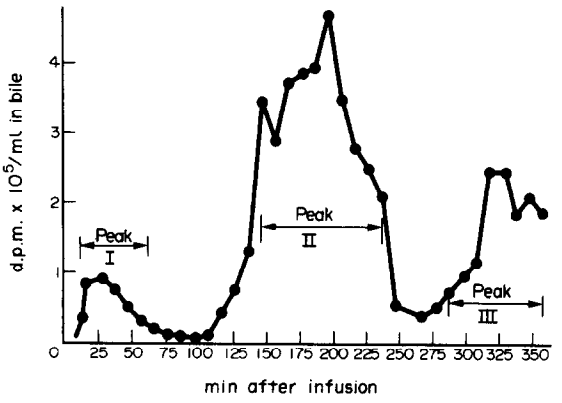

Fig. 2. Levels of radioactivity in bile of a bull after duodenal infusion of 1·45 mCi of [^{3}H]-estrone.

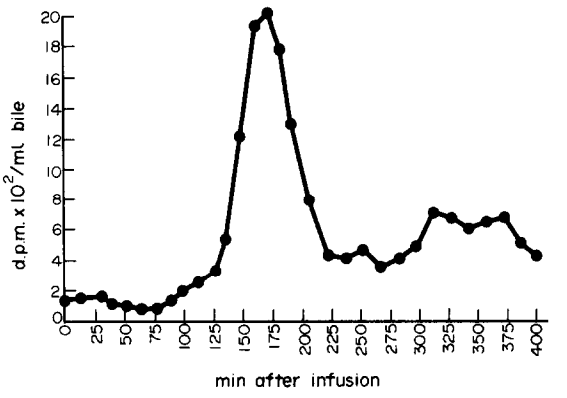

Fig. 3. Radioactivity in bile of a bull after duodenal infusion of 1·2 μCi of biliary metabolites of injected [16-^{14}C]-estrone.

radioactive metabolites in bile were found to appear in the conjugated fraction since less than 0·1% was extracted with ether or eluted from the amberlite column in the free steroid fraction.

A curve representing the excretion of radioactivity in bile after duodonal infusion of [^{3}H]-estrone is shown in Fig. 2. To facilitate the analysis and identification of metabolites, the several bile samples were combined according to the peak of radioactivity with which they had been associated as indicated in Fig. 2. The results of the analysis are shown in Table 1.

As seen in Fig. 3, the curve representing the excretion of radioactivity in the bile collected after duodenal infusion of radioactive metabolites of [^{14}C]-estrone is similar to the one shown in Fig. 2. However, only the bile collected between 127–222 min (Peak II) contained enough radioactivity to permit identification of metabolites. Results of this analysis are shown in Table 2.

Radio-estrone and radio-17α-estradiol were identified by chromatographic behavior, by crystallization with carrier from different solvents and by derivative formation as described earlier [5]. Some radioactive materials isolated from hydrolyzed bile were not identified.

Table 1. Composition of bile obtained from a bull after duodenal infusion of 1·45 mCi of [^{3}H]-estrone

Peak number (See Fig. 2)	I	II	III
Time after infusion (minutes)	13–63	148–238	288–388
Bile collected (ml)	224	223	160
Radioactivity extracted (μc)‡	10·6	46·2	14·2
Estrone (%)	32	29	14
[17α]-Estradiol (%)	0	69	63
Unknown II (%)	38	0	0
Unknown III (%)	3	0	0
Unknown IV (%)	8	0	0
Unknown V (%)	4	0	0
Polar material (%)	15	1	9

‡ The radioactivity was extracted with ether after treatment of the diluted bile with β-glucuronidase.

Estrogens were separated from ether extract by paper on or by column chromatography. The percentages were calculated on the basis of the recovery from the bile extract. (Detail of method see ref. 5).

Characterization of estrone-3-glucuronide. After partial purification by column chromatography with Amberlite XAD-2 and alumina, separation of estrone glucuronide from other metabolites by paper chromatography (System 1) was achieved. The estrone conjugate was identified as follows: This conjugate was easily hydrolyzed with β-glucuronidase to yield a steroid moiety, the mobility of which was identical with that of authentic estrone in several paper chromatography systems (Systems 3–6 Table 3). The steroid moiety gave a Turnbull-blue color with ferric chloride–potassium ferricyanide reagent [11] and a violet color with Zimmermann reagent [12]. The estrone conjugate itself was negative to ferric chloride-potassium ferricyanide reagent and positive with Zimmermann reagent (violet spot). Further identification of estrone was provided by formation of an acetate derivative and by crystallization with authentic estrone and estrone acetate to constant S.A. respectively. The results were similar to those reported previously [5]. In addition, an oxime derivative was made [13] and crystallized from ethyl acetate to constant S.A. That estrone and its oxime derivative were in good crystalline form is indicated by their melting points which were 256–258 C corr. and 232–234 corr., respectively; each agrees well with the literature [14]. After 76 hr of hydrolysis with β-glucuronidase (0·2 mg of conjugate/5000 units) over 96% of the isolated estrone conjugate was converted to estrone. The hydrolysis of a duplicate sample was inhibited by 5·4 mg

Table 2. Composition of bile obtained from a bull after duodenal infusion of 1·2 μCi of biliary metabolites of injected [16-^{14}C]-estrone

Time and infusion (minutes) (See Fig. 3)	127–222
Bile collected (ml)	142
Radioactivity extracted (dpm)‡	161,000
Conjugated	99·9%
Estrone*	16%
17α-Estradiol	83%
Polar material	0%
Non-polar material	1%

‡ The radioactivity was extracted with ether after treatment of the diluted bile with β-glucuronidase.

* The percentages of estrogens and unkonwns are calculated on the basis of recovery from the bile extract.

Table 3. Chromatography systems for estrogens (by vol.)

A. Systems used for paper chromatography
For estrogen conjugates
1. Isopropyl ether:n-butanol (1:3) vs. ammonium hydroxide:water (1:4)
For carbohydrates
2. n-Butanol:ethyl acetate:acetate:acetic acid water (3:3:2:2)
For free estrogens
3. Benzene vs. 55% methanol in water
4. Isooctane:toluene (1:3) vs. water:methanol (1:4)
5. Isooctane:toluene (2:1) vs. water:methanol (1:4)
6. Skelly-Solve H vs. methanol:water (85:15)
B. Systems used for thin-layer chromatography
For estrogen conjugates
a. n-Butanol:t-butanol:water:acetic acid (150:50:100:20)
b. sec-Butanol saturated with deionized water
For free estrogens
c. Benzene:ethanol (95:5)
d. Ethyl acetate:cyclohexane:ethanol (45:45:10)

of saccharo-1,4-lactone (Cal Biochem, California) to give only 14·5% hydrolysis. Adsorption maximum of estrone conjugate was at 275 nm with a shoulder 283 nm while extrone adsorbs maximally at 281 nm with a shoulder at 287 nm. These results suggest that the estrone is conjugated at the phenolic ring.

After hydrolysis of this conjugate with dilute acid in a sealed tube [9], the carbohydrate moiety was chromatographed in System 2. When sprayed with benzidine–periodate [15], the only spots developed were those that ran identically with D-glucuronic acid standard and its lactone. Subsequent to hydrolysis by B-glucuronidase and removal of the protein by a Sephadex G-10 column, the glucuronic acid was quantitatively estimated by the carbazole method to be in a ratio with the estrone moiety of 0·98 to 1·0.

The estrone conjugate was further purified by converting it to a barium salt and then reconverting it to the free acid [16]. The acidic solution was then passed through an Amberlite column and eluted as described. The methanol eluate was further purified by thin-layer chromatography in Systems *a* and *b*. Attempts to crystallize the conjugate in various solvents or mixtures of solvents were unsuccessful. At this stage, an I.R. spectrum in a KBr pellet was obtained with a Perkin–Elmer 457 I.R. spectrophotometer, Fig. 4.

DISCUSSION

Enterohepatic circulation. Following a single i.v. injection estrone, 99·9% of the biliary metabolites appeared as glucuronides of 17α-estradiol, estrone and certain unidentified minor conjugated metabolites. When either free [^{3}H]-estrone or conjugated metabolites of [^{14}C]-estrone were infused into the duodenum, the curves representing the excretion of radioactivity in bile and the composition thereof were similar (Figs. 2 and 3) and demonstrating the exclusive presence of glucuronides. These experiments give direct evidence that estrogens, in the form of glucuronides, participate in the enterohepatic cycling in the bull. The physiological significance of this process, however, remains obscure.

The structure of the compounds involved in the actual absorption process in the intestine is not known. Certain possibilities can be considered. First, direct absorption of free estrone may occur at a certain section of the intestine. This possibility is suggested by the biliary excretion patterns from the infusions of [^{3}H]-estrone and conjugated metabolites of [^{14}C]-estrone. A small peak of radioactivity was present (Peak I, Fig. 2) from the infusion experiment of [^{3}H]-estrone but was absent in the infusion of conjugated metabolites. It appears that soon after the entry of free steroids into the intestinal wall, most of them are converted to conjugated forms either prior to or after metabolism of the steroid moiety. Secondly, both free and conjugated estrogens can enter the intestinal wall in their original forms, followed by subsequent metabolism, including the formation of conjugated metabolites. It is known that estrogens can be converted to glucuronides by the intestinal wall both in human and in experimental animals [18–21]. Obviously, our results need to be enlarged by experiments *in vitro* from incubation of these isolated glucuronides with different sections of gut.

It is clear from various chromatography, color reactions, derivatives of the aglycones, and stoichometry of the steroid moiety and carbohydrate moiety,

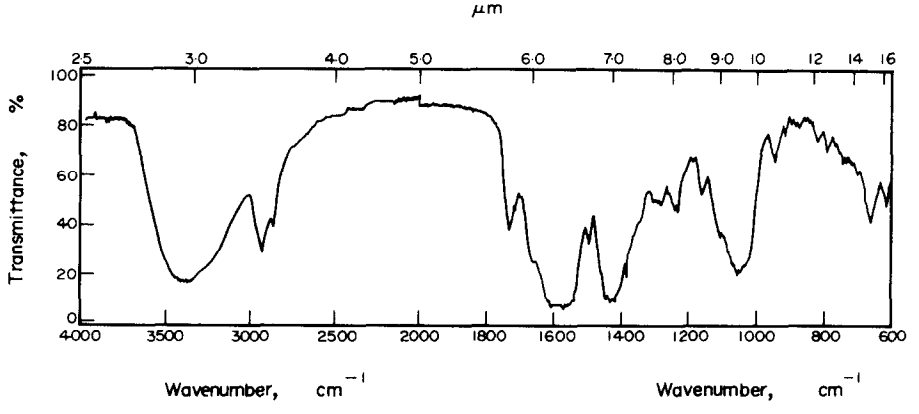

Fig. 4. Infrared spectrum of [^{3}H]-estrone-3-glucuronide (in K Br) isolated from bile of a bull injected with [^{3}H]-estrone.

that estrone conjugate exists in the form of estrone-3-glucuronide (E_1-3-G). This form is further supported by the resemblance of the infrared spectrum of E_1-3-G to that of synthetic estriol-3-glucuronide [22], and the sodium salt of cholesterol glucuronide [23]. The strong broad peak near 3400 cm.$^{-1}$ due to polyhydroxy groups, a strong peak at 1620–1550 cm.$^{-1}$ due to asymmetrical stretching of the carboxylate ion [24] are also exhibited by monovalent salts of D-glucuronic acid. The strong band at 1100–1000 cm.$^{-1}$ due to the skeletal vibration of C-O-C of the glycosidic linkage is known to be extremely sensitive to environmental influences due to interaction with neighboring bands [25], and the profile of this peak was described by Smakula *et al.*[26] to be a result of the glycosidic linkage to the steroid at the C-3 position of the aromatic ring. The C-H stretching in the general region of 300–2840 cm.$^{-1}$ is given by authentic standards of 17α-estradiol, estrone, and D-glucuronic acid. The sharp peak at 1730 cm.$^{-1}$, representing the carbonyl of estrone, can only be demonstrated by E_1-3-G and not by 17α-estradiol glucuronide, cholesterol glucuronide or estriol glucuronide.

After a single injection of either radio-estradiol or estrone, radioactivity were excreted preferentially to the bile than to urine. Presumably, a great portion is being removed from the feces. We have found in earlier studies with the bull that biliary metabolites of [^{14}C]-testosterone are also conjugated [27] while the fecal metabolites are free. The fecal materials comprise over 10% of the injected testosterone in the first 24 h of collection. Although unequivocal identification of the 17α-estradiol conjugates in this study awaits completion, preliminary results indicated that they are similar to the biliary metabolites of 17β-estradiol, identified recently as 17α-estradiol-3β-D-glucuronide and estradiol-3, 17α-β-D-diglucuronide [6]. Identification of these conjugates will be presented elsewhere shortly.

Acknowledgements—This investigation was supported in part by the National Institutes of Health, Grant Number 5 T01 GM 00963, Grant Number 5 R01 AM 03124 and Grant Number 1 SO 5FRO7127. The authors wish to thank Doctor L. C. Fulkner and Doctor W. V. Lumb for their help in preparing the animals for this investigation.

REFERENCES

1. Rakoff A. E., Cantarow A., Paschkis K. E., Hansen L. P. and Walkling A. A.: *Endocrinology* **34** (1944) 370–375.
2. Cantarow A., Rakoff A. E., Paschkis K. E., Hansen L. P. and Walkling A. A.: *Proc. Soc. exp. Biol. Med.* **52** (1943) 256–257.
3. Martin R. P., Loriaux D. L. and Farnham G. S.: *Steroids* Suppl II (1965) 149–163.
4. Martin R. P.: *Fedn Proc.* **22**(2) (1963) 331 (Abstract).
5. Pearson J. R. and Martin R. P.: *Endocrinology* **78** (1966) 914–918.
6. Leung B. S. and Martin R. P.: *Fedn. Proc.* **31** (1972) 880 (Abstract).
7. Lumb W. V., Faulkner L. C., Leung B. S. and Martin R. P. (Unpublished data).
8. Bradlow H. L.: *Steroids* **11** (1968) 265–272.
9. Jirku H. and Levitz M.: *J. clin. Endocr. Metab.* **29** (1969) 615–637.
10. Gauthier P. B. and Kenyon A. J.: *Biochem. biophys. Acta* **130** (1966) 551–553.
11. Barton B. M., Evans R. S. and Gardner J. A. F.: *Nature, Lond.* **170** (1952) 249–250.
12. Lisboa B. P. and Diczfalusy E.: *Acta endocr. Copenh.* **43** (1963) 545–560.
13. Shriner R. L., Fuson R. C. and Curtain D. Y.: *The Systematic Identification of Organic Compounds.* John Wiley & Sons, New York, London, (1960) p. 254, Method A.
14. Stecher P. G.: *The Merck Index.* Merck & Co. (1968) p. 422.
15. Viscontini M., Hoch D. and Karrer P.: *Helv. chim. Acta* **38** (1955) 642–645.
16. Cohen S. L. and Marrian G. F.: *Biochem. J.* **30** (1936) 57–65.
17. Marsh C. A., Alexander F. and Levvy G. A.: *Nature* **170** (1952) 163–164.
18. Dahm K. and Breuer H.: *Z. Klin. Chem.* **4** (1966) 153–157.
19. Diczfalusy E., Franksson C., Lisboa B. P. and Martinsen B.: *Acta endocr., Copenh.* **40** (1962) 527–551.
20. Smith F. R., Tapley D. F. and Ross J. E.: *Biochim. biophys. Acta* **69** (1963) 68–73.
21. Lisboa B. P., Drosse I. and Breuer H.: *Hoppe-Seyler's Z. physiol. Chem.* **342** (1965) 106–131.
22. Ladany S.: *Steriods* **12** (1968) 717–721.
23. Nagayama F., Saito A. and Idler D. R.: *Can. J. Biochem.* **44** (1966) 1109–1114.
24. Silverstein R. M. and Bassler G. C.: In *Spectrometric identification of Organic Compounds.* John Wiley & Sons, New York, London (1967) p. 91.
25. Bellamy L. J.: *The Infrared Spectra of Complex Molecules.* Methuen, London (1954) p. 466.
26. Smakula E., Leftin J. H. and Wotiz H. H.: *J. Am. chem. Soc.* **81** (1959) 1708–1715.
27. Martin R. P.: *Endocrinology* **78** (1966) 907–913.