Sexual Differences in Hepatic Metabolism and Intracellular Distribution of Corticosterone Studied by Pulse Labeling with [1,2,6,7-^{3}H]Corticosterone†

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ABSTRACT: The subcellular distribution of corticosterone and its metabolites in liver was studied 5, 30, and 90 min after injection of [1,2,6,7-^{3}H]corticosterone in adult male and female rats that were adrenalectomized or hypophysectomized; 5 min after administration of isotope, the adrenalectomized male rats contained ten times as much labeled unconjugated corticosterone, 5α-dihydrocorticosterone, and 3α- and 3β,11β,21-trihydroxy-5α-pregnan-20-one in the nuclear fraction than the corresponding female rats. The metabolites of corticosterone in the soluble fraction of liver from adrenalectomized females occurred as about 90% steroid monosulfates and disulfates already 5 min after administration of isotope. In contrast, the soluble fraction of liver from males contained only 38% labeled monosulfate 5 min after injection of [1,2,6,7-^{3}H]corticosterone. The individual labeled metabolites from the different subcellular fractions were identified by thin-layer and radio-gas chromatography. The major metabolites found in the female were mono- and disulfurylated 3α,11β,15,21-tetrahydroxy-5α-pregnan-20-one, 3α,15,21-trihydroxy-5α-pregnane-11,20-dione, and 3α,11β,21-trihydroxy-5α-pregnan-20-one. The predominant metabolites in the male were 5α-pregnane-3α(and 3β),11β,20β,21-tetrol and 3β,11β,21-trihydroxy-5α-pregnan-20-one which mainly occurred as mono- and disulfates. Hypophysectomized female rats showed a corticosterone metabolite pattern with almost no 15-hydroxylated metabolites but with large amounts of isomers of pregnane-3,11β,20β,21-tetrol, *i.e.*, a "masculinized" pattern. It is concluded that hepatic intracellular metabolism and transport of corticosterone *in vivo* in rats are characterized by large sexual differences which are at least partly under hypophyseal control.

In order to elucidate the mechanism of action of corticosteroids in the liver it is essential to have a detailed knowledge about the distribution and uptake of these steroids by different cellular subfractions and about the metabolic transformation of the steroids *in vivo*. During recent years several investigations have been published on the hepatic metabolism and binding of ^{14}C- and ^{3}H-labeled cortisol and corticosterone in the rat (Litwack *et al.*, 1963; Morris and Barnes, 1967; Morey and Litwack, 1969; Beato *et al.*, 1969; Litwack *et al.*, 1973). In general, these studies have demonstrated a rapid uptake and metabolism of corticosteroids in liver tissue, and the radioactive metabolites have been recovered from the microsomal, mitochondrial, cytosol, and nuclear fractions of liver cells. Especially the work of Litwack *et al.* (1973) has revealed the occurrence of several hepatic carrier proteins involved in the binding of specific metabolites of corticosterone, probably also sulfurylated metabolites. However, in spite of all the information gained on the metabolism *in vivo* of radioactive corticosteroids in liver tissue, the major part of radioactive hepatic metabolites have remained unidentified. Since it is possible that some of these metabolites may be the physiologically active forms of cortisol/corticosterone in certain metabolic reactions (Morey and Litwack, 1969) it is important to have a more complete understanding of the metabolism *in vivo* in liver of corticosteroid hormones. The present paper describes a series of pulse-labeling experiments where [1,2,6,7-^{3}H]corticosterone has been administered to adrenalectomized rats and the distribution and identity of radioactivity in liver have been determined at various intervals after administration of the isotope. The experiments have been carried out on both male and female rats in view of the large sexual differences in the biliary, fecal, and urinary patterns of corticosteroid metabolites in the rat (Gustafsson, 1970; Eriksson and Gustafsson, 1971; Gustafsson and Gustafsson, 1974) and also on hypophysectomized rats in view of the recently discovered hypophyseal control of hepatic sex-dependent steroid metabolism in the rat (Gustafsson and Stenberg, 1974).

Experimental Procedure

Animal Experiments. Male and female rats of the Sprague-Dawley strain were operated under ether anaesthesia at 8 weeks of age. Adrenalectomy was carried out from the dorsal side and hypophysectomy was carried out from the ventral side. The animals were given a pellet diet *ad libitum*. The adrenalectomized animals received 0.9% (w/v) saline and the hypophysectomized animals 0.9% (w/v) saline in 5.5% (w/v) glucose to drink; 2 days after adrenalectomy or 7 days after hypophysectomy the animals received a single intraperitoneal injection of 250 μCi of [1,2,6,7-^{3}H]corticosterone (specific activity: 102 Ci/mmol; Radiochemical Centre, Amersham, England) dissolved in 100 μl of propane-1,2-diol, at 7.30 a.m. Before injection, 5 μl of this solution was taken off and diluted with 5.0 ml of methanol; 0.1 ml of this solution was determined for radioactivity in a liquid scintillation spectrometer (Packard Model 4322) using Instagel® as scintillation fluid.

Five, 30, or 90 min after the injection (30 min only for the hypophysectomized animals), the animal was killed by a

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blow across the neck and the liver was immediately perfused *in situ, via* the portal vein with 50 ml of ice-cold homogenizing buffer (pH 7.4) containing 0.01 M Tris, 0.01 M potassium chloride, and 0.001 M EDTA (sodium salt).

Preparation of Liver Subfractions. After perfusion, the liver was quickly removed, placed on ice, and rinsed in 25 ml of the homogenizing buffer. The liver was cut in small pieces and homogenized in 25 ml of the buffer in a Potter-Elvehjem glass homogenizer equipped with a loosely fitting Teflon pestle. The homogenate was centrifuged at 20,000*g* for 20 min at 4°, in an MSE High-Speed 18 refrigerated centrifuge. The supernatant from this centrifugation was further centrifuged at 105,000*g* for 70 min at 2°, in a Beckman Model L3-50 ultracentrifuge. The pellet obtained by this centrifugation was resuspended in 24 ml of a modified Bucher medium (Bergström and Gloor, 1955) and homogenized. This represented the microsomal fraction of the liver cells. The supernatant was also saved.

The pellet from the 20,000*g* centrifugation was resuspended in 20 ml of 0.88 M sucrose solution, homogenized, and centrifuged once more at 20,000*g* for 20 min. The supernatant obtained was saved and the pellet was resuspended in 20 ml of 2.2 M sucrose solution, homogenized, and centrifuged at 58,000*g* for 90 min in an SW 25.1 rotor in the Beckman ultracentrifuge. The pellet obtained represented the crude nuclear fraction. This was resuspended in 10 ml of 0.88 M sucrose solution, homogenized, and layered carefully on 10 ml of 2.2 M sucrose solution. This was then centrifuged at 58,000*g* for 30 min in the SW 25.1 rotor. The pellet was resuspended in 1.5 ml of the modified Bucher medium. This represented the purified nuclear fraction of the liver cells.

Extraction of Steroids. Aliquots from the microsomal and nuclear fractions and from the 105,000*g* and the second 20,000*g* supernatants were taken off for determination of radioactivity. These four pools were then extracted with 10 volumes of chloroform–methanol, 2:1 (v/v), for 18 hr at 37° on a shaking water bath. The extracts were filtered through glass wool in order to remove the precipitated proteins. The glass wool was rinsed well with the chloroform–methanol mixture. The filtrate was evaporated to dryness.

Separation of Steroid Conjugates. The steroid conjugates were separated by column chromatography on Sephadex LH-20 (Pharmacia, Uppsala, Sweden) and eluted with chloroform–methanol 1:1 (v/v), 0.01 M with respect to sodium chloride (Sjövall and Vihko, 1966). The extracts of the microsomal fraction and of the 105,000*g* supernatant fraction were separated on a 40-g column. The nonconjugated steroids and steroid glucuronides were eluted between 70 and 200 ml (pool I). The steroid monosulfates were eluted between 270 and 460 ml (pool II). The steroid disulfates were eluted with 400 ml of methanol (pool III).

The extracts from the nuclear fraction and from the second 20,000*g* supernatant were dissolved in water and applied on a 10-g XAD-2 column. The inorganic salts and sucrose were eluted first with water. The steroids were then eluted with ethanol. The ethanol eluate was evaporated to dryness and applied to a 10-g Sephadex LH-20 column, eluted as described above. Pool I was eluted between 20 and 60 ml, pool II between 80 and 130 ml, and pool III with 100 ml of methanol.

Pools I, II, and III from the second 20,000*g* supernatant were pooled with pools I, II, and III, respectively, from the 105,000*g* supernatant. This represented pools I, II, and III from the soluble fraction of the liver cells. Aliquots were taken from all of the pools eluted from the columns for radioactivity determinations and then the pools were evaporated to dryness. Pool I was partitioned between ethyl acetate (pool IA) and 8.4% (w/v) sodium bicarbonate solution (pool IB) in order to separate the free steroids from the steroid glucuronides (Begue *et al.*, 1973). The sodium bicarbonate phase was neutralized with 1 M HCl and applied to a 10-g XAD-2 column (see above). The ethanol eluate was evaporated to dryness. A portion of the ethanol eluate was applied to a thin-layer silica gel plate and developed in the solvent system ethyl acetate–ethanol–ammonium hydroxide, 5:5:1 (by volume), in order to control the separation of free steroids and steroid glucuronides.

Hydrolysis of Conjugates and Separation of Steroids. Ethyl acetate was acidified by partition with 2 M sulfuric acid for 24 hr. Pools II and III were dissolved in 60 ml of acidified ethyl acetate and hydrolyzed for 18 hr at 37° on a shaking water bath. The ethyl acetate was then neutralized by partition with 8.4% (w/v) sodium bicarbonate solution and the ethyl acetate phase was evaporated to dryness.

Pool IB was dissolved in an acetate buffer (pH 4.6) and hydrolyzed for 48 hr at 37° using Ketodase® (beef liver glucuronidase). The organic salts were removed by chromatography on XAD-2 and the ethanol eluate was evaporated to dryness.

Pool IA and the hydrolyzed conjugate pools were applied to thin-layer silica gel plates (250 μ, Merck A.G., Darmstadt, Germany). The thin-layer plates were developed twice in the solvent system chloroform–ethanol, 9:1 (v/v). The zones of radioactivity on the thin-layer plates were located by scanning with a Berthold thin-layer scanner, Model II (Berthold, Wildbad, Germany). These zones were scraped off and the steroids were eluted from the silica gel with methanol. Aliquots from each of the eluates were determined for radioactivity.

Identification of Steroids. Each of the methanol eluates was evaporated to dryness and (trimethyl)silylated. The silyl ethers were analyzed on a Hewlett-Packard Model 402 gas chromatograph equipped with a Barber-Colman Radioactivity Monitoring System, Model 5190. The stationary phases used were 1.5% SE-30 and 1% OV-17. The conditions used were: oven temperature, 290°, flash heater and detector temperature, 310°; argon flow, 20 ml/min.

A steroid was considered identified if it had the same retention times, relative to 5α-cholestane (t_R), on SE-30, and relative to corticosterone (t_S), on OV-17, as the reference steroids.

A summary of the experimental procedure employed in the present investigation is shown in Figure 1.

Preparation of Reference Steroids for Radio-Gas Chromatography. An adult male and an adult female bile-fistula rat were each injected with 125 μCi of [1,2,6,7-^{3}H]corticosterone and bile was collected for 24 hr. The two bile pools were analyzed as described previously (Begue *et al.*, 1973) and a part of each pool was analyzed as usual by gas chromatography and by gas chromatography–mass spectrometry to control the identities of the different steroids and they were found to be the same as described previously (Cronholm *et al.*, 1971, 1972). A part of each of the hydrolyzed conjugate pools was analyzed by radio-gas chromatography. The rest of the pools were separated by thin-layer chromatography and each of the radioactive zones was analyzed by radio-gas chromatography.

Statistical analysis was carried out using Student's t-test. The significance level was set at $P = 0.05$.

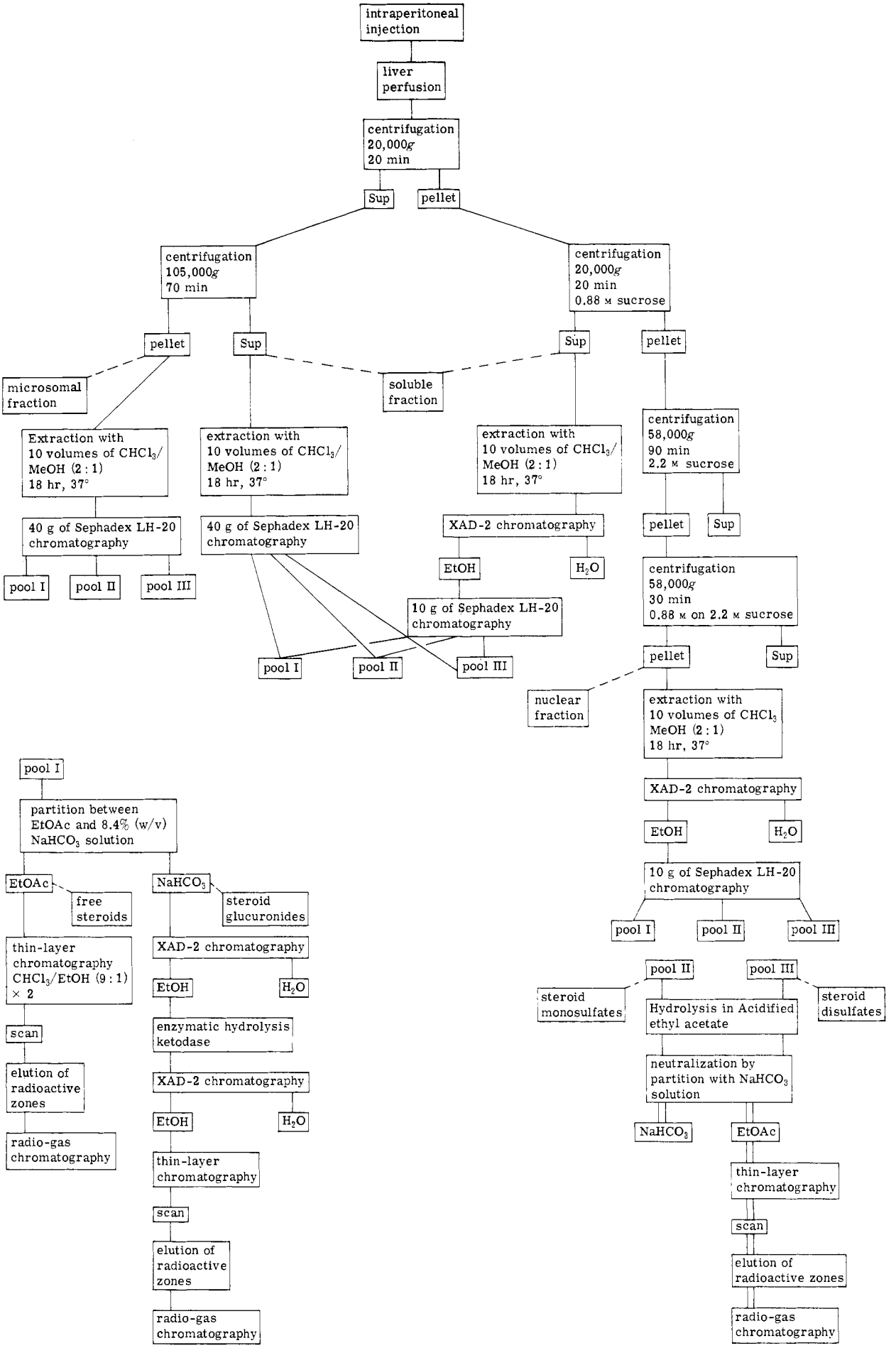

FIGURE 1: Flow chart of the experimental procedure used in the present investigation.

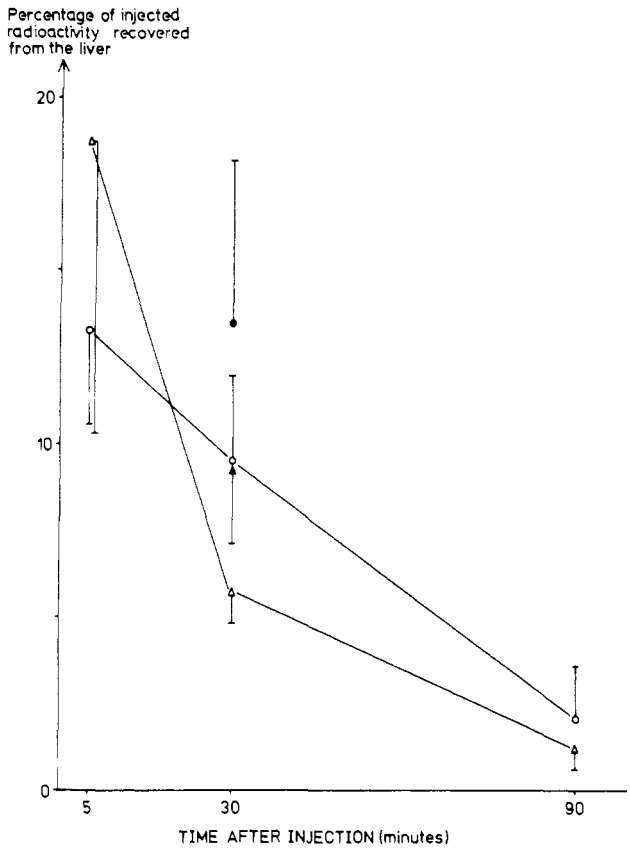

FIGURE 2: Recovery of injected radioactivity from the liver. The points represent the mean value of each group of three animals. The bars arising from each point represent ± one standard deviation. (○) Adrenalectomized male animals; (●) hypophysectomized male animals; (△) adrenalectomized female animals; (▲) hypophysectomized female animals.

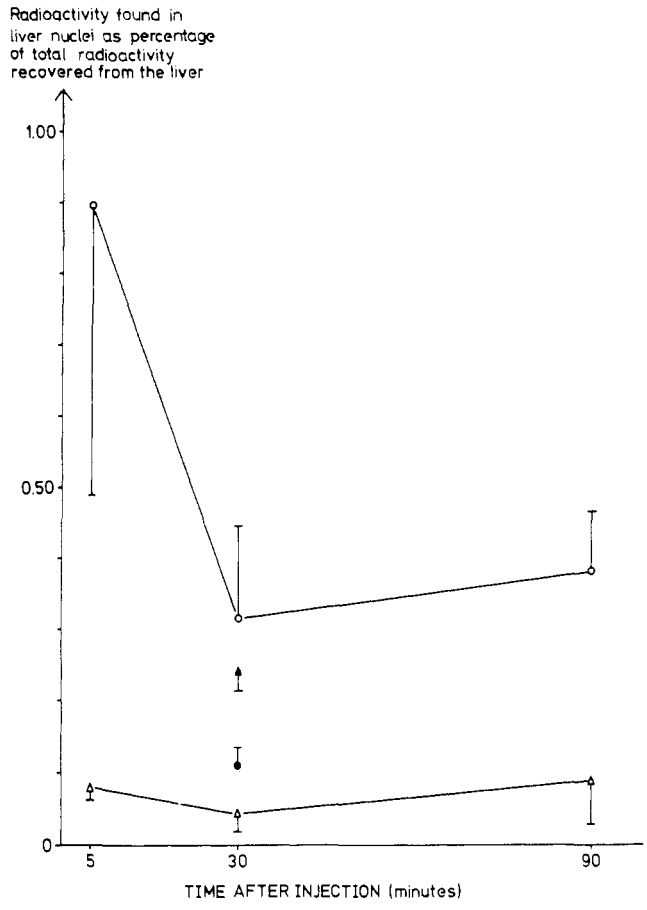

FIGURE 3: Radioactivity found in liver nuclei expressed as per cent of total radioactivity recovered in the liver. The points represent the mean value of each group of three animals. The bars arising from each point represent ± one standard deviation. (○) Adrenalectomized male animals; (●) hypophysectomized male animals; (△) adrenalectomized female animals; (▲) hypophysectomized female animals.

Table I: Distribution of Recovered Radioactivity in The Soluble and Microsomal Fraction of Rat Liver.[a]

	Percentage of Total Recovered Radioactivity from Liver	
	Microsomal Fraction	Soluble Fraction
♂ 5	19.2 ± 6.5	79.9 ± 6.8
♂ 30	13.7 ± 5.8	86.0 ± 5.8
♂ 90	17.6 ± 2.5	82.0 ± 2.5
HX ♂ 30	11.0 ± 1.6	88.9 ± 1.6
♀ 5	8.9 ± 0.9	91.1 ± 1.0
♀ 30	7.8 ± 2.2	92.2 ± 2.3
♀ 90	10.5 ± 1.0	81.1 ± 13.6
HX♀ 30	9.1 ± 2.2	90.7 ± 2.2

[a] The figures given represent the mean ± one standard deviation for three animals in each group. Abbreviations: ♂5, 30, and 90 = adrenalectomized male animals examined after 5, 30, and 90 min, respectively; ♀5, 30, and 90 = adrenalectomized female animals examined after 5, 30, and 90 min, respectively; HX♂30 = hypophysectomized male animals examined after 30 min; HX♀30 = hypophysectomized female animals examined after 30 min.

Results

The radioactivity recovered from the liver of both male and female adrenalectomized animals was maximal after 5 min and decreased with time (Figure 2). The differences between male and female animals were not significant at any of the three times. The radioactivity recovered from the liver of male and female hypophysectomized animals 30 min after injection tended to be higher than that recovered from male and female adrenalectomized animals, respectively, under the same conditions. Neither of these differences was significant.

Table I shows the distribution of the recovered radioactivity between the soluble and microsomal fractions. The percentage of recovered radioactivity found in these two fractions did not change significantly with time either in male or female adrenalectomized animals. The difference between the percentages of recovered radioactivity in the microsomal fraction 5 min after injection from male and from female rats was significant ($\delta > \varphi$; $P < 0.05$). Similarly the amount of radioactivity in the microsomal fraction of liver from adrenalectomized male animals was significantly higher ($P < 0.05$) than that recovered from the corresponding fraction from female animals, 90 min after injection. There were no significant differences between hypophysectomized and adrenalectomized rats of either sex.

Figure 3 shows the radioactivity recovered in the nuclear fraction as a percentage of the total radioactivity recovered from the liver. Male adrenalectomized animals concentrated more radioactivity in the nuclei than the corresponding female animals 5 min ($P < 0.005$), 30 min ($P < 0.05$), and 90 min ($P < 0.01$) after injection of the isotope. The effects

Table II: Radio-Gas and Thin-Layer Chromatographic Properties of The Reference Radioactive Steroids.[a]

	t_R on SE-30	t_s on OV-17	R_s
5β-[^{3}H]Pregnane-3α,11β,20β,21-tetrol[b]	2.31		0.26
5α-[^{3}H]Pregnane-3α,11β,20β,21-tetrol[b] (I)	2.47	0.39	0.26
5α-[^{3}H]Pregnane-3β,11β,20β,21-tetrol[b] (II)	3.24	0.54	0.26
3α,15,21-Trihydroxy-5α-[^{3}H]pregnane-11,20-dione[b] (III)	2.33	0.83	0.39
3α,11β,15,21-Tetrahydroxy-5α-[^{3}H]pregnan-20-one[b] (IV)	2.29	0.53	0.45
3β,20,21-Trihydroxy-5α-[^{3}H]pregnan-11-one[b] (V)	2.94	0.63	0.50
3α,11β,21-Trihydroxy-5β-[^{3}H]pregnan-20-one[b] (VI)	1.96	0.40	0.81
3β,11β,21-Trihydroxy-5α-[^{3}H]pregnan-20-one[b] (VII)	2.61	0.58	0.84
3α,11β,21-Trihydroxy-5α-[^{3}H]pregnan-20-one[b] (VIII)	2.05	0.43	0.87
[1,2,6,7-^{3}H]Corticosterone (IX)	2.97	1.00	1.00
[^{3}H]-5α-Dihydrocorticosterone[b] (X)	2.41	0.80	1.09
21-Hydroxy-4-[1,2-^{3}H]pregnene-3,11,20-trione[c]	2.47	1.09	1.15
21-Hydroxy-4-[1,2-^{3}H]pregnene-3,20-dione[d]	2.16		1.27

[a] For preparation, see Experimental Procedure. Abbreviations: t_R = retention time on 1.5% SE-30 relative to 5α-cholestane; t_s = retention time on 1.0% OV-17 relative to [1,2,6,7-^{3}H]corticosterone; R_s = mobility on thin-layer silica gel plates relative to corticosterone. Roman numerals are given to compounds shown in Figures 4–6. [b] Identified by gas chromatography–mass spectrometry after injection of [1,2,6,7-^{3}H]corticosterone into bile fistula rats and collection of bile for 24 hr which was analyzed as described previously (Begue *et al.*, 1973). [c] 21-Hydroxy-4-[1,2-^{3}H]pregnene-3,11,20-trione was purchased from IRE, Mol, Belgium. [d] 21-Hydroxy-4-[1,2-^{3}H]pregnene-3,20-dione was purchased from the Radiochemical Centre, Amersham, England.

of hypophysectomy were to reduce the uptake into male nuclei and to increase the uptake into female liver nuclei ($P < 0.001$). After chromatography on Sephadex LH-20, the nuclear radioactivity was shown to consist of a minimum of 90% nonconjugated steroids 5 min after injection and 95% 30 min after injection. The conjugated steroids were mainly monosulfates. The nonconjugated steroids recovered from the nuclei were identified as corticosterone, 11β,21-dihydroxy-5α-pregnane-3,20-dione (5α-dihydrocorticosterone), and 3α,11β,21-trihydroxy-5α-pregnan-20-one (see Table II), from both male and female rats. 3β,11β,21-Trihydroxy-5α-pregnan-20-one was also tentatively identified in the free steroid fraction from male nuclei. Figure 4 shows the scan from a thin-layer plate after chromatography of the free steroid fraction from the liver nuclei from a male animal examined 5 min after injection. It can be seen that corticosterone made up the greatest part of the nuclear steroids. Also shown in Figure 4 is the radio-gas chromatogram of the silylated extract of the thin-layer chromatographic zone containing [^{3}H]corticosterone. The small radioactive peak eluted just before the large corticosterone peak was tentatively identified as 3β,11β,21-trihydroxy-5α-pregnan-20-one (t_R on SE-30 = 2.61; see Table II). However, there was insufficient radioactivity to confirm this identification on OV-17.

The steroid monosulfates recovered from the nuclei of adrenalectomized males 5 min after injection were identified as corticosterone (60% of total nuclear monosulfates), 5α-dihydrocorticosterone (10%), and 3β,11β,21-trihydroxy-5α-pregnan-20-one (30%).

The type of conjugation of the radioactive steroid metabolites extracted from the microsomal and soluble fractions of the liver is shown in Table III. The subcellular pools from hypophysectomized male and female animals were pooled, respectively, before chromatography on Sephadex LH-20. There were several significant differences in the conjugation pattern of steroids at different times after administration of isotope between adrenalectomized male and female animals. The male animals contained significantly

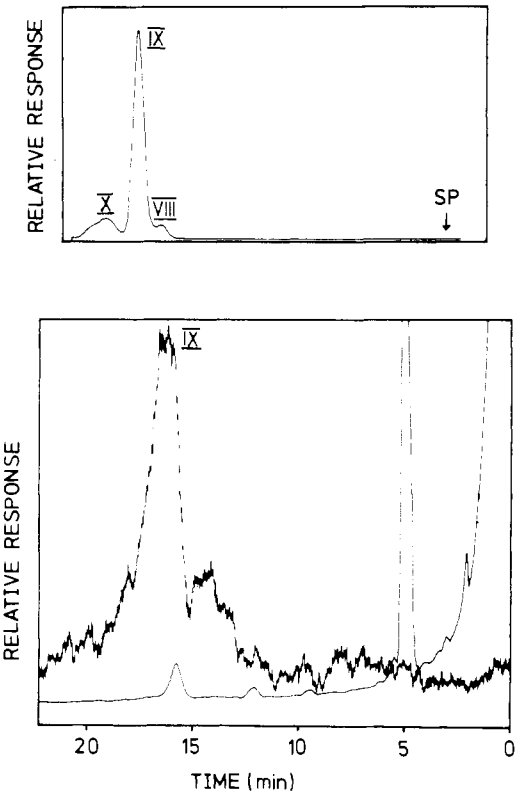

FIGURE 4: Steroids identified in the nuclear fraction of liver from adrenalectomized male animals. The upper panel of the figure shows the scan of the thin-layer chromatography of the free steroid pool from the nuclear fraction from an adrenalectomized male rat examined 5 min after injection of [1,2,6,7-^{3}H]corticosterone. The lower panel of the figure shows the radio-gas chromatographic analysis of the silylated methanol eluate of zone IX from the thin-layer chromatogram shown in the upper panel. The steady trace (lower trace) represents the flame-ionization detection. The unsteady (upper) trace represents the radioactive detection. Abbreviations: SP, starting point on the thin-layer plate; VIII, 3α,11β,21-trihydroxy-5α-pregnan-20-one; IX, corticosterone; X, 5α-dihydrocorticosterone. The small radioactive peak before corticosterone in the lower part of the figure was tentatively identified as 3β,11β,21-trihydroxy-5α-pregnan-20-one.

Table III: Conjugation of Radioactive Steroids Recovered from The Microsomal and Soluble Fractions of Rat Liver.[a]

		Percentage of Total Radioactivity Eluted from the Sephadex LH-20 Column			
		Free	Gluc	MoS	DiS
♂ 5	Mic	39.8 ± 8.3	15.1 ± 2.8	37.1 ± 8.0	7.6 ± 2.8
	Sol	30.0 ± 2.1	16.0 ± 1.1	36.7 ± 4.9	14.9 ± 4.1
♂ 30	Mic	27.5 ± 11.9	20.8 ± 7.8	40.5 ± 13.0	11.7 ± 5.9
	Sol	19.8 ± 11.7	27.5 ± 4.2	32.7 ± 2.6	18.8 ± 5.1
♂ 90	Mic	45.1 ± 16.7	27.2 ± 0.8	27.1 ± 0.6	8.8 ± 5.4
	Sol	11.9 ± 8.0	37.1 ± 7.2	34.6 ± 7.3	12.5 ± 3.6
HX♂ 30	Mic	44.1	21.9	24.2	8.8
	Sol	23.1	37.7	26.2	12.1
♀ 5	Mic	6.2 ± 2.0	3.5 ± 0.9	67.8 ± 21.2	22.2 ± 18.7
	Sol	3.3 ± 1.1	4.0 ± 0.7	69.6 ± 13.1	21.0 ± 14.7
♀ 30	Mic	6.5 ± 2.1	10.9 ● 4.5	63.0 ± 3.0	18.3 ± 9.0
	Sol	2.3 ± 0.1	9.2 ± 2.3	72.6 ± 5.9	14.4 ± 7.3
♀ 90	Mic	24.2 ± 6.1	31.0 ± 2.4	29.6 ± 7.9	8.8 ± 3.2
	Sol	11.0 ± 4.3	42.7 ± 3.6	35.1 ± 4.3	5.2 ± 0.4
HX♀ 30	Mic	17.1	12.8	34.7	32.8
	Sol	12.1	17.6	35.3	32.2

[a] The figures given represent the mean ± one standard deviation for three animals in each group. The subcellular fractions from the three hypophysectomized animals were pooled before chromatography on Sephadex LH-20. Abbreviations: Free = free steroids; Gluc = steroid glucuronides; MoS = steroid monosulfates; DiS = steroid disulfates; Mic = microsomal fraction; Sol = soluble fractions; for further explanations, see Table I.

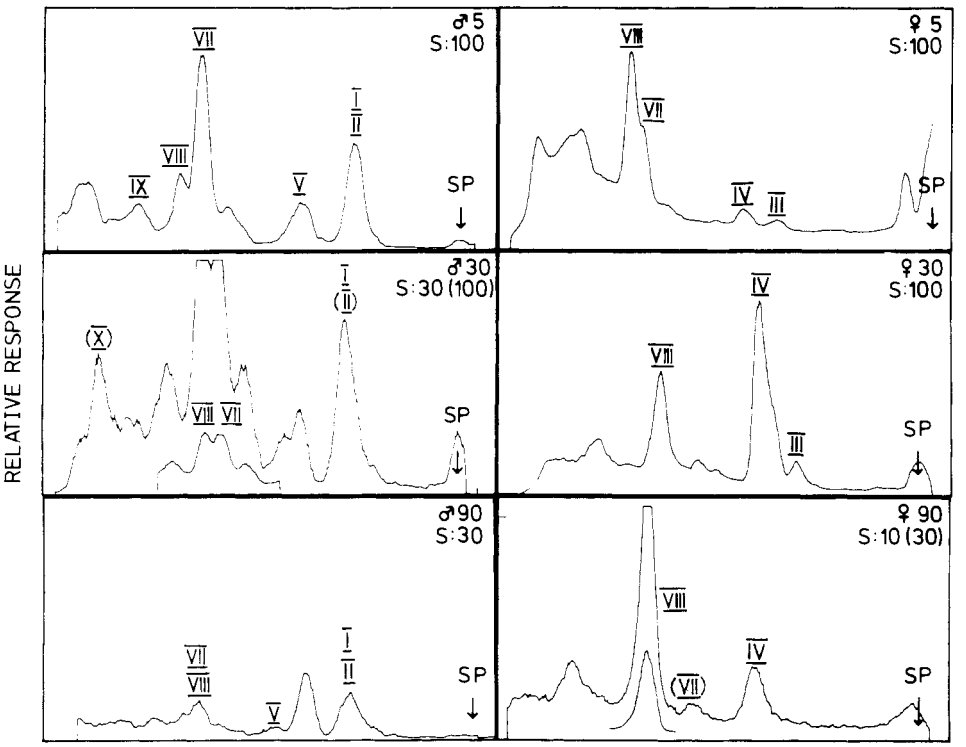

FIGURE 5: Thin-layer chromatogram scans of the steroid monosulfate pools from the soluble fractions of adrenalectomized animals. Abbreviations: S, measuring range; where two values are given, the value in parentheses refers to the lower of the scans where a peak that is off-scale has been rescanned; ♂ 5, 30, or 90, adrenalectomized male animals studied after 5, 30, or 90 min, respectively; ♀ 5, 30, or 90, adrenalectomized female animals studied after 5, 30, or 90 min, respectively; SP, starting point for the thin-layer chromatography; I, 5α-pregnane-3α,11β,20β,21-tetrol; II, 5α-pregnane-3β,11β,20β,21-tetrol; III, 3α,15,21-trihydroxy-5α-pregnane-11,20-dione; IV, 3α,11β,15,21-tetrahydroxy-5α-pregnan-20-one; V, 3β,-20,21-trihydroxy-5α-pregnan-11-one; VII, 3β,11β,21-trihydroxy-5α-pregnan-20-one; VIII, 3α,11β,21-trihydroxy-5α-pregnan-20-one; IX, corticosterone; X, 5α-dihydrocorticosterone.

more free steroids and steroid glucuronides in the microsomal fraction than female animals both 5 min ($P < 0.001$ for each) and 30 min ($P < 0.01$ for each) after injection. Furthermore, the female animals contained significantly more steroid monosulfates in the microsomal fraction after 30 min ($P < 0.05$). Similar sexual differences were found in the soluble fraction: after 5 min, male rats contained more free steroids ($P < 0.001$) and steroid glucuronides ($P <$

Table IV: Occurrence of Radioactive Steroids Identified in The Different Conjugate Pools from the Microsomal and Soluble Fractions of Liver from Adrenalectomized Animals.

		Animals Examined 5 min after Injection				Animals Examined 30 min after Injection				Animals Examined 90 min after Injection			
		Free	Gluc	MoS	DiS	Free	Gluc	MoS	DiS	Free	Gluc	MoS	DiS
Soluble Fraction													
♂	B	*****	***	*		****				****			
	5αdHB	*		Trace									
	3α5αTHB	*	***	*	**	**	***	***	*	*	**	**	**
	3β5αTHB	**		***	***	**	***	***	***	***		**	*
	3α5βTHB		****				****				**		
	3β5αHHA			**	*			*				*	
	3α5α20βHHB			**			*	***	*			**	Trace
	3β5α20βHHB			**	***		*	**	***			*	***
	3α5β20βHHB			Trace			Trace	Trace					
♀	B	*				***				***			
	5αdHB	*				***							
	3α5αTHB	*****	***	***		***	***	***	*	***	*****	****	****
	3β5αTHB	***	*****	*	****	*	**	*	***				
	3α5βTHB		*				*						
	15HO3α5αTHB			*				****	*			**	*
	15HO3α5αTHA			*				*	*			*	
Microsomal Fraction													
♂	B	*****	****	*		****				***			
	5αdHB	*								*			
	3α5αTHB	*	**	**		**	***	***			**		
	3β5αTHB	**		****	****	***	*	***	****	*		**	
	3α5βTHB						****						
	3β5α20βHHB			*	**			*	**			*	
	3α5α20βHHB			*				*					
♀	B	*				***				***			
	5αdHB	*				*				*			
	3α5αTHB	***	***	****		**	***	***			****	***	
	3β5αTHB	**	***	**	*****		**		****				
	15HO3α5αTHB			*				*				*	
	15HO3α5αTHA			*				*					

Abbreviations: B = corticosterone; 5αdHB = 5α-dihydrocorticosterone, 3α5αTHB = 3α,11β,21-trihydroxy-5α-pregnan-20-one; 3β5αTHB = 3β,11β,21-trihydroxy-5α-pregnan-20-one; 3α5βTHB = 3α,11β,21-trihydroxy-5β-pregnan-20-one; 3β5αHHA = 3β,20,21-trihydroxy-5α-pregnan-11-one; 15HO3α5αTHB = 3α,11β,15,21-tetrahydroxy-5α-pregnan-20-one; 15HO3α5αTHA = 3α,15,21-trihydroxy-5α-pregnane-11,20-dione; 3α5α20βHHB = 5α-pregnane-3α,11β,20β,21-tetrol; 3β5α20βHHB = 5α-pregnane-3β,11β,20β,21-tetrol; 3α5β20βHHB = 5β-pregnane-3α,11β,20β,21-tetrol; ***** = steroid occurs as more than 60% of the conjugate pool; **** = steroid occurs as 40–60% of the conjugate pool; *** = steroid occurs as 20–40% of the conjugate pool; ** = steroid occurs as 10–20% of the conjugate pool; * = steroid occurs as less than 10% of the conjugate pool; for further explanations, see Table III.

0.001) than female rats whereas female animals contained more steroid monosulfates ($P < 0.05$). After 30 min males still contained more steroid glucuronides than females ($P < 0.01$) and females contained more steroid monosulfates than males ($P < 0.001$). After 90 min the soluble fraction from male rats contained more steroid disulfates than the corresponding fraction from females ($P < 0.05$).

The distribution of the corticosterone metabolites identified in the conjugate pools from the soluble and microsomal fractions from adrenalectomized animals can be seen in Table IV. In addition, thin-layer chromatogram scans from the hydrolyzed steroid monosulfate pools from the soluble fractions from adrenalectomized animals studied at the three different times are shown in Figure 5. Sexual differences are readily apparent. The male animals were characterized by the presence of 5α-pregnane-3α,11β,20β,21-tetrol which occurred mainly as monosulfate and of 5α-pregnane-3β,11β,20β,21-tetrol which occurred both as mono- and disulfate. No isomers of pregnane-3,11,20,21-tetrol were found in female rats. The males were further characterized by the occurrence of larger amounts of 3β,-11β,21-trihydroxy-5α-pregnan-20-one than of 3α,11β,21-trihydroxy-5α-pregnan-20-one and by the occurrence of 3α,11β,21-trihydroxy-5β-pregnan-20-one as glucuronide, very little of which was found in the female.

The presence of 3α,11β,15,21-tetrahydroxy-5α-pregnan-20-one and 3α,15,21-trihydroxy-5α-pregnane-11,20-dione was specific for female rats. These metabolites occurred mainly as monosulfates but also as disulfates. 3α,-11β,15,21-Tetrahydroxy-5α-pregnan-20-one did not appear in appreciable quantities at 5 min but 30 min after administration of isotope it was quantitatively the most important metabolite.

The female rats were further characterized by 3α,-11β,21-trihydroxy-5α-pregnan-20-one monosulfate (quantitatively predominant metabolite after 5 min) with very lit-

Table V: Occurrence of Radioactive Steroids Identified in the Different Conjugate Pools from the Microsomal and Soluble Fractions of Liver from Hypophysectomized Animals.[a]

		Free	Gluc	MoS	DiS
Soluble Fraction					
HX♂ 30	B	***			
	5αdHB	Trace			
	3α5αTHB	**		*	***
	3β5αTHB	***	*	**	*
	3α5βTHB		**		
	3β5αHHA	Trace		*	
	3α5α20βHHB	*		***	*
	3β5α20βHHB	*		***	***
	3α5β20βHHB	Trace		Trace	
HX♀ 30	B	**			
	5αdHB	**		*	
	3α5αTHB	**		**	***
	3β5αTHB	**	****	***	**
	3β5αHHA			*	
	15HO3α5αTHB			*	
	3α5α20βHHB	Trace	***	**	
	3β5α20βHHB		**	*	**
	3α5β20βHHB		*		
Microsomal Fraction					
HX♂ 30	B	****			
	5αdHB	*			
	3α5αTHB	*	*	*	**
	3β5αTHB	***	**	*	*
	3β5αHHA	*		*	
	3α5α20βHHB			**	*
	3β5α20βHHB			**	**
HX♀ 30	B	**			
	5αdHB	*			
	3α5αTHB	**			*
	3β5αTHB	**	**	**	***
	3α5α20βHHB	*	**	*	
	3β5α20βHHB	*	**		*

[a] The microsomal and soluble fractions from three animals were pooled, respectively, before chromatography. The results from these two pools are given below. Abbreviations: HX♂30 = hypophysectomized male animals examined 30 min after injection; HX♀30 = hypophysectomized female animals examined 30 min after injection; for further explanations, see Table IV.

tle 3β,11β,21-trihydroxy-5α-pregnan-20-one monosulfate. However, the main constituent of the disulfate pools from female animals was 3β,11β,21-trihydroxy-5α-pregnan-20-one disulfate.

The steroids identified from the microsomal and soluble fractions from hypophysectomized males and females are given in Table V and thin-layer chromatogram scans of the hydrolyzed monosulfate pools from the soluble fraction of hypophysectomized male and female animals are shown in Figure 6. The male animals contained more 5α-pregnane-3α,11β,20β,21-tetrol and 5α-pregnane-3β,11β,20β,21-tetrol than the corresponding adrenalectomized animals. The females contained only a trace of 3α,11β,15,21-tetrahydroxy-5α-pregnan-20-one monosulfate. Also in the females, there occurred significant amounts of 5α-pregnane-3α (and 3β),11β,20β,21-tetrol monosulfates and 5α-pregnane-3β,11β,20β,21-tetrol disulfate. Furthermore, the hypophysectomized females contained large quantities of 3β,-11β,21-trihydroxy-5α-pregnan-20-one in all conjugate pools. The large peak seen close to the starting point of the thin-layer chromatogram scan of the monosulfurylated metabolites from hypophysectomized female rats (Figure 6) did not show as a peak when analyzed by radio-gas chromatography and could not be identified.

Discussion

In contrast to other cellular subfractions, liver nuclei concentrated predominantly unconjugated metabolites of [^{3}H]corticosterone. Part of the nuclear radioactivity was identified as unmetabolized corticosterone but the nuclei also contained the reduced metabolites, 5α-dihydrocorticosterone and 3α- and 3β,11β,21-trihydroxy-5α-pregnan-20-one. The finding of hepatic nuclear uptake of corticosterone is in agreement with the hypothesis that unmetabolized corticosterone binds to a specific cytoplasmic receptor protein in the liver cell and that the formed steroid–receptor complex is transported into the nuclei where it interacts with the chromatin (Koblinsky *et al.*, 1972; Litwack *et al.*, 1973). The presence of reduced metabolites of corticosterone in the liver nuclear fraction may only represent unspecific binding of these compounds to the nuclei but may also indicate the existence of specific intranuclear transfer of physiologically active metabolites of corticosterone. Metabolic activation of a steroid hormone is well known from studies on androgen action; the physiologically active androgen in prostatic tissue is 5α-dihydrotestosterone formed

by predominantly microsomal 5α reduction of testosterone and transferred into the nuclei by a specific receptor protein ("β protein") (Bruchovsky and Wilson, 1968; Fang *et al.*, 1969; Mainwaring, 1969). Also the further reduced testosterone metabolite 5α-androstane-3β,17β-diol has been suggested to play a role in androgen action in prostate (Robel *et al.*, 1971; Gittinger and Lasnitzki, 1972). Thus, on the basis of the present results, it may be relevant to speculate about the possibility that corticosterone action in liver is mediated not only by unmetabolized corticosterone but also by reduced metabolites of corticosterone.

At all intervals studied liver nuclei from male rats were more efficient in concentrating radioactivity than nuclei from female animals. This suggests the possibility that corticosterone may normally be more active in male than in female rat liver. The sexual difference in nuclear concentration of corticosterone and corticosterone metabolites decreased following hypophysectomy indicating that the hypophysis may influence the intranuclear transfer of corticosteroids in liver.

The major part of the radioactivity recovered from the liver was found in the microsomal and soluble fractions. The relative distribution of radioactivity between these fractions was similar in all experiments. The slightly higher ratio of soluble/microsomal radioactivity found in female than in male rats may depend upon the presence of more polar metabolites of corticosterone in female than in male rat liver.

The large sexual differences characterizing the pattern of labeled conjugates recovered from the microsomal and soluble fractions of liver agree with previous studies showing a higher sulfurylating activity *in vitro* in female than in male rat liver (Roy, 1956; Wengle, 1963; Carlstedt-Duke and Gustafsson, 1973) and a higher capacity of the male than the female rat liver to form steroid glucuronides (Eriksson and Gustafsson, 1971). The sexual differences observed in the present investigation were most pronounced 5 and 30 min after administration of [^{3}H]corticosterone; 90 min after injection of the isotope, the sexual differences in conjugation of radioactive metabolites had almost disappeared. Interestingly, the ratio of labeled steroid sulfates/steroid glucuronides decreased successively with time, possibly indicating a more efficient clearance of sulfates than glucuronides from female liver.

The proportion of corticosterone which was free in the soluble and microsomal fractions of the male rat liver was much higher than in the female rat liver (*cf.* Table IV). It is possible that the greater nuclear binding of corticosterone in male rats pointed out above is related to the larger concentration of free corticosterone in the soluble fraction. The reason for the slower metabolism of corticosterone in male rats could possibly be a high degree of binding to specific receptor proteins. In fact, preliminary results from investigations in progress in our laboratory on sexual differences in protein-mediated intracellular transport of corticosterone seem to indicate that male rat liver cytosol contains higher concentration of high-affinity corticosterone-binding proteins than female liver cytosol (Carlstedt-Duke, Gustafsson, Gustafsson, and Wrange, unpublished observations).

The most conspicuous sexual differences in the corticosterone metabolite pattern in the microsomal and soluble fractions in liver were the sex-specific occurrence of 15-hydroxylated metabolites (3α,11β,15,21-tetrahydroxy-5α-pregnan-20-one and 3α,15,21-trihydroxy-5α-pregnane-11,20-dione) in female rats and of isomers of pregnane-3,11,20,21-tetrol (5α-pregnane-3α- and 3β,11β,20β,21-tetrol and 5β-pregnane-3α,11β,20β,21-tetrol) in male rats. The sex-specific presence of 15-hydroxylated metabolites of corticosterone in female rats has previously been described in bile, feces, and urine (Gustafsson, 1970; Cronholm *et al.*, 1971) and the occurrence of isomers of pregnane-3,11,20,21-tetrol in bile and feces from male rats but not from female rats has also been demonstrated (Eriksson, 1971; Cronholm *et al.*, 1972). In consideration of the fact that about 90% of the radioactivity recovered from female rat liver is made up of sulfurylated metabolites already 5 min after administration of isotope, the pronounced increase in relative quantitative importance of the 15-hydroxylated sulfurylated metabolites between 5 and 30 min after isotope administration indicates that the 15-hydroxylase system involved is active on sulfurylated substrates rather than on free steroids. The presence of a liver microsomal steroid sulfate-specific hydroxylating system has recently been demonstrated and partially characterized in female rats (Gustafsson and Ingelman-Sundberg, 1974). Finally, the ratio of monosulfurylated 3β,11β,21-trihydroxy-5α-pregnan-20-one to 3α,11β,21-trihydroxy-5α-pregnan-20-one in both the soluble and microsomal fractions of liver was much higher in male than in female rats. These results agree well with previous reports in literature which have described a higher ratio in male than in female rats of 3β- to 3α-hydroxylated metabolites of steroids administered *in vivo* or incubated *in vitro* with homogenates and microsomal preparations of liver tissue (Schriefers, 1967; Schriefers *et al.*, 1973; Hoff and Schriefers, 1973).

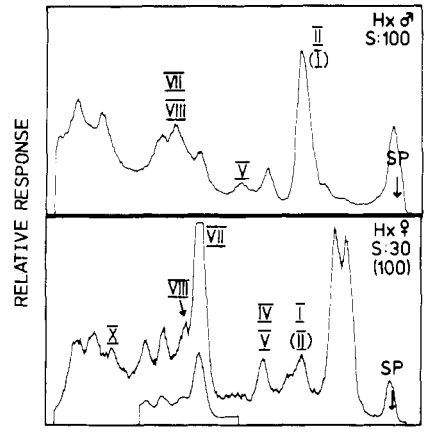

FIGURE 6: Thin-layer chromatogram scans of the steroid monosulfate pools from the soluble fractions of hypophysectomized animals. Abbreviations: Hx♂, hypophysectomized male animals studied after 30 min; Hx♀, hypophysectomized female animals studied after 30 min. The soluble fraction from the three animals in each group were pooled, respectively, before chromatography on Sephadex LH-20. For further explanations, see legend to Figure 4.

Hypophysectomy resulted in an almost total disappearance of 15-hydroxylated corticosterone metabolites from female rat liver. Instead, formation of isomers of pregnane-3,11,20,21-tetrol became an important metabolic pathway in female rats. This "masculinizing" effect of hypophysectomy upon the metabolism of corticosterone in female rat liver agrees well with the recently suggested hypothesis regarding the secretion of a hypophyseal "feminizing factor" in female rats regulating the activities of hepatic sex-dependent enzymes (Gustafsson and Stenberg, 1974).

The present investigation has given several examples of sexual differences in hepatic intracellular metabolism and

transport of corticosterone. The more efficient nuclear concentration of corticosterone and corticosterone metabolites in male than in female rats may indicate the existence of sex-dependent intracellular transport proteins, perhaps specific for certain metabolites of corticosterone. Work is now in progress in our laboratory to characterize these proteins.

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