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# REGULATION OF PLASMA LEVELS OF ALDOSTERONE AND ITS METABOLITES DURING THE LATENT PERIOD OF ALDOSTERONE

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## SUMMARY

Castration of male rats led to both increased biliary excretion of aldosterone metabolites and clearance of aldosterone radiometabolites from the plasma at rates similar to those of intact females and the elimination of the previously observed sex-dependence of these processes. Ovariectomy of female rats did not cause any effects in these processes. Treatment of both castrated male and ovariectomized female rats with testosterone reintroduced the sex-dependence so that the radiometabolites of aldosterone were cleared from the plasma and excreted *via* the bile of these animals at a slower rate typical of males. These findings suggest that the presence of androgens plays a major role in regulating the routes of hepatic metabolism of aldosterone, the subsequent rates of excretion of aldosterone metabolites *via* the bile and hence the concentration of aldosterone and the composition of the metabolites of aldosterone attained in the plasma during the latent period of the hormone.

Hypophysectomy of female rats significantly altered both the biliary excretion of aldosterone metabolites and the clearance from the plasma of aldosterone metabolites to rates typically observed in males. However, hypophysectomy of male rats did not cause changes in either of these two processes. These findings suggest that the presence of an intact pituitary is required for the sex-dependence of these processes in rats.

## INTRODUCTION

From previous studies it has been shown that the antinatriuretic and kaliuretic components of the physiological response to aldosterone in the kidneys of rats are separable [1]. It has also been demonstrated that each component of the physiological response to aldosterone is sex-dependent [1], the males showing the greater response. Similar to the findings with many other steroid hormones [2–10], the hepatic metabolism of aldosterone is also sex-dependent in rats [11].

We have reported recently that following administration of physiological quantities of [$^3$H]-aldosterone to both adrenalectomized and intact rats, significant sex differences occur in the plasma levels of aldosterone and its metabolites. The polar metabolites of aldosterone (NEPD) reached peak levels in the plasma of the males during the latent period of the hormone [12, 13]. In contrast, these metabolites were rapidly cleared from the blood of the females.

The biliary route of excretion of aldosterone [14, 15], corticosterone, cortisol and many other steroids [16–20] has been demonstrated to be the major pathway for the excretion of these steroids in rats. The differences in the rates of clearance of aldosterone and its metabolites from the plasma of male and female rats were then shown to be due to the sex-dependent excretion of aldosterone metabolites *via* the bile in these animals [13, 15].The biliary excretion of aldosterone as polar metabolites of aldosterone was rapid and significantly greater in female than in male rats.

Similar to the findings with corticosterone, it appears that the concentration of aldosterone and the composition of the metabolites of aldosterone in the plasma are regulated to a large extent by the sex-dependent routes of metabolism of this hormone in the liver. The effects of the sex hormones on the biliary excretion and plasma levels of aldosterone and its metabolites were therefore studied in order to understand further their role in the regulation of the pathways and rates of metabolism of aldosterone in the liver. The findings from these experiments might help understand not only the sex-dependence of the physiological response to aldosterone in rats, but also the physiological role of the polar metabolites of aldosterone during the latent period of the hormone.

### *Biliary excretion studies in castrated male and female rats*

Orchiectomy and ovariectomy of intact male and female Sprague–Dawley rats were performed at 24 days of age [26]. Bile duct cannulations were performed [15] on all groups of rats 45–48 days of age, 21–24 days following gonadectomy. Following the cannulation of the bile duct, 10 $\mu$Ci of chromatographically pure [$^3$H]-aldosterone (0.1 $\mu$g) was administered intravenously. All animals were injected on a given day with the same fresh preparation of [$^3$H]-aldosterone.

Castration caused marked changes in the rate of excretion of radiometabolites of aldosterone into the bile of male rats. Orchiectomized male rats excreted (Fig. 1) significantly greater quantities of $^3$H-radioactivity into the bile than their respective male controls

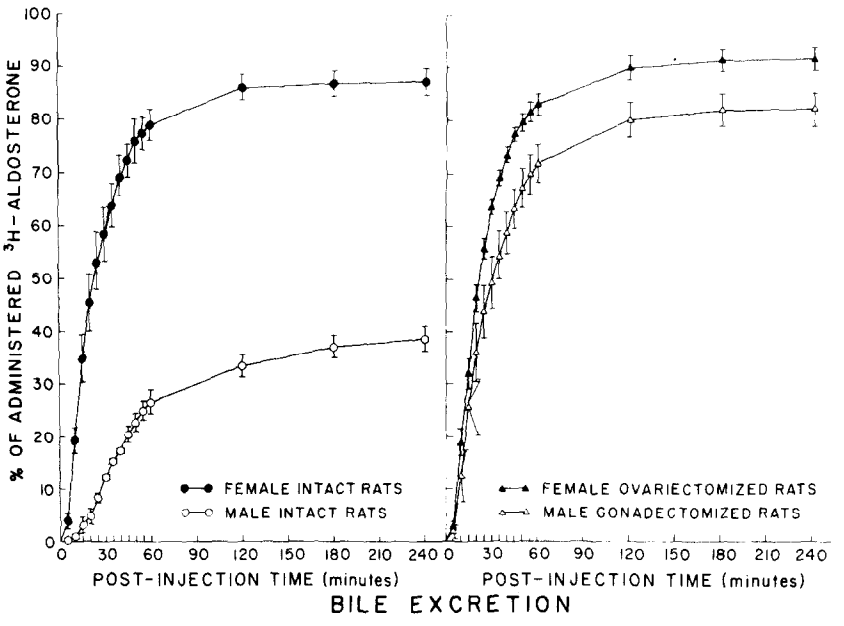


Fig. 1. *Left.* Cumulative bile excretion of $^3$H-radioactivity following intravenous injection of 0.1 μg [$^3$H]-aldosterone (10 μCi) into *intact* male and female rats. *Right.* Cumulative bile excretion of $^3$H-radioactivity in *gonadectomized* male and *ovariectomized* female rats. Each point represents mean ± SE (n = 5). (By permission of *Endocrinology* 26.)

($P < 0.005$). Although the biliary excretion of $^3$H-radioactivity was greater in ovariectomized female rats than in intact female rats, this increase was not significant ($P > 0.05$). The quantities of $^3$H-radioactivity excreted into the bile of castrated male rats were not significantly different from those excreted by intact female rats ($P > 0.05$). Thus the rate of excretion of aldosterone metabolites *via* the bile was markedly increased in males by castration, whereas ovariectomy in female rats did not substantially increase the rate of excretion of this hormone. The increased rate of excretion of aldosterone metabolites caused by castration of the males led to rates of biliary excretion similar to those in intact females and the elimination of the previously observed sex-dependence of this process [13, 15]. Thus both castrated male and female rats show a "female" rate of biliary excretion of this hormone.

*Biliary excretion studies in castrated male and female rats treated with testosterone*

Male and female castrated rats were treated daily

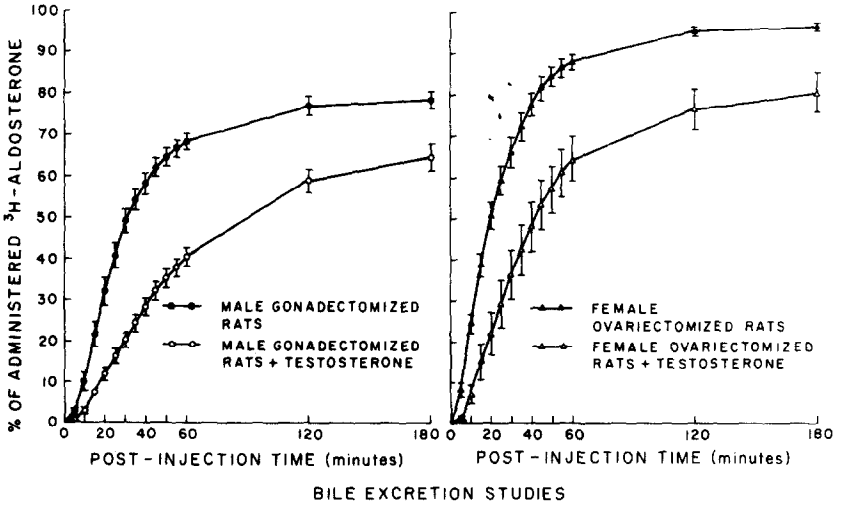


Fig. 2. *Left.* Cumulative bile excretion of $^3$H-radioactivity following intravenous injection of 0.1 μg [$^3$H]-aldosterone (10 μCi) into *gonadectomized* male rats treated with testosterone and untreated *gonadectomized* male control rats. *Right.* Cumulative bile excretion of $^3$H-radioactivity in *ovariectomized* female rats treated with testosterone and untreated *ovariectomized* female control rats. Each point represents mean ± SE (n = 5). (By permission of *Endocrinology* 26.)

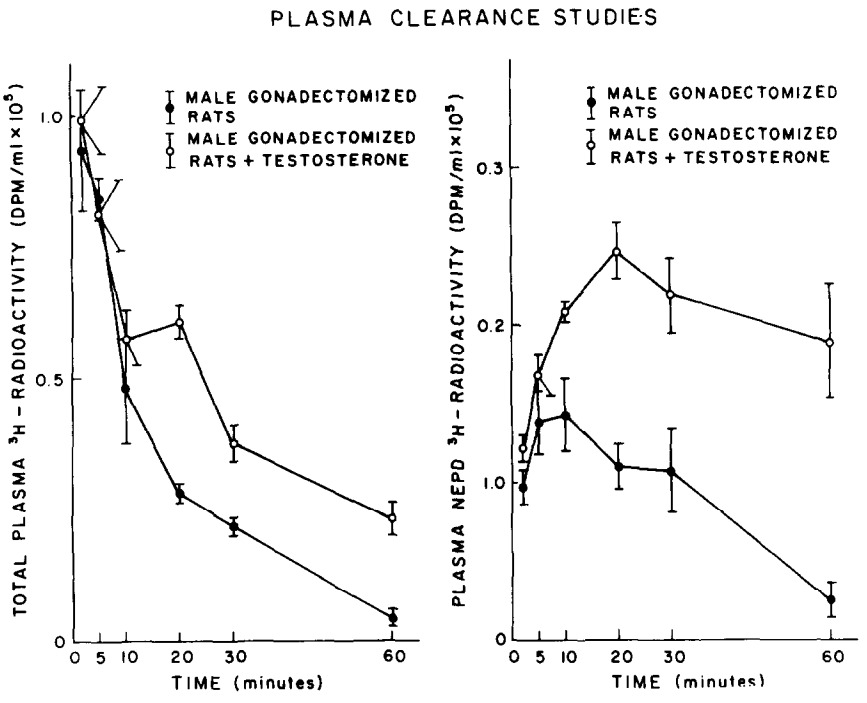


Fig. 3. Disappearance of (LEFT) the total $^3$H-radioactivity and (RIGHT) the Non-Extractable $^3$H-radioactivity (NEPD) from the plasma of *gonadectomized* male rats treated with testosterone and untreated *gonadectomized* male control rats. Blood was taken from rats following intravenous injection of 0.1 $\mu$g [$^3$H]-aldosterone (10 $\mu$Ci). Each point represents mean $\pm$ SE (n = 4).

for 14 days with testosterone cyclopropionate (kindly supplied by Upjohn), 5 mg/100g body wt. commencing 8–10 days post-castration [26]. Treatment of castrated male rats with testosterone (Fig. 2) reintroduced the sex-dependence so that these animals excreted the aldosterone metabolites *via* the bile at a slower rate. Similarly, treatment of ovariectomized female rats with testosterone (Fig. 2) also significantly slowed the biliary excretion of aldosterone metabolites. Thus the rate of biliary excretion of aldosterone radiometabolites in gonadectomized male and female rats was significantly slower following treatment with testosterone and approached the "male" rate of excretion.

*Plasma clearance rate studies in castrated male and female rats*

Castrated male and female rats were injected intravenously with 10 $\mu$Ci [$^3$H]-aldosterone (0.1 $\mu$g) and blood was collected from these animals into heparinized syringes [12] at 2, 5, 10, 20, 30 and 60 min post-injection times. Four rats were used for each time-interval.

As can be seen in Figs. 3 and 4, the total plasma radioactivity rapidly dropped with time in both castrated male and female rats. The rates of clearance of the total plasma radioactivity in both groups of castrated rats were similar to those previously demonstrated in intact female rats [13]. The very small quantities of the polar metabolites of aldosterone (NEPD; non-extractable in dichloromethane) (Figs. 3 and 4) in the plasma of both castrated male and female rats were also similar to those present in the plasma of intact female rats [13]. Only small quantities of tetrahydroaldosterone were shown to be present in the plasma of these rats. These findings indicate that these metabolites are rapidly cleared from the plasma into the bile of these animals at a "female" rate [13].

When the castrated male and female rats were treated with testosterone, however, the rates of clearance of the total plasma radioactivity were significantly slower than the untreated male and female castrated rats (Figs. 3 and 4). Interestingly, the quantities of the non-extractable polar metabolites of aldosterone (NEPD) in the plasma were significantly greater in the testosterone treated animals than in their respective controls. The quantities of these non-extractable polar metabolites of aldosterone reached peak levels by 20 min post-injection, similar to the findings with intact male rats [13]. The treatment with testosterone resulted in the appearance of greater quantities of tetrahydroaldosterone in the plasma than observed in the untreated castrated male and female control rats.

It is of interest that the quantities of [$^3$H]-aldosterone present in the dichloromethane extractable fraction of the plasma were similar in both the gonadectomized male and female rats and were not significantly altered following the treatment with testosterone. In previous studies [13] similar quantities of [$^3$H]-aldosterone were demonstrated to be present in

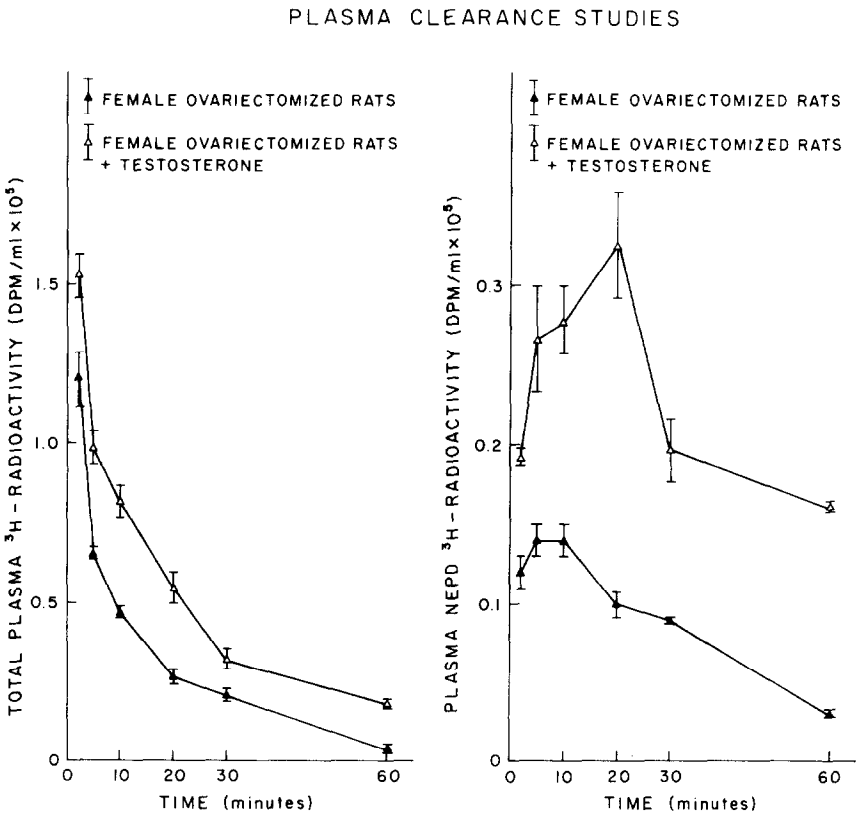


Fig. 4. Disappearance of (LEFT) the total $^3$H-radioactivity and (RIGHT) the Non-Extractable $^3$H-radioactivity (NEPD) from the plasma of *ovariectomized* female rats treated with testosterone and untreated *ovariectomized* female control rats. Blood was taken from rats following intravenous injection of 0.1 μg [$^3$H]-aldosterone (10 μCi). Each point represents mean ± SE (n = 4).

the plasma of both male and female intact rats during the first 60 min post-injection of the aldosterone.

These findings correlate well with the biliary excretion studies on these animals. The rates of biliary excretion of these polar metabolites were significantly slower in the testosterone treated animals, resulting in a slower clearance rate of the aldosterone metabolites from the plasma at a rate typical of males.

*Biliary excretion studies in Hypophysectomized male and female rats*

Intact male and female rats were hypophysectomized at 48 days of age and two weeks later bile duct cannulation experiments were performed. Hypophysectomy of female rats significantly lowered the excretion of the aldosterone radiometabolites *via* the bile to rates which were not significantly different ($P > 0.05$) from those observed in the intact male controls (Fig. 5). There were also no significant differences between the rates of biliary excretion of $^3$H-radioactivity of the hypophysectomized males and those of the intact male controls. The small differences between the hypophysectomized male and female values, which were significant during the first 60 min ($P < 0.005$), became insignificant ($P > 0.05$) after one hour post-injection of the aldosterone. Thus both hypophysectomized male and female rats excreted the radiometabolites of aldosterone *via* the bile at a similar slower "male" rate.

*Plasma clearance rate studies in hypophysectomized male and female rats*

When the rates of clearance of total plasma radioactivity, following intravenous administration of 0.1 μg [$^3$H]-aldosterone were investigated in hypophysectomized rats, the total plasma radioactivity was consistently higher in the females than in the males at all times studied (Fig. 6). This finding is opposite to the usual sex-dependence of this process in the plasma of intact rats [13]. Of note, peak levels of the non-extractable polar metabolites of aldosterone (NEPD) were reached 20 min post-injection (Fig. 6) in both hypophysectomized male and female rats. Considerable quantities of tetrahydroaldosterone were also present in the plasma of these rats. Both the bile excretion and the plasma clearance studies indicate that hypophysectomy of female rats causes the radiometabolites of aldosterone to be cleared from the plasma *via* the bile at rates typical of males.

*Regulation of aldosterone and its metabolites in plasma*

Biliary excretion of aldosterone [14, 15], corticos-

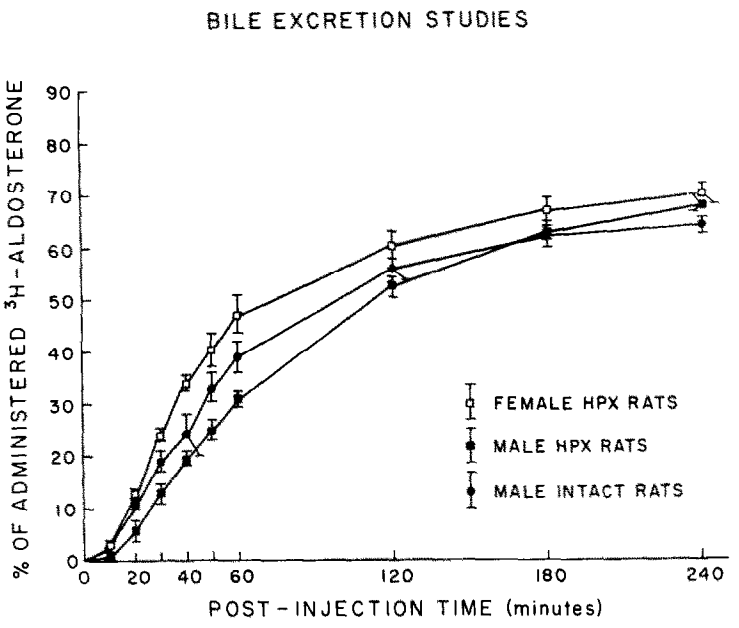


Fig. 5. Cumulative bile excretion of $^3$H-radioactivity following intravenous injection of 0.1 $\mu$g [$^3$H]-aldosterone (10 $\mu$Ci) into *hypophysectomized* male and female rats and *intact* male control rats. Each point represents mean ± SE (n = 5).

terone, cortisol and other steroid hormones [16–20] has been shown to be the major route of excretion of these steroids in rats. The routes by which many of these steroid hormones are metabolized in the liver, particularly in the case of corticosterone [7, 19, 22, 23], have been clearly demonstrated to be different in male and female rats.

The enzymatic *A*-ring reduction [7, 9, 19, 21] and

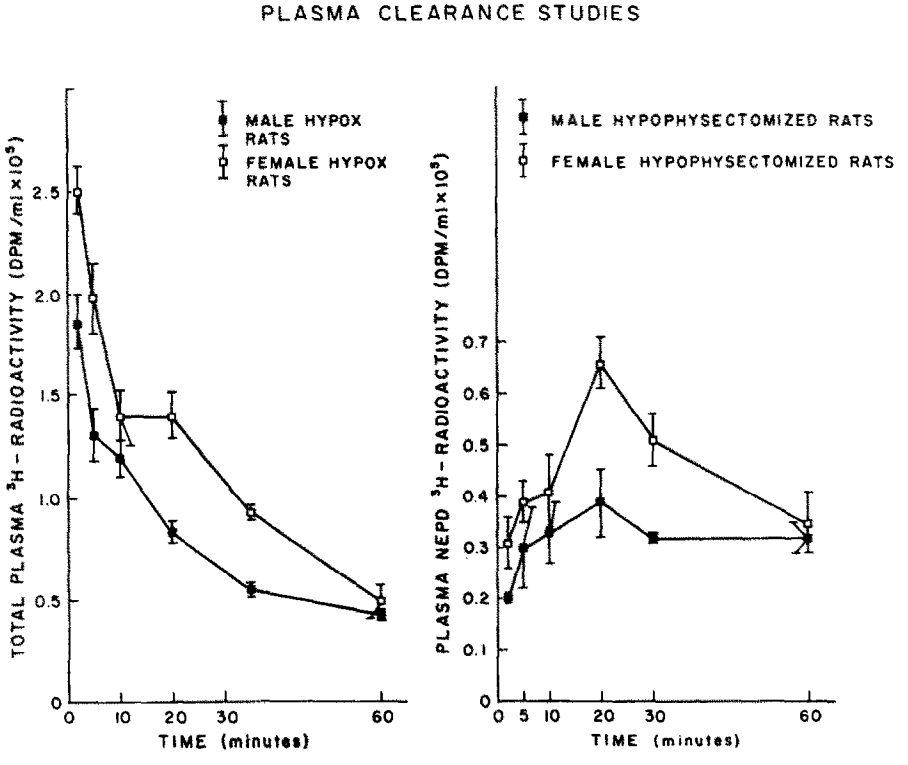


Fig. 6. Disappearance of (LEFT) the total $^3$H-radioactivity and (RIGHT) the Non-Extractable $^3$H-radioactivity (NEPD) from the plasma of *hypophysectomized* male and female rats. Blood was taken from rats following intravenous injection of 0.1 $\mu$g [$^3$H]-aldosterone (10 $\mu$Ci). Each point represents mean ± SE (n = 4).

the synthesis of various hydroxylated products of corticosterone in the liver [19, 22, 23] have been shown to be regulated by the sex hormones both *in vitro* and *in vivo*. Conjugation of steroid hormone metabolites, an important step for their excretion *via* the bile, is also known to be sex-dependent in the liver of rats [5, 19, 22, 24, 25]. It appears that differences in the metabolites synthesized in the liver, together with differences in their conjugation, result in the sex-dependence of the clearance rates of corticosterone from the plasma of these animals. Recent studies both *in vivo* and with the isolated perfused liver, have clearly demonstrated that the sex hormones regulate the metabolites of corticosterone excreted *via* the bile in rats [19, 22, 23].

The findings described in this paper indicate that castration of male rats led to biliary excretion of aldosterone metabolites and clearance of aldosterone and its metabolites from the plasma at rates similar to those of intact females and the elimination of the previously observed sex-dependence of these processes [12, 13]. However, ovariectomy of female rats did not cause any notable effects on these processes. Similarly, castration of male rats has been reported to shorten the plasma half-life of corticosterone, demonstrating that the lack of androgens led to a more rapid rate of hepatic clearance of this hormone, typical of females [7, 9]. Other investigators have demonstrated *in vivo* that the mixture of corticosterone metabolites excreted *via* the bile by male rats is altered to a "female" pattern following castration [19, 22].

Treatment of castrated male rats with testosterone reintroduced the sex-dependence so that aldosterone and its metabolites were cleared from the plasma and excreted *via* the bile of these animals at a slower rate typical of males. Similarly, treatment of ovariectomized female rats with testosterone significantly slowed both the rate of clearance of aldosterone and its metabolites from the plasma and their excretion *via* the bile. Treatment of both gonadectomized male and female rats with testosterone also reintroduced into the plasma the appearance of (a) considerable quantities of tetrahydroaldosterone and (b) a peak of polar metabolites of aldosterone, during the latent period of aldosterone; findings which are characteristically observed in the plasma of males [13].

The findings with aldosterone presented in this report suggest that the presence of androgens plays a major role in regulating (a) the routes of hepatic metabolism of aldosterone, (b) the rates of excretion of aldosterone metabolites *via* the bile, and (c) the concentration of aldosterone and the composition of the metabolites of aldosterone attained in the plasma during the latent period of the hormone.

Recently, it has been suggested that regulation of the hepatic metabolism of corticosterone [21, 22, 27] and other steroids (28–32) by androgens and estrogens in the rat requires the presence of an intact pituitary. It has been clearly demonstrated that the hepatic metabolism of steroid hormones in hypophysectomized female rats leads to metabolites characteristic of male rats. In the studies reported here, hypophysectomy of female rats significantly altered both the biliary excretion of aldosterone metabolites and the clearance from the plasma of aldosterone and its metabolites to rates typically observed in males. However, hypophysectomy of male rats did not cause changes in either of these two processes. Like the findings reported with other steroid hormones, our findings suggest that interactions between hypophyseal and gonadal factors may also be important in the regulation of the hepatic metabolism of aldosterone in rats. The physiological role of such interactions which regulate the plasma levels of aldosterone and its metabolites remains, as yet, unclear and requires further detailed investigations.

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## DISCUSSION

*Johnsen.* I wonder, could it not be that also in respect to your findings that rats are peculiar animals. I am referring to the fact that in the rat there is a marked sex difference in liver metabolism for example of drugs. Male rats have a very rapid drug metabolism which is not found in the females. If you castrate male rats they behave in this respect like females and if you give them testosterone they are again like males. But in man you have no such sex difference in drug metabolism. My question is therefore: Do you have any indications that your sex differences in rat aldosterone metabolism has any parallel in man?

*Morris.* First of all the whole purpose of the design and the use of the rat model and its sex-dependence was to use what is known about the pharmacology of other drugs in order to decide whether the hepatic metabolism of aldosterone could in any way be involved in its mechanism of action. Of course, we know quite well about the work with barbiturates and so on. From what we've shown I doubt if the male metabolizes aldosterone at a greater rate than the female. In fact what we have shown (contrary to our first conclusion) was that when we measured the concentration of receptors in the kidney of male and female rats at the same doses as I showed you in the first 2 slides, we found that the receptor labelling was not different in male and female rats but the amount of metabolites in the target tissue, kidney, was in a ratio of 5 to 1 and so we thought that the male must metabolize aldosterone at a faster rate. But it turns out that is not the truth. What this work shows is that the routes of hepatic metabolism of aldosterone are different and therefore we cannot say anything about the rates. The types of metabolites of aldosterone which are conjugated in the liver are such that those synthesized by the female happen to be cleared more rapidly from the body. The fact that this sex difference hasn't been discovered in man or doesn't appear to be important is not relevant. This research is trying to use the idea that rats can be used as a model so as to explain why the males have a greater response to aldosterone than the females. There is every possibility and some of the work we are doing now shows that the routes of metabolism of aldosterone are remarkably different in male and female rats and therefore the male attains certain metabolites in the plasma that the female cannot sequester. We are currently working on this

*Taylor.* The problem here is that you are dealing with a number of things which are going on at the same time, many of which are the results of the rather extreme procedures to which you subject your rats. For example, it is well known that female rats produce mainly 5α-reduced metabolites from delta-4-3-oxosteroids, whereas the male rat produces both 5α- and 5β-metabolites. Part of the reason for the sex difference in biliary secretion of steroid metabolites may be due to the "affinity"—or whatever word you use to describe it—of the biliary secretory mechanism for 5α- and 5β-steroids. Then there is the problem of a possible sex difference in the conjugation of these steroid metabolites. I don't know about aldosterone metabolites, but certainly most other metabolites of neutral steroids are converted more readily to glucuronides by female rats, and to sulphates by male rats, at least *in vitro*. Generally speaking, steroid sulphates appear to be preferentially excreted in bile and glucuronides in urine. So there are many possible complications. Added to these are the problems of the biliary secretory capacity for particular steroids at any given time, and, now that we know a little more about what goes on in the biliary tree, it is quite possible that at least part of the highly polar metabolites may be reabsorbed as they pass down the bile ductules and ducts.

*Morris.* Absolutely, this is a very fast hemodynamic situation and I could comment on 2 or 3 points here. One is that we have shown that with aldosterone, the conjugates in the bile of the females principally co-chromatograph with steroid mono- and disulfates. In the case of the male they are principally neutral, glucuronides and mono sulfates, and it is obvious that once one can get at the mixture of metabolites before conjugation these are probably the molecules which are going to reach the end organ, the kidney. That is what we are currently studying. It will be the sex differences of the types of metabolites of aldosterone which are hitting the target tissues which may be of some physiological significance. We have done preliminary studies on the enterohepatic circulation of aldosterone and its metabolites; both the male and the female do reabsorb at least 15% of the biliary metabolites which we have introduced into the duodenum. So far there does not appear to be quantitative sex differences, but there may be different types of molecules which are reabsorbed.

*Taylor.* When you feminised the male rats by gonadectomy and *vice versa*, did you measure the different types of conjugates formed?

*Morris.* Yes, preliminary experiments have shown marked conjugation differences, but we are not yet ready for publication of this data.

*Ulick.* Were the metabolites in the plasma during the latent period the same as those in the bile?

*Morris.* This question is being examined very carefully. So far we have shown at least 5 bands of chromatographically separable metabolites of aldosterone in the plasma of male rats. The molecules that we believe are sequestered in the plasma are such that the majority were unable to be conjugated at a fast enough rate and be excreted into the bile. Hence they tend to back up in the plasma. The question may be how many of them are sulfates, how many of them are neutral metabolites, acidic metabolites, and so on. That is what we are now working on. I expect them to be very much different from the metabolites in the bile of course.

*Crabbé.* Is it not surprising that you cannot reproduce with hypophysectomy what you observe after gonadec-

tomy? It is well known if one performs hypophysectomy early enough after birth in rats, they behave in terms of enzyme activity and so on like females don't they?

*Morris.* Yes in the studies that I've described so far and the rest that are in process we have not studied the biliary excretion of animals that have been both gonadectomized and hypophysectomized; the results of such experiments will be of great interest.

*Crabbé.* You told us that you were led into these studies by the fact that females seem to be less responsive to a given dose of aldosterone in terms of the kaliuretic effect, yet, if I got it right, the receptor labelling was identical in both sexes after injection of aldosterone. Would there be some kind of interference on the part of the metabolites but at a step different from receptor binding or how do you visualize the situation?

*Morris.* That's highly speculative. I would say that we can always see the appearance of the receptors in the kidney when we have antinatriuresis and we have never seen a rat that produced antinatriuresis in which the receptor labelling is not visible. There is the possibility, however, that these aldosterone metabolites could well be related to the kaliuretic effect or to the regulation of the antinatriuretic effect, and this remains to be investigated in detail.