PRODUCTION, CLEARANCE RATES AND METABOLIC FATE OF ESTRADIOL-17β IN THE PLASMA OF THE LAYING HEN

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ABSTRACT

A single dose of tritiated estradiol-17β (^{3}H-$E_{2}\beta$) was injected i.v. into 5 high egg producing White Leghorn hens, 31 weeks of age, at 19.2 ±2.1 (mean ±S.D.) hr before oviposition. Blood (2 ml) was sampled at approximately 5 min intervals over 40 min. Whenever possible, metabolites were monitored and identified by the double isotope technique with the addition of the corresponding ^{14}C-labelled standards to plasma prior to analysis. The metabolic half-life and clearance rate of ^{3}H-$E_{2}\beta$ in plasma were 10.9 ± 1.9 min and 118 ±18 ml/min/kg body weight, respectively. The calculated production rate of $E_{2}\beta$ at 19.2 hr before oviposition was 19.5± 5.7 ng/min based on the plasma level (93±22 pg/ml) measured at that time. The relative concentrations (% of plasma radioactivity) of the major metabolites isolated at 5.7 ± 0.6 min post injection were, in descending order: estradiol-17β-3-sulfate ($E_{2}\beta$-3S : 14.9± 2.7), estradiol-17α-3-sulfate ($E_{2}\alpha$-3S; 5.7 ±0.3), estrone (E_{1}; 4.6 ±0.5), estrone sulfate ($E_{1}S$; 2.2 ± 0.5), and estradiol-17α ($E_{2}\alpha$; 1.2 ±0.4). As time proceeded, the relative concentration of $E_{2}\alpha$-3S gradually increased so that by 43.2 ± 1.0 min it became the most abundant identifiable metabolite (12.3 ± 1.1) followed by $E_{2}\beta$-3S (9.1 ± 1.7), $E_{1}S$ (1.2 ± 0.6), E_{1} (0.7 ± 0.4) and $E_{2}\alpha$ (0.3 ± 0.2). These findings are consistent with the view that one of the major pathways of $E_{2}\beta$ metabolism in the circulation of the hen is via $E_{2}\beta \rightleftharpoons E_{2}\beta\text{-}3S \rightleftharpoons E_{1}S \rightleftharpoons E_{2}\alpha\text{-}3S$.

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INTRODUCTION

A number of investigators have measured plasma concentrations of estradiol-17β($E_2\beta$) relative to the ovulatory cycle of the laying hen (1-7). These studies have shown a major peak at 2 to 7 hr and possibly a minor peak 16-20 hr before ovulation. The role of the $E_2\beta$ peaks with respect to ovulation is not clear and, so far, all attempts to induce premature ovulation with exogenous $E_2\beta$ have resulted in delayed ovulation (8) or no response (4). The $E_2\beta$ peaks, conceivably, have an influence on the calcification of the egg because estrogen is known to stimulate medullary bone formation (9), increase protein bound calcium (Ca) in plasma (10,11) and induce or enhance formation in the shell gland of a Ca-binding protein believed to be involved in Ca transport (12). Recently, we have demonstrated that young laying hens actively immunized against $E_2\beta$ lay eggs with decreased egg shell quality (13).

In order to investigate the role of $E_2\beta$ in the mobilization of Ca for egg calcification we found it desirable to know the clearance rate and production rate of $E_2\beta$ because many past studies of $E_2\beta$ functions involved non-physiological doses of the hormone. While the metabolites of $E_2\beta$ in the urine (14,15) of the laying hen have been investigated, there is little information on the plasma clearance and production rates of $E_2\beta$ nor of the kinetics of the metabolites. When this investigation was in progress, Johnson and van Tienhoven (16) reported the clearance and production rates of $E_2\beta$ in 5 laying hens, but they did not study the metabolites.

The present investigation was conducted to study the production

rate, clearance rate and metabolic fate of $E_2\beta$ in the plasma of a strain of White Leghorn laying hens, which had been selected for high egg production over 25 years (17,18) and which we used for egg shell quality research.

MATERIALS AND METHODS

Hens

Five 31-week-old high egg producing White Leghorns (Strain 1) of the Animal Research Centre (17,18) were caged individually in a windowless room. The hens had been under a light regime of 16 hr light and 8 hr dark since they were 20 weeks of age. Feed and water were provided ad libitum and the temperature was maintained at 13°C. The hens had laying cycles ranging between 24 and 26 hr and had laid consecutively at least 2 eggs immediately prior to the experiment. Their hen-day egg production averaged 95% around 31 weeks of age. The tracer injection was given between 17 and 23 hr before the next oviposition (see below and Table 1 for details).

Solvents and Reagents

All solvents were reagent grade and were used as obtained. Unless otherwise stated, radioactive and non-radioactive steroids were obtained commercially and their purity was checked by thin-layer chromatography (TLC).

Estrone-4-^{14}C sulfate, ammonium salt (^{14}C-E_1S), used as internal tracer, was synthesized from estrone-4-^{14}C (52 mCi/mmol) as described by Longcope (19), and purified by TLC (20). Estradiol-17β-4-^{14}C-3-sulfate (^{14}C-$E_2\beta$-3S), used as internal tracer, was prepared by reduction of ^{14}C-E_1S with sodium borohydride (21).

Administration of tritiated labelled estradiol-17β (^{3}H-$E_2\beta$)

Immediately prior to the tracer injection, a 2 ml blood sample was taken from the brachial vein for radioimmunoassay of $E_2\beta$ (7,21,22). Then, 10 µCi of ^{3}H-$E_2\beta$ (2,4,6,7-^{3}H; 90 Ci/mmol), dissolved in 0.05 ml propylene glycol plus 0.45 ml of 0.9% saline, were injected into the left brachial vein. The net amount injected averaged 9.7 µCi, with 0.3 µCi left in the syringe. Blood (2 ml) was withdrawn from the right brachial vein with a heparinized syringe at approximately 5 min intervals over 40 min. Within 20 min the plasma was separated by centrifugation (20) and stored at -20°C until assayed.

Counting of Radioactivity

Plasma (0.02 ml), labelled standards or isolated metabolites were counted in a Beckman model LS-8000 scintillation spectrometer with automatic quench compensation as described previously (30).

Extraction of estrogens from plasma

Prior to extraction, known amounts of ^{14}C-labelled estrone (E_1), $E_2\beta$, E_1S and $E_2\beta$-3S were added to each plasma sample as internal standards for correction of procedural losses for the corresponding ^{3}H-labelled metabolites. Because ^{14}C-labelled estradiol-17α($E_2\alpha$) and estradiol-17α-3-sulfate ($E_2\alpha$-3S) were not available, the losses of these two ^{3}H-labelled metabolites were estimated from the losses of the corresponding ^{14}C-labelled 17β isomers.

Plasma (0.5-1.0 ml) was partitioned in sequence, with benzene and then tetrahydrofuran:ethyl acetate (1:1;THFE) to extract the unconjugated estrogens and estrogen monosulfates essentially as described (22) except that 1 ml of 18% NaCl solution was used instead of sodium bicarbonate.

Chromatographic separation of estrogens

Unconjugated E_1, $E_2\alpha$, and $E_2\beta$ from the benzene fraction were isolated and identified essentially as described previously (20) except that the identity of ^{3}H-$E_2\alpha$ was based on chromatographic mobility with unlabelled standard, because of the unavailability of ^{14}C-$E_2\alpha$,. However, from a previous study using the double isotope technique (20) there was good evidence that the radiochemical purity of $E_2\alpha$ approached 90% after Step 1 (20) of the purification procedure.

The sulfate fraction was purified on TLC using ethyl acetate: ethanol:ammonium hydroxide (90:90:1.8) which was a modification of the system reported by Sarfaty and Lipsett (23) and which separated the steroid monosulfates, glucuronides and unconjugated steroids as classes. By developing the thin-layer plate in the modified system 4 times under conditions described (22) it was possible to separate the 3-sulfate from the 17-sulfate of estradiol-17(α,β), but the 3-sulfate of estradiol-17(α,β) and of estrone still remained together. The area containing E_1S plus E_2(α,β)-3S was eluted and the eluent hydrolysed with 20% acetic acid in methanol (21). The unconjugated estrogens released by hydrolysis were isolated as described for the benzene fraction. The 17-sulfate of E_2(α,β) resisted hydrolysis by acetic acid; radioactivity in this spot was counted without correction for procedural losses, and no further identification was made. Thus the identify of this (these) metabolite(s) was (were) tentative.

Calculation of clearance rate and production rate of $E_2\beta$

The disappearance curve of ^3H-$E_2\beta$ in plasma after i.v. injection was found to best fit the two-compartmental model described by Rizkallah et al (24), and the metabolic clearance rate was calculated based on this model.

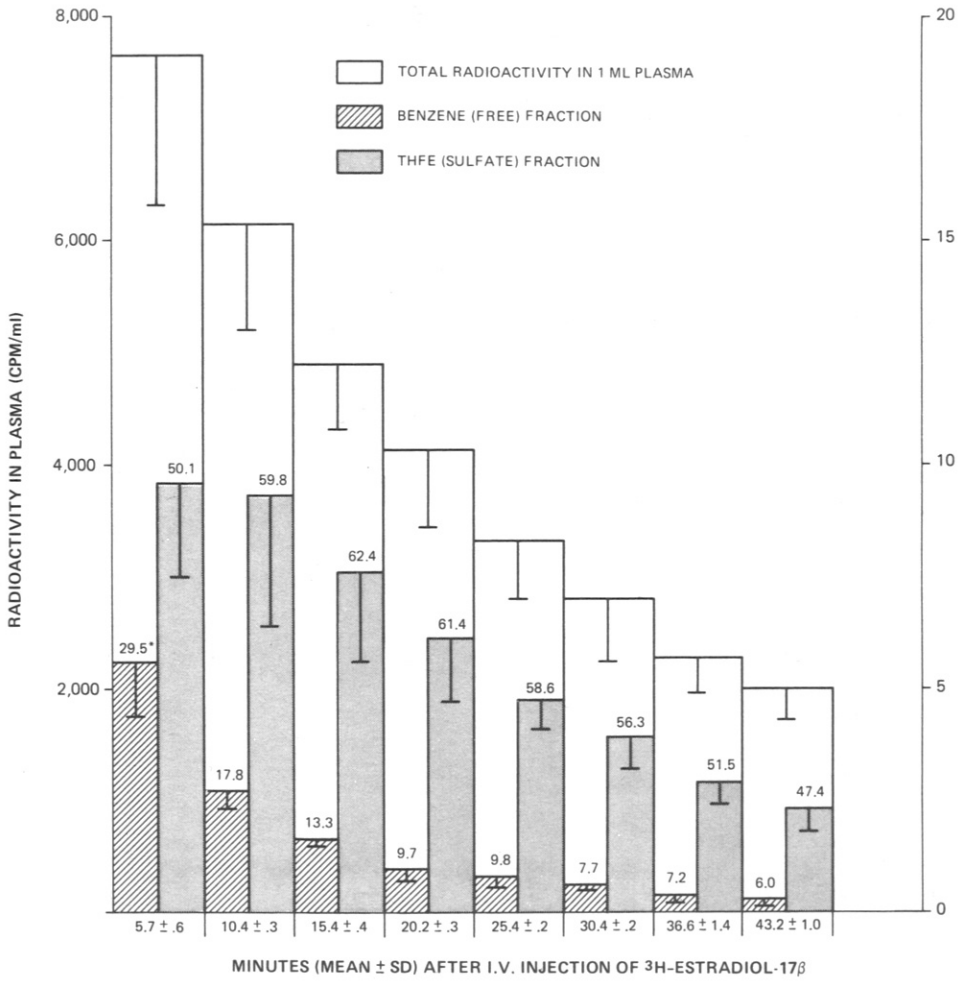

FIG. 1. DISTRIBUTION OF FREE AND MONOSULFATE ESTROGENS IN THE PLASMA OF 5 HENS AFTER ^3H-$E_2\beta$ INJECTION.
*Figures indicate the mean percent of radioactivity in plasma; bars represent standard deviations.

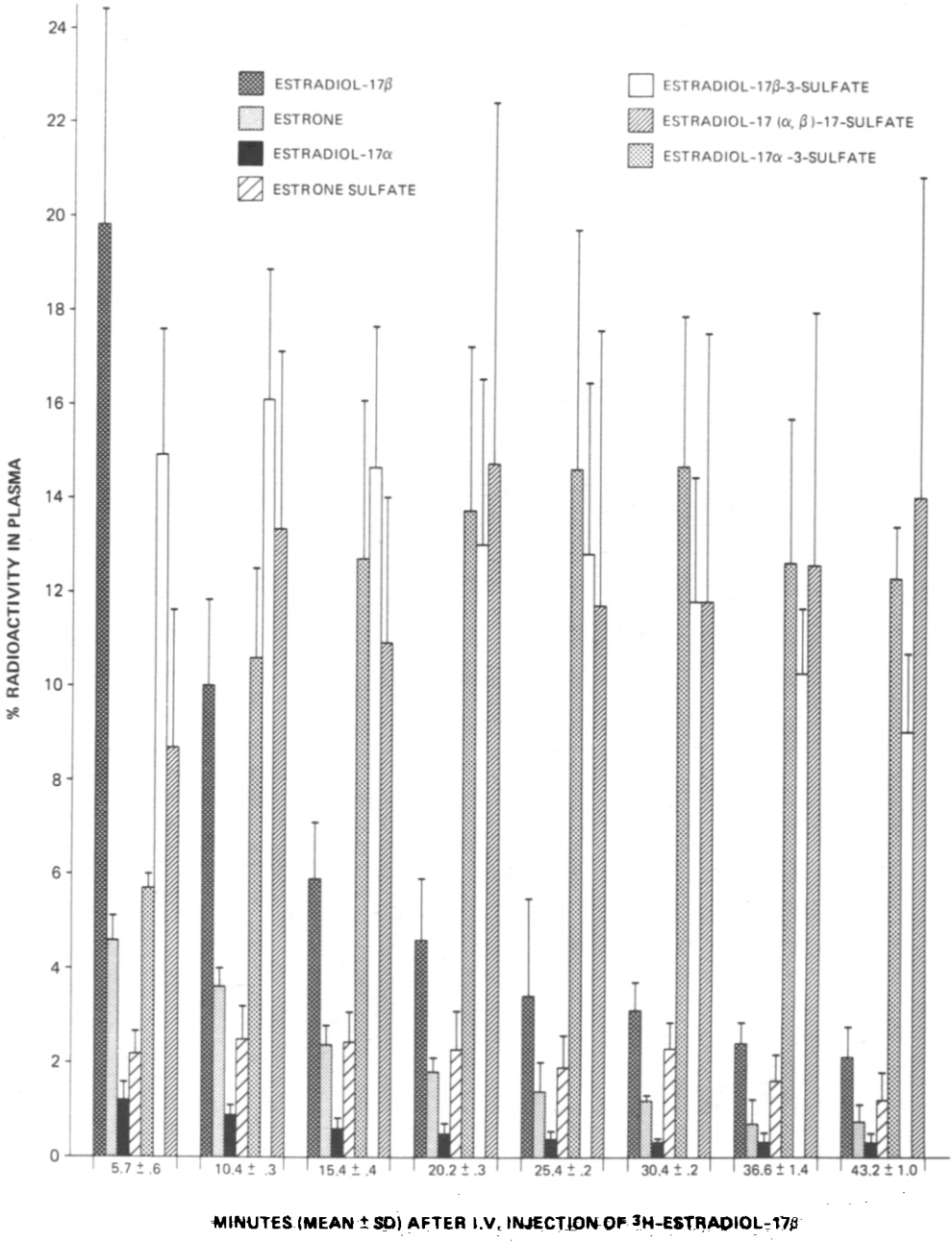

FIG. 2. CHANGES IN RELATIVE PLASMA CONCENTRATIONS OF METABOLITES OF ^{3}H-$E_{2\beta}$ OVER TIME AFTER IV INJECTION OF ^{3}H-$E_{2\beta}$

RESULTS

All results represent the mean ± SD (standard deviation) from 5 hens. Fig. 1 shows the disappearance of radioactivity from the plasma, and the distribution of the unconjugated (free) and the sulfate fractions as a function of time after i.v. injection of ^{3}H-E$_2\beta$. At around 6 minutes post injection, about 50% of the radioactivity in plasma was already in the THFE or sulfate fraction, and only 30% in the benzene or free fraction, indicating a rapid conversion of the injected free hormone into the monosulfate metabolites. By 15 minutes, the relative concentration of the sulfate fraction increased to a peak value of 62% and then slowly declined to 47% by 43 minutes. In contrast, the free-fraction continued to decline rapidly so that by 15 min and 43 min it accounted for only about 13% and 6% of plasma radioactivity respectively. It is also of interest to note that the total radioactivity recovered by the benzene and THFE extractions, after corrections for procedural losses, accounted for 79.6% (29.5% + 50.1%) of the plasma radioactivity at 6 min post injection. By 43 min post injection, the figure had dropped to 53.4% (6% + 47.4%). This would indicate that as time progressed more polar metabolites, such as the disulfates, were formed and remained in the aqueous phase. The exact nature of this relatively polar fraction was not investigated in this study.

The relative plasma concentrations of the isolated ^{3}H-labelled free estrogens and estrogen monosulfates are shown in Fig. 2.

^{3}H-E$_2\beta$. This injected substrate accounted for only about 20% of the total plasma radioactivity by 6 min post injection; by 43 min it

had decreased to 2%. The disappearance of absolute radioactivity (not shown) was typically exponential and fitted best the two-compartmental model on which the metabolic clearance rate (MCR) and related parameters were calculated (Table 1).

TABLE 1. The metabolic clearance rate (MCR) and production rate (PR) of estradiol-17β in the laying hen

Hen	Body Weight (kg)	MCR (ml/min)	MCR_b (ml/min/Kg)	Plasma $E_2\beta$ (ng/ml)	PR (ng/min)	Half-life $t_{\frac{1}{2}}\beta$ (min)	*Hr to next oviposition
66	1.60	223	139	0.124	27.7	12.1	22.5
75	1.90	217	114	0.087	18.9	8.2	16.5
76	1.79	217	121	0.066	14.3	13.0	19.7
83	1.71	216	126	0.103	22.2	11.4	18.6
86	1.83	167	91	0.085	14.2	10.0	16.8
Mean	1.77	208	118	0.093	19.5	10.9	19.2
S.D.	0.12	23	18	0.022	5.7	1.9	2.1

Age of birds: 31 weeks

*Time of injection of ^3H-$E_2\beta$ with reference to oviposition.
MCR_b = MCR per kg body weight.

^3H-E_1. An oxidized metabolite of ^3H-E_2, the relative concentration of this metabolite was highest at 6 min post injection (5% of plasma radioactivity) and declined to a low level of only 0.7% by 43 min.

^3H-$E_2\alpha$. This was the least abundant of all the metabolites detected. Its highest concentration was 1% of plasma radioactivity at 6 min post injection and progressively decreased to 0.3% by 43 min. It

was derived from ^{3}H-E_1 by enzymic reduction (15,27).

^{3}H-$E_2\beta$-3S. This was by far the most abundant metabolite 6 min post injection, accounting for 15% of the total plasma radioactivity, compared with 6% in the case of ^{3}H-$E_2\alpha$-3S and 9% in the case of ^{3}H-$E_2(\alpha,\beta)$-17S. It remained the predominant metabolite for the first 15 min. Then, its level slowly declined to below that of ^{3}H-$E_2\alpha$-3S. The most direct route of the formation of this metabolite was by 3-sulfoconjugation of ^{3}H-$E_2\beta$.

^{3}H-E_1S. In contrast to ^{3}H-$E_2\beta$-3S, ^{3}H-E_1S was the least abundant of the monosulfate metabolites, with relative concentrations ranging from 3% of plasma radioactivity at 10 min, to 1% at 43 min post injection. It was most likely formed by oxidation of ^{3}H-$E_2\beta$-3S and/or 3-sulfoconjugation of ^{3}H-E_1.

^{3}H-$E_2\alpha$-3S. This was quantitatively the third most important metabolite in the first 15 min and became the second most abundant metabolite at 43 min (12% plasma radioactivity). It was derived by reduction of ^{3}H-E_1S and/or 3-sulfoconjugation of ^{3}H-$E_2\alpha$.

^{3}H-$E_2(\alpha,\beta)$-17S. The area corresponding to $E_2\beta$-17S likely contained a mixture of ^{3}H-$E_2\beta$-17S and ^{3}H-$E_2\alpha$-17S. In spite of the fact that procedural losses were not corrected, this fraction ranked second in relative concentration in the first 15 min and tended to become the predominant fraction (14%) by 43 min. The large standard deviation probably reflect variations due to procedural losses.

DISCUSSION

The results indicate that ^{3}H-$E_2\beta$ in the circulation of the laying hen was metabolized rapidly. The mean plasma metabolic half-life ($t_{\frac{1}{2}}\beta$) of ^{3}H-$E_2\beta$ in the 5 hens was 10.9 min and the MCR was 208 ml plasma/min/hen or 118 ml plasma/min/kg body weight. The estimated mean PR of $E_2\beta$, based on the mean plasma concentration of 93 pg/ml measured about 19 hr to oviposition, was 19.5 ng/min (Table 1). The rapidity of metabolism is also seen by the fact that as early as 5 to 6 min after intravenous injection only 20% of the plasma radioactivity remained as ^{3}H-$E_2\beta$ while 50% was in the conjugated fraction in the form of monosulfates. The rapid disappearance of ^{3}H-$E_2\beta$ from plasma agrees well with the data of Hawkins and Taylor (25) who found that 3.75 min after i.v. injection of ^{3}H-$E_2\beta$ into a strain of White Leghorn laying hens 24-20 hr before ovulation, only 25% of the plasma radioactivity was associated with ^{3}H-$E_2\beta$. Johnson and van Tienhoven (16) reported a mean $t_{\frac{1}{2}}\beta$ of 27.5 min and MCR of 73.7 ml/min/kg for $E_2\beta$ in a strain of 5 White Leghorn laying hens 26 to 24 hr prior to ovulation. Their calculated plasma PR of $E_2\beta$, based on the mean plasma concentration of 55 pg/ml, was 6.4 ng/min. These values of Johnson and van Tienhoven reflect a slower metabolism of $E_2\beta$. Several factors such as differences in strain, age, physiological state and methodology could have contributed to the differences.

As mentioned before, our experimental Strain 1 White Leghorn hens were high egg producers which at the time of the experiment (31 weeks of age) had 93% hen-day production. Johnson and van Tienhoven

mentioned that the age of their hens was between 30 and 40 weeks and with a clutch size of 4 to 7 eggs, which would be equivalent to 80% to 88% hen-day production. Since $E_2\beta$ is primarily produced by the follicles of the ovary (26) it is conceivable that a strain of high egg producers will have a higher rate of $E_2\beta$ production, perhaps compensated by a higher rate of clearance. The time of tracer injection in relation to ovulation or oviposition is also important.

Johnson and van Tienhoven administered ^3H-$E_2\beta$ about 26 to 24 hr prior to ovulation when plasma levels of $E_2\beta$ (reported to be 55 pg/ml) were expected to be basal. In our case, injection was given between 17 and 22 hr before oviposition when the mean concentration of $E_2\beta$ was 93 pg/ml, which might have been close to the minor peak and would have resulted in a higher calculated PR even if the MCR had remained unchanged (PR=MCR x concentration). The higher concentration of $E_2\beta$ measured in our hens could have been due to differences in strain and/or physiological stage. At present, we are not certain whether or not the MCR is also influenced by the ovulatory cycle.

Johnson and van Tienhoven did not attempt to separate ^3H-$E_2\beta$ from ^3H-$E_2\alpha$. Mathur and Common (15) clearly demonstrated the conversion of ^{14}C-$E_2\beta$ to ^{14}C-$E_2\alpha$ via ^{14}C-E_1 (see Fig. 3). Later, Chan and Common (27) showed that E_1-4-^{14}C injected intramuscularly into the laying hen was reduced to ^{14}C-$E_2\alpha$ and ^{14}C-$E_2\beta$ in plasma. In the present study, we confirmed that ^3H-$E_2\alpha$ was a minor metabolite of ^3H-$E_2\beta$. The radioactivity of ^3H-$E_2\alpha$ was 8% to 13% of that of ^3H-$E_2\beta$ beyond 10 min

post injection. Thus, if ^{3}H-$E_2\beta$ had not been separated from ^{3}H-$E_2\alpha$, the calculated $t_{½\beta}$ would have been greater. However, the contamination of ^{3}H-$E_2\beta$ by ^{3}H-$E_2\alpha$ is probably not sufficient to account for the considerable lower metabolism of $E_2\beta$ in the work of Johnson and van Tienhoven. We believe that the differences in strain and blood sampling time in relation to ovulation are the primary factors.

Mathur et al (28) have shown that estrogens are present in the urine of the hen mostly as monosulfates and disulfates. We have recently reported $E_2\beta$-3S as quantitatively the most important plasma estrogen measurable by radioimmunoassay in the laying hen (7). Chan and Common (27) indicated that a large part of E_1-4-^{14}C injected into 2 hens appeared in plasma in the 'bound' fraction which they believed was the monosulfates of estrogens and from which they isolated ^{14}C-$E_2\alpha$ and ^{14}C-$E_2\beta$ upon solvolysis. They did not follow the kinetics of the metabolites.

The present results confirm that the estrogen monosulfates constitute a major portion of the estrogen metabolites. The kinetic data further indicate that 3-sulfoconjugation is one of the most important steps in the metabolism of $E_2\beta$ as is evident from the fact that by far the predominant metabolite 5-6 min post injection of ^{3}H-$E_2\beta$ was ^{3}H-$E_2\beta$-3S. The present data provide no direct evidence that ^{3}H-$E_2\beta$-3S can be oxidized to ^{3}H-E_1S without prior hydrolysis of the sulfate group. However, the elegant work of Mathur (29) clearly demonstrated that ^{3}H-E_1-3-^{35}S injected into the hen was converted to ^{3}H-$E_2\alpha$-3-^{35}S and ^{3}H-$E_2\beta$-3-^{35}S directly, i.e.,

without prior hydrolysis of the sulfate ester. If the reverse reaction can be assumed to occur, then the present kinetics suggest that $E_2\beta \rightleftharpoons E_2\beta\text{-}3S \rightleftharpoons E_1S \rightleftharpoons E_2\alpha\text{-}3S$ represents a major pathway for $E_2\beta$ metabolism (Fig. 3). This is evident from the initial accumulation of $^3H\text{-}E_2\beta\text{-}3S$. However, the level of $^3H\text{-}E_2\alpha\text{-}3S$ gradually increased so that by the end of the experiment it had surpassed the level of $^3H\text{-}E_2\beta\text{-}3S$ (see Fig. 2). In contrast, the relative concentration of $^3H\text{-}E_1S$ remained relatively stable, consistent with the role of an intermediate metabolite (Fig. 3). Undoubtedly, $E_2\beta$ is also metabolized to E_1 and $E_2\alpha$, each of which can then be 3-sulfoconjugated. However, the relative levels of $^3H\text{-}E_1$, and $^3H\text{-}E_2\alpha$ (in particular) were relatively low at all times in comparison with $^3H\text{-}E_2\beta\text{-}3S$ suggesting that their direct contribution to the corresponding 3-sulfates is limited.

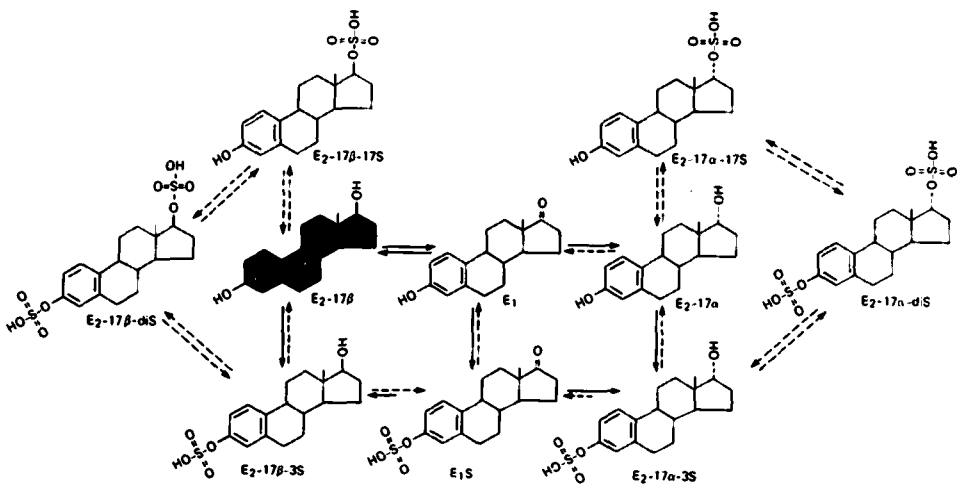

FIG. 3. METABOLISM OF ESTRADIOL-17β IN THE HEN.
Reactions well established are indicated by ——; reactions which are likely or theoretically possible but not unequivocally demonstrated *in vivo* are indicated by ---.

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