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The Determination of Oestradiol and Oestrone in the Plasma of the Domestic Fowl by a Method involving the Use of Labelled Derivatives

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1. A method involving the use of triple-labelled derivatives has been developed for the determination of total oestrone and oestradiol in the plasma of the domestic fowl. The double-labelling technique devised by Svendsen (1960) for the determination of free oestrogens in human plasma was modified to enable the total oestrogen recovery to be determined for each sample. 2. [6,7-$^3H_2$]Oestradiol-17β is added to the plasma samples (1–10ml.), which are hydrolysed with acid and the phenolic steroids then extracted and partially purified. The extract is esterified with iodobenzene-*p*[^{35}S]-sulphonyl chloride of high specific activity. After addition of standard oestrogen [^{131}I]iodobenzene-*p*-sulphonates the esters are finally purified by paper chromatography. 3. The oestrogens are determined by comparing the $^3H/^{35}S$ and $^{131}I/^{35}S$ ratios in the purified esters with similar ratios of appropriate standards. 4. With this procedure the recoveries of oestrone and oestradiol after hydrolysis were 70–85% and 72–84% respectively, and after hydrolysis and preliminary purification 38–53% and 39–51% respectively. With this procedure up to 500ng. of oestradiol can be determined. The sensitivity of the technique for oestrone is 3·0ng. and for oestradiol 2·1ng. 5. The ranges of oestradiol and oestrone concentrations found in six plasma samples were 8·3–21·4ng./ml. and 15·2–31·6ng./ml. respectively.

The steroid oestrogens have long been known to have considerable and varied effects on the metabolism of the domestic fowl (Sturkie, 1966; Van Tienhoven, 1961). In particular, the growth of the oviduct and the synthesis of lipids and proteins by the liver are grossly stimulated by oestrogen administration (Dorfman & Dorfman, 1948; Entenman, Lorenz & Chaikoff, 1940; Heald & McLachlan, 1965; Heald & Rookledge, 1964).

The developing ovary is considered to be the source of the oestrogens necessary for the stimulation of yolk formation in the bird (Van Tienhoven, 1961). Oestrogenic secretion by the avian ovary has been demonstrated by Marlow & Richert (1940). Oestrone, oestradiol and oestriol have been isolated from the ovary of the laying hen (Layne, Common, Maw & Fraps, 1958). Early attempts to isolate and identify the plasma oestrogens of the laying hen met with little success, although oestrone was tentatively stated to be present as a conjugate by Layne *et al.* (1958). Ozon (1965), in a fluorimetric study of the oestrogens of plasma of the laying fowl, estimated that the concentration of oestrone was 15·9ng./ml. and that of oestradiol 2·2ng./ml. Ozon's (1965) study was limited, however, in that only two plasma samples (60ml. each) were examined and no estimate was made of losses of oestrogens during the extraction and purification of the individual samples.

As part of a study of the mechanism of ovulation in the fowl, a quantitative method for the determination of plasma oestrogens was required. A major difficulty in such studies, however, was that the colorimetric, fluorimetric and chromatographic methods available were unsuitable through lack of sensitivity and specificity for the determination of small quantities of oestrogen in the presence of the extensive amounts of lipid and protein that are found in the plasma of the laying hen.

With the advent of methods involving the use of double-labelled derivatives for the determination of plasma oestrogens (Svendsen, 1960), it became possible to isolate and identify oestrone and oestradiol in plasma from the laying domestic fowl (O'Grady & Heald, 1965; O'Grady, 1966).

In these studies, the oestrogens were purified by solvent partition (Svendsen, 1960), methylated with [^{3}H]dimethyl sulphate and purified by chromatography on alumina columns (Brown, 1955) and by thin-layer chromatography (Lisboa & Diczfalusy, 1960). However, attempts to develop this technique into a quantitative procedure were unsuccessful,

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since the methylation reaction could not be made to give either a quantitative or constant yield in the presence of the small amounts of high-specific-activity [^{3}H]dimethyl sulphate available for the assay. Since the methylation procedure had such a low reproducibility, it was replaced by a [^{35}S]pipsyl chloride (iodobenzene-*p*[^{35}S]-sulphonyl chloride) esterification procedure, which was originally devised by Bojesen (1956) and developed into a quantitative technique for oestrogen esterification and assay by Svendsen (1960).

In Svendsen's (1960) procedure the phenolic steroids contained in samples of human plasma were extracted and purified by a series of solvent partitions. This steroid extract was esterified with [^{35}S]pipsyl chloride. The products of esterification were mixed with standard [^{131}I]pipsyl esters and purified by paper-chromatographic techniques similar to those developed by Bush (1952). The oestrogens were estimated by comparing the ^{35}S/^{131}I ratios of the sample oestrogen pipsyl esters with those of the labelled pipsyl esters of known amounts of oestrogen. The addition of the ^{131}I-labelled ester allows the esters to be located during chromatographic purification and also gives an estimate of the chromatographic losses of the [^{35}S]oestrogen ester. However, Svendsen's (1960) method does not permit any assessment of the oestrogen losses incurred in each determination before esterification. This difficulty has now been overcome by adding to the plasma before processing a tritiated oestrogen of high specific activity to allow an estimate of total oestrogen recovery to be made. By this technique, the oestradiol and oestrone contents of 1·0ml. of plasma from the laying domestic fowl have been determined. This paper describes a method for measuring the total oestrone and oestradiol in plasma from the domestic fowl by a modification of Svendsen's (1960) assay technique with double-labelled derivatives.

METHODS

The following procedure is based on Svendsen's (1960) method. In the interests of clarity, much of the detail of the original procedure has been repeated below.

Hydrolysis and extractions of plasma (*Scheme* 1)

Stage 1. [6,7-$^3H_2$]Oestradiol-17β (60000 disintegrations/min.) in ethanolic solution was pipetted into a 100ml. round-bottomed flask and the ethanol evaporated in a stream of dry N_2 at room temperature (25°). Simultaneously, identical portions of the [6,7-$^3H_2$]oestradiol-17β solution were pipetted into glass-stoppered test tubes and, after evaporation of the solvent, stored at 2° until required for measurement of radioactivity. Plasma (1vol., i.e. 1–10ml.) was pipetted into the flask and, after dilution with water (10vol.), conc. HCl (1·75vol.) was added and the whole was heated under reflux for 30min. After being cooled, the hydrolysate was quantitatively transferred to a 250ml. separating funnel and extracted with 20ml. of chloroform that had previously been used to wash the hydrolysis flask and the transfer funnel. The chloroform extract was transferred to a 100ml. round-bottomed flask and the extraction of the original aqueous phase was repeated with two further 20ml. volumes of chloroform. Emulsions tended to form during this procedure but were readily broken on standing for 10min. Any emulsion finally remaining was separated by centrifugation for 5min. at 2000*g*. The total chloroform extract was dried by the addition of anhydrous Na_2SO_4 (3–5g.) and, after being transferred to a 100ml. round-bottomed flask with the aid of 5ml. of fresh chloroform, dried in a rotary evaporator at 40°. The dried extract was stored in the stoppered flask overnight at 2°.

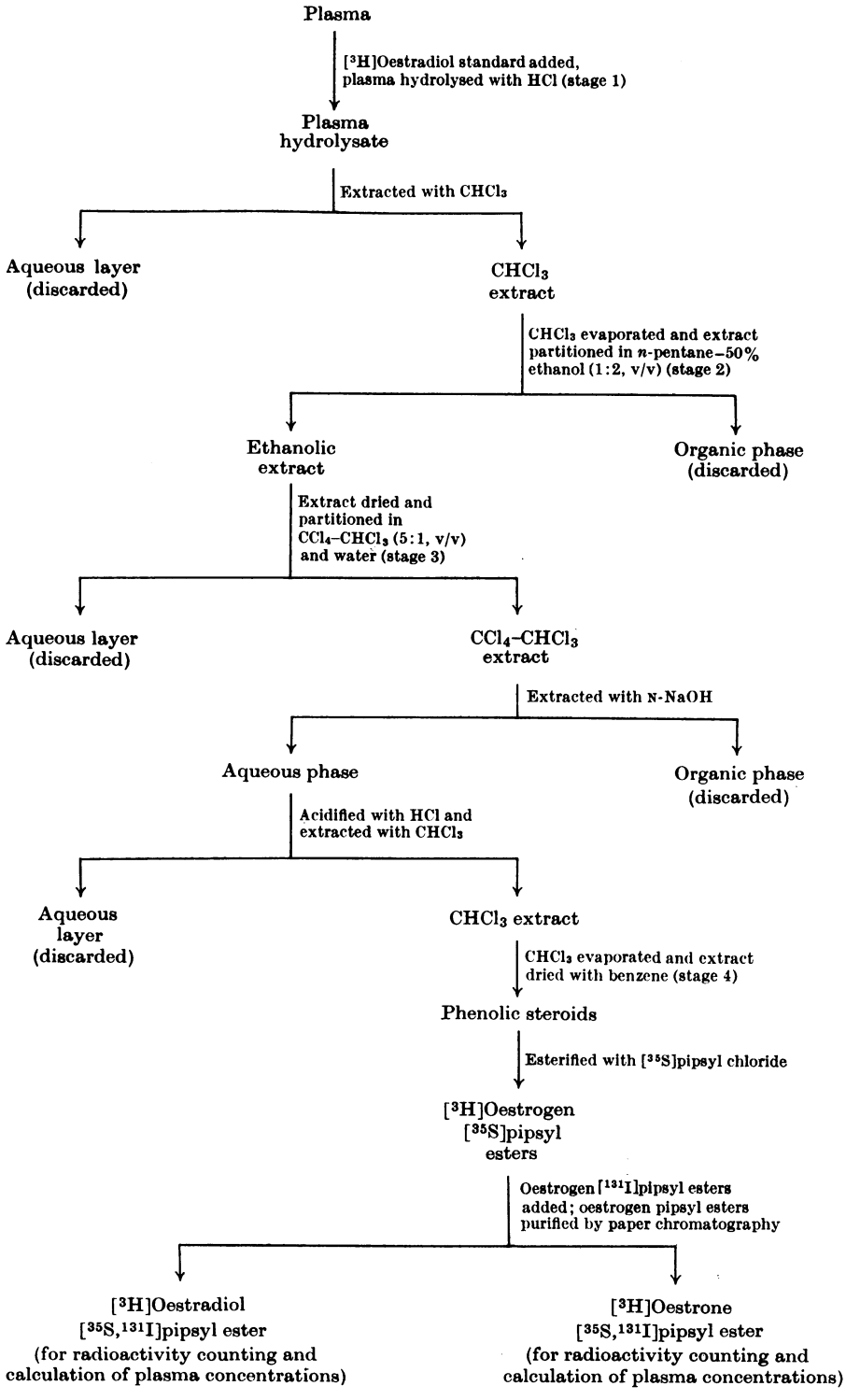
Preliminary purification of the chloroform extract (*Scheme* 1)

Stage 2. The dried extract was transferred to a test tube (105mm.×12mm.) by means of chloroform–methanol (1:1, v/v) and the solution was evaporated to dryness in a stream of N_2 at 40°. Ethanol (0·5ml.), water (0·5ml.) and *n*-pentane (0·5ml.) were added to the residue and the system was thoroughly shaken. On standing for 5min. the two phases usually separated and the pentane layer, which contained plasma lipid, was removed and discarded. The aqueous solution was washed twice more with 0·5ml. volumes of *n*-pentane.

Stage 3. After the final aqueous extract had been dried under N_2 at 40°, the residue was dissolved in carbon tetrachloride–chloroform (5:1, v/v) and washed twice with water (0·5ml.). The aqueous phase was discarded and the organic phase was extracted by shaking three times with 0·5ml. portions of N-NaOH. The total NaOH solution was acidified with 0·15ml. of 11N-HCl and extracted with three 0·5ml. volumes of chloroform. The combined chloroform extracts were washed once with 0·1M-Na_2HPO_4 (0·5ml.) and once with water (0·5ml.). After washing, the chloroform solution was evaporated in a test tube (8mm.×75mm.) under N_2 at 40°. The final extract was dissolved in 0·5ml. of benzene and again evaporated. This procedure was repeated twice more and the tube was stoppered and, if necessary, stored overnight at 2°.

Esterification and determination of the oestrogens (*Scheme* 1)

Stage 4. (i) Esterification of the purified plasma extract. The purified extract was dissolved in 0·1N-NaOH (25μl.), and acetone (25μl.) and water (25μl.) were added. After shaking to ensure complete solution of the extract [^{35}S]-pipsyl chloride (0·2mg.) dissolved in acetone (25μl.) was added. The inside of the tube was rinsed with acetone (25μl.) and shaking was continued for 30min. Standard solutions of oestrone and oestradiol [^{131}I]pipsyl ester indicators were prepared immediately before use and adjusted to give 20000 counts/100sec./0·2ml. of chloroform solution. The [^{131}I]pipsyl esters were added to the esterification mixture (0·200ml. of each). The indicator solutions (0·200ml.) were also pipetted into separate test tubes and, after evaporation of the chloroform, stored at 2° until required for measurement of radioactivity at the end of the procedure. After thorough mixing of the ^{131}I-labelled standards with the esterification solution, the chloroform


Plasma
[3H]Oestradiol standard added, plasma hydrolysed with HCl (stage 1)
Plasma hydrolysate
Extracted with CHCl3
Aqueous layer (discarded)
CHCl3 extract
CHCl3 evaporated and extract partitioned in n-pentane–50% ethanol (1:2, v/v) (stage 2)
Ethanolic extract
Organic phase (discarded)
Extract dried and partitioned in CCl4–CHCl3 (5:1, v/v) and water (stage 3)
Aqueous layer (discarded)
CCl4–CHCl3 extract
Extracted with N-NaOH
Aqueous phase
Organic phase (discarded)
Acidified with HCl and extracted with CHCl3
Aqueous layer (discarded)
CHCl3 extract
CHCl3 evaporated and extract dried with benzene (stage 4)
Phenolic steroids
Esterified with [35S]pipsyl chloride
[3H]Oestrogen [35S]pipsyl esters
Oestrogen [131I]pipsyl esters added; oestrogen pipsyl esters purified by paper chromatography
[3H]Oestradiol [35S,131I]pipsyl ester (for radioactivity counting and calculation of plasma concentrations)
[3H]Oestrone [35S,131I]pipsyl ester (for radioactivity counting and calculation of plasma concentrations)

Scheme 1.

phase was twice washed with water (0·25 ml.), twice with methanol–water (1:1, v/v) and twice again with water (0·25 ml.). The chloroform was then evaporated under N_2.

(ii) Purification and isolation of the radioactive oestrogen pipsylates. The paper-chromatographic purification was carried out by the descending technique. The esters were transferred to the paper chromatograms with a small amount of chloroform. The papers were placed in the tank and allowed to equilibrate for 3 hr. before irrigation with the solvent for 1·25–1·75 hr., during which time the solvent front had advanced about 20 cm. After development the chromatograms were wrapped in aluminium foil. The radioactive areas were located by means of an IDL chromatogram scanner adjusted to detect only ^{131}I and then cut out, and the paper corresponding to this area was lodged halfway down a test tube with the aid of forceps. The esters were each eluted by repeated washing with a 1 ml. portion of chloroform–methanol (1:4, v/v). The paper was finally washed with a further 0·5 ml. of chloroform–methanol (1:4, v/v), and the total eluate (1·5 ml.) evaporated to dryness. The esters were transferred to the origin of the next chromatogram with a small amount of chloroform and, after separation, radioautography and a chromatogram scanner were used to locate the esters. The first chromatographic step separated the oestrone and [^3H]oestradiol [^{35}S,^{131}I]pipsyl esters [system I of Svendsen (1960): light petroleum (b.p. 100–120°)–water (1:1, v/v)]. The oestrone ester was purified by running in system II [light petroleum (b.p. 100–120°)–methanol–water (10:9:1, by vol.)] and finally in system III [light petroleum (b.p. 100–120°)–methanol–water (10:1:9, by vol.)]. The oestradiol ester was purified by using the same systems, except that separation in system III and then in system II was employed. Radioautography was used to locate the esters after the first and last chromatographic purifications and chromatogram scanning after the second. The spots of oestrogen located in the last chromatogram were divided into an upper and lower section and eluted separately. The eluates were dried under N_2 and the radioactivity was measured by liquid-scintillation counting. An oestradiol [^{35}S]pipsyl ester standard containing a known number of moles of oestradiol was prepared at the time of counting. This standard was prepared from the [^{35}S]pipsyl chloride used in the esterification technique.

(iii) Radioactivity counting. A 2 ml. volume of scintillation fluid [NE220; Nuclear Enterprises (G.B.) Ltd., Sighthill, Edinburgh] was added to each tube containing the dried eluates and standards. After shaking the scintillator was transferred to silica counting vials coated with aluminium reflector. The tubes were washed with a further 2 ml. of scintillation fluid and the total extract was sealed inside the vials with internally silvered plastic screw caps. The vials were placed in a three-channel automatic scintillation counter [Nuclear Enterprises (G.B.) Ltd.] and triplicate 400 sec. counts were made. The vials were recounted in duplicate for 400 sec. with a new setting for the third channel. Channel 1 (0·4–1·7 v) counted over the range of the ^3H spectrum, although some counts from both ^{35}S and ^{131}I are included. Channel 2 (1·7–6·7 v) excluded any counts from ^3H, but included all of the ^{35}S spectrum and part of the ^{131}I spectrum. Channel 3 (9·5–9·8 v) excluded both ^3H and ^{35}S but included part of the high-energy ^{131}I spectrum. Channel 4 (a re-setting of the third channel during the re-count to 9·8–10·5 v) included the highest recordable part of the ^{131}I spectrum. The counts due to ^3H, ^{35}S and ^{131}I in the samples and standards were then determined by the channels-ratio method of Hendler (1964). A check for quenching was made by reference to the channel 3/channel 4 ratio. This ratio was found to be invariably constant for a given assay.

Calculations

Abbreviations. The following abbreviations are used: TO, radioactivity of added standard [^3H]oestradiol; TL, radioactivity of sample [^3H]oestradiol pipsyl ester; SO, radioactivity of standard oestradiol [^{35}S]pipsyl ester; SL, radioactivity of sample oestradiol [^{35}S]pipsyl ester; SN, radioactivity of sample oestrone [^{35}S]pipsyl ester; I_O^L, radioactivity of standard oestradiol [^{131}I]pipsyl ester; I_O^N, radioactivity of standard oestrone [^{131}I]pipsyl ester; IL, radioactivity of sample oestradiol [^{131}I]pipsyl ester; IN, radioactivity of sample oestrone [^{131}I]pipsyl ester; P, percentage pre-chromatographic oestradiol recovery.

Oestradiol.

Oestradiol in sample (ng.) =

$$\text{wt. of oestradiol equivalent to SO} \times \frac{\left(\frac{\text{SL.TO}}{\text{TL}}\right)}{\text{SO}}$$

Oestrone. It is assumed, in this calculation, that the pre-chromatographic recovery of oestrone in a given sample is identical with that of oestradiol.

$$P = \left(\frac{TL}{TO} \times 100\right)\frac{I_O^L}{IL}$$

Oestrone in sample (ng.) =

$$\text{wt. of oestrone equivalent to SO} \times \frac{\left(\frac{\text{SN}.I_O^N}{\text{IN}}\right) \times \frac{100}{P}}{\text{SO}}$$

MATERIALS

All the reagents used in this study were of analytical grade and all the solvents were redistilled before use.

Radiochemicals. Radiochemicals were obtained from The Radiochemical Centre, Amersham, Bucks., and possessed the following specific radioactivities: [6,7-$^3H_2$]oestradiol (14·8 c/m-mole); [4-^{14}C]oestrone (10 mc/m-mole); [^{35}S]pipsyl chloride (100–150 mc/m-mole); [^{131}I]pipsyl chloride (20–60 mc/m-mole). Oestrogen [^{35}S]- and [^{131}I]-pipsylates were prepared and purified and their specific activities determined as described by Svendsen (1960).

Chromatography paper. Whatman no. 1 paper, cut into 3 cm. × 40 cm. strips, was washed with methanol and toluene before use.

Materials for radioautography. Ilford Industrial G (10 in. × 12 in.) X-ray film was used for radioautography; an exposure period of 18 hr. was employed with this film, aluminium foil being interposed between the chromatograms and the film, so that only the high-activity ^{131}I radiation was recorded. The film was developed with Ilford X-ray film developer.

Birds. The birds used were a White Leghorn cross (LRM × CCA) supplied by Thornber Bros. Ltd., Mytholmroyd, Halifax, Yorks. They were maintained on a commercial ration (British Oil and Cake Mills Ltd.) in a windowless room under artificial light for 16 hr. in each

24hr. and at an average temperature of 55° F. Food and water were supplied *ad libitum*.

Blood samples. Blood was collected from the wing vein into a syringe wetted with aqueous heparin; approx. 5ml. of blood was obtained each time. Plasma was obtained by centrifugation and was used immediately whenever possible, although occasionally samples that had stood overnight at −20° were employed.

RESULTS

Examination of the method in avian plasma. Plasma from the laying hen contains large and variable amounts of lipids and proteins (Sturkie, 1966; Heald & Rookledge, 1964). The application of the extraction and purification procedures described by Svendsen (1960) to acid hydrolysates of such plasmas led to low and variable recoveries of hormone (Table 1). Thus, when 2mμc of [4-^{14}C]-oestrone (10mc/m-mole) was added to plasma and processed as described in the Methods section, 70–85% of the added steroid was recovered in the initial chloroform extract (stages 1–2; see Scheme 1). With each successive step, the recovery diminished and the variability increased so that the overall mean recoveries from before acid hydrolysis (stage 1) to the final benzene drying (stage 4) had a range of 53–38% with standard deviations of as much as 17% for five observations. The variability and low recovery of oestrone during processing was not related to the volume of plasma employed in the hydrolysis (Table 1). Similar results were obtained when 2mμc of [16-^{14}C]oestradiol-17β (10mc/m-mole) was processed with plasma of the laying hen (Table 2). The original procedure as described by Svendsen (1960) only permits an estimate of losses of oestrogen that occur after the preliminary purification, since it is only at this stage that the ^{131}I-labelled standard is added. Consequently, this procedure could not be directly applied to avian plasma, since the high variability of recovery before addition of this standard clearly made accurate determination of the original plasma concentrations impossible to achieve. To overcome this difficulty, and to estimate total oestrogen loss during the entire procedure, an internal standard of [6,7-^{3}H$_2$]-oestradiol-17β of high specific activity was added since the addition of substantial radioactivity was

Table 1. [4-^{14}C]*Oestrone recovery during the hydrolysis, extraction and preliminary purification of plasma oestrogens*

[4-^{14}C]Oestrone (10mc/m-mole) was added to plasma samples and treated as described in the Methods section. Samples were taken at the appropriate stages and estimates made of oestrone recovery during these procedures. The results are expressed as percentage of added [4-^{14}C]oestrone recovered ±S.D., with the numbers of observations in parentheses.

Plasma volume (ml.)	Recovery at various stages (% of amount added)		
	Stages 1–2	Stages 1–3	Stages 1–4
10 (5)	85±7	62±12	47±7
5 (5)	70±4	61±9	38±9
2·5 (5)	71±9	58±6	51±16
1 (5)	81±6	70±6	53±15

Table 2. [16-^{14}C]*Oestradiol-17β recovery during the hydrolysis, extraction and preliminary purification of plasma oestrogens*

[16-^{14}C]Oestradiol-17β (10mc/m-mole) was added to plasma samples and treated as described in the Methods section. Samples were taken at the appropriate stages and estimates made of oestradiol-17β recovery during these procedures. The results are expressed as percentage of added [16-^{14}C]oestradiol-17β recovered ±S.D., with the numbers of observations in parentheses.

Plasma volume (ml.)	Recovery at various stages (% of amount added)		
	Stages 1–2	Stages 1–3	Stages 1–4
10 (5)	84±6	66±8	42±15
5 (5)	72±5	59±8	51±13
1·5 (5)	82±9	73±7	44±10
1 (5)	79±6	72±11	39±9

Table 3. [6,7-^{3}H$_2$]*Oestradiol-17β recovery during hydrolysis and preliminary purification and the purity of the* [6,7-^{3}H$_2$]*oestradiol-17β recovered after these procedures*

[6,7-^{3}H$_2$]Oestradiol-17β (14·2c/m-mole) was added to plasma prepared for acid hydrolysis. After purification to stage 4, a sample was taken to determine the ^{3}H recovered, which was expressed as a percentage of the ^{3}H added. The remaining extract was chromatographed on the B1 system (Bush, 1952) with unlabelled oestradiol-17β standards. The ^{3}H appearing with oestradiol-17β after chromatography was expressed as a percentage of the total ^{3}H added to the paper. Numbers of observations are given in parentheses.

Plasma volume (ml.)	Oestradiol-17β added (ng.)	^{3}H recovered after hydrolysis and purification to stage 4 (%)	^{3}H added appearing in the oestradiol-17β position after chromatography (%)
10 (1)	—	49·5	98·0
10 (1)	10	42·0	89·2
5 (1)	—	51·2	95·1
5 (1)	10	54·6	94·7
1 (1)	—	42·7	97·3
1 (1)	10	45·1	88·1
0 (3)	2	47·0	90·9

possible without making any appreciable increase in the total oestrogen content of the plasma.

Validity of the use of [6,7-$^3H_2$]*oestradiol-17β as an internal standard.* Because of the extremely high specific activity of the added tritiated oestradiol, it was essential to show that no tritiated material giving rise to spurious counts was present in the samples. For although any such materials may not have become esterified they could conceivably run to the same chromatographic positions as the oestrogen esters and consequently interfere in the final calculation of the oestrogen concentration. [6,7-$^3H_2$]Oestradiol-17β (approx. 200000 counts/100 sec.) was added to 2·5 ml. of plasma of the laying hen. The plasma was hydrolysed and the oestrogens were purified and dried ready for pipsylation as described in the Methods section. This material was dissolved in chloroform and a sample taken for measurement of radioactivity. Further samples were subjected to paper chromatography in the B1 system of Bush (1952). The chromatograms were cut into 2 cm. fractions and eluted with chloroform. The tritium recovery, after the preliminary purification, varied between 42·0 and 54·0% (Table 3). These values were similar to the low recoveries reported in Table 2. Nevertheless, 95–98% of the tritium recovered from the chromatograms ran to the position normally occupied by oestradiol (Table 3). The remainder of the activity eluted from the chromatogram did not run to any specific spot. About 2–8% of the tritium added to the chromatograms was lost on elution. Losses of up to 10% on elution are not unusual in these systems. The tritium recovery and the homogeneity of the ^3H-labelled material recovered after preliminary purifications was not affected by the plasma volume (1–10 ml.) or the addition of unlabelled oestradiol (10 ng.) to the plasma (Table 3).

Examination of the purification of derivatives. In the chromatographic purification procedure described by Svendsen (1960), the oestrogen pipsyl esters were found to be homogeneous after the third chromatographic step. This was shown to be the case by the constancy of the $^{35}S/^{131}I$ ratios in the two halves of the spot derived from the third chromatogram. Additional evidence in support of

Table 4. *Purification by paper chromatography of the* [6,7-$^3H_2$]*oestradiol-17β* [^{35}S,^{131}I]*pipsyl esters derived from pure oestradiol*

[6,7-$^3H_2$]Oestradiol-17β was added to various quantities of pure unlabelled oestradiol, pipsylated with [^{35}S]-pipsyl chloride, mixed with standard oestradiol [^{131}I]pipsyl ester as described in the Methods section and purified by chromatography in five steps as described in the text. The products of the fifth chromatographic transfer were divided into an upper and a lower half.

	$^{35}S/^{131}I$ ratio					
Chromatographic step	1	2	3	4	5	
					System II	
Unlabelled oestradiol-17β added (ng.)	System I	System III	System II	System III	Upper half	Lower half
10	2·82	2·40	2·51	2·64	2·37	2·34
100	3·82	3·71	3·02	3·24	3·14	3·21
100	0·61	0·55	0·47	0·53	0·52	0·57

Table 5. *Purification by paper chromatography of the* [6,7-$^3H_2$]*oestradiol-17β* [^{35}S,^{131}I]*pipsyl esters derived from pure oestradiol*

The esters were prepared and purified as described in Table 4.

	$^{35}S/^{131}I$ ratio					
Chromatographic step	1	2	3	4	5	
					System III	
Unlabelled oestradiol-17β added (ng.)	System I	System II	System III	System II	Upper half	Lower half
10	201	1·37	0·08	0·08	0·08	0·08
100	30·6	1·82	0·74	0·76	0·79	0·80
100	18·2	1·24	0·77	0·78	0·74	0·78

Table 6. *Purification by paper chromatography of the* [6,7-$^3H_2$]*oestradiol-17β* [^{35}S,^{131}I]*pipsyl chloride esters derived from extracts of plasma from the laying hen*

The esters were prepared and purified as described in Table 4, with the exception that the unlabelled oestrogen was replaced by plasma of the laying hen that had been treated and purified as described in the Methods section. The results for 1 ml. plasma samples are the means of duplicates.

Chromatographic step	$^{131}I/^3H$ ratio					
	1	2	3	4	5 System II	
Plasma volume (ml.)	System I	System III	System II	System III	Upper half	Lower half
10	1·28	1·40	1·37	1·22	1·24	1·20
10	6·08	6·23	6·20	6·19	6·16	6·01
5	1·21	1·04	1·20	1·14	1·04	1·09
5	0·69	0·63	0·53	0·61	0·62	0·66
1	0·70	0·72	0·76	0·77	0·72	0·75
1	3·11	3·06	3·17	3·10	3·01	3·12

this contention was afforded by the observations that rechromatography of the esters gave no change in the $^{35}S/^{131}I$ ratio.

The capacity of the chromatographic systems to purify the esters derived from pure oestrogens and from avian plasma oestrogens was examined in a similar fashion. [6,7-$^3H_2$]Oestradiol-17β (16·8 c/m-mole) (25 000 counts/100 sec.; approx. 0·5 ng.) was added to pure unlabelled oestradiol or pure unlabelled oestrone in a test tube. Similar quantities of tritiated oestradiol were also added to extracts (1–4; see Scheme 1) derived from various volumes of plasma of the laying hen (1–10 ml.). All these oestrogens were esterified with high-activity [^{35}S]-pipsyl chloride and treated as described in the Methods section. The esters were mixed with standard amounts of oestrogen [^{131}S]pipsyl esters and purified by chromatography as in the final method. After each elution a sample was taken for radioactivity measurement. The oestrone pipsyl esters derived from the third chromatography were not divided into two halves but were rechromatographed after elution in system II. The esters derived from this chromatogram were eluted and sampled as before. The remaining ester, after sampling, was finally purified by chromatography in system III. The ester spot from this chromatogram was divided into an upper and a lower fraction and eluted. After drying, the radioactivity in these final eluates was determined. The oestradiol pipsyl esters were similarly treated after the third chromatography, except that the fourth chromatographic system was system III and the fifth was system II. The $^3H/^{131}I$ ratios of the oestradiol esters derived from the pure oestrogen were constant through all the chromatographic steps (Table 4). This indicates that all the tritium running to the ester positions was derived from oestradiol pipsyl ester and was not due to any degradation products of [^3H]oestradiol. It is unlikely that radiochemical homogeneity could have been maintained through all the chromatographic steps if the tritium was derived from a non-oestradiol ester source. The $^3H/^{35}S$ ratios of the oestradiol pipsyl esters derived from the pure oestrogens were decreased during the first and second chromatographies, [^{35}S]pipsyl chloride breakdown products and non-oestradiol esters being separated from the oestradiol ester. The isotopic ratios of the pipsyl esters, after the third chromatographic steps, attained values that remained constant throughout all the subsequent purifications. The divided halves of the final ester spot have isotopic ratios identical with one another and with those of the products of the third and fourth chromatographies (Table 5). All these observations indicate that radiochemical homogeneity and therefore a high chemical purity had been reached after the third chromatographic purification.

Identical results were obtained in the chromatography of oestrone and oestradiol pipsyl esters derived from plasma extracts (Tables 6, 7 and 8). When judged by the constancy of their $^{131}I/^{35}S$ or $^3H/^{35}S$ ratios, the pipsyl esters were found to be homogeneous after three chromatographic transfers, irrespective of the plasma volume employed, i.e. 5 ml. and 15 ml. of plasma for oestrone and 1–10 ml. of plasma for oestradiol. The pipsyl esters formed from the oestradiol contained in duplicate 1 ml. plasma samples gave similarly constant $^3H/^{35}S$ ratios after the third chromatography and also during the subsequent purification (Table 7). These results would appear to justify the use of only three chromatographic steps in the purification of the esters derived from avian plasma. These

Table 7. *Purification by paper chromatography of the* [6,7-$^3H_2$]*oestradiol-17β* [^{35}S,^{131}I]*pipsyl esters derived from extracts of plasma of the laying hen*

The esters were prepared and purified as described in Table 6. The results for 1 ml. plasma samples are the means of duplicates.

Chromatographic step	^{35}S/^{3}H ratio					
	1	2	3	4	5 System II	
Plasma volume (ml.)	System I	System III	System II	System III	Upper half	Lower half
10	101	4·34	1·30	1·33	1·38	1·35
10	17·5	2·67	0·45	0·44	0·42	0·41
5	11·1	8·47	0·18	0·19	0·12	0·19
5	54·2	0·80	0·41	0·37	0·32	0·37
1	16·3	0·87	0·11	0·16	0·12	0·15
1	50·2	0·34	0·20	0·19	0·17	0·17

Table 8. *Purification by paper chromatography of the oestrone* [^{35}S,^{131}I]*pipsyl esters derived from extracts of plasma from the laying hen*

Plasma samples were treated as described in Table 6, except that oestrone [^{131}I]pipsyl chloride was added in place of the oestradiol-17β ester. The oestrone ester alone was purified and examined.

Chromatographic step	^{35}S/^{131}I ratio					
	1	2	3	4	5 System III	
Plasma volume (ml.)	System I	System II	System III	System II	Upper half	Lower half
15	3·14	1·23	0·86	0·89	0·82	0·84
15	14·6	0·52	0·19	0·15	0·17	0·12
5	92·1	4·9	3·17	3·06	3·12	3·00
5	12·1	3·14	0·53	0·55	0·51	0·54
5	5·67	0·99	0·71	0·66	0·69	0·67
5	9·74	6·00	0·83	0·89	0·81	0·86

experiments also show that it is not necessary to further oxidize or prepare further derivatives of the esters to attain a high purity as Svendsen & Sørensen (1964*a*,*b*) did during the quantitative determination of human plasmas containing very small amounts of oestrogen.

Application of the final procedure to plasma of the domestic fowl. The concentrations of oestrone and oestradiol in plasma from the laying hen were determined by the procedure described above. Plasma samples (1–10 ml.) from the same large blood sample were examined, the variation in plasma volume having no obvious effect on the recorded oestrogen concentration. The oestradiol concentration of this sample had a range of 39·1–34·1 ng./ml. (mean 36·6 ng./ml.). The plasma oestrone concentrations of the same blood sample had a range of 29·4–24·2 ng./ml. (mean 27·0 ng./ml.) (Table 9). The total oestradiol recoveries were between 9·1 and 14·9% in these experiments. A further series of oestrogen determinations were carried out to determine the recoveries of various amounts (10–500 ng.) of non-radioactive oestradiol added to plasma of the laying hen. Two of a series of seven identical 1 ml. plasma samples were determined without the addition of unlabelled oestradiol. The remaining five samples were augmented with 10, 100 or 500 ng. of unlabelled oestradiol. The oestrone and oestradiol concentrations in all the samples were then determined by the normal procedure. The mean plasma oestradiol concentrations of the untreated samples were subtracted from the oestradiol concentrations of the treated plasmas and the remaining oestradiol was expressed as a percentage of the added oestradiol (Table 10). The mean untreated plasma oestradiol concentration was 41·8 ng./ml. of plasma. The mean percentage added oestradiol recovery

was 97·1%. The mean plasma oestrone concentration was 26·7ng./ml. The observed plasma oestrone concentration was unaffected by the addition of non-radioactive oestradiol. The total oestradiol recoveries calculated from the recovery of the added [^{3}H]oestradiol varied in the range 5·1–15·4% (mean 10·4%), and was not related to the amount of additional non-radioactive oestradiol (Table 10).

An estimate was made of the normal range of oestrone concentration to be found in plasma of the laying hen. Six hens with normal laying patterns were chosen at random from a large flock and the oestrogens determined. The range of oestradiol concentration was 8·3–21·4ng./ml. (mean ± S.E.M. 14·1 ± 1·9ng./ml.). The plasma oestrone concentrations were of a similar order with a range of 15·2–31·6ng./ml. (mean ± S.E.M. 22·8 ± 2·7ng./ml.) (Table 11).

DISCUSSION

The procedure described provides a method for the determination of the total oestrone and oestradiol concentrations of plasma of the laying hen. The criteria that must be considered when assessing the reliability of such a technique have been outlined by Diczfalusy (1957). These criteria are the precision, accuracy, specificity and sensitivity.

Precision. Precision is determined by an evaluation of the variations observed between duplicate

Table 9. *Determination of oestrone and oestradiol in plasma of the laying hen*

Plasma of a single laying hen was treated as described in the Methods section, and the oestrone and oestradiol concentrations were determined. The total oestradiol recovery was the ^{3}H counts of the purified [6,7-$^3H_2$]oestradiol-17β [^{35}S,^{131}I]pipsyl ester expressed as a percentage of the count of the added [6,7-$^3H_2$]oestradiol-17β.

Plasma volume (ml.)	Oestradiol recovered (%)	Plasma oestradiol concn. (ng./ml.)	Plasma oestrone concn. (ng./ml.)
10	10·2	34·1	27·6
5	8·7	36·2	29·4
5	14·9	37·2	26·9
1	9·1	39·1	24·2
Mean	10·7	36·1	27·0

Table 11. *Determination of oestrone and oestradiol-17β in plasma from six laying hens*

Duplicate plasma samples (2ml.) from six individual hens were treated as described in the final procedure of the Methods section, and their oestrone and oestradiol concentrations were determined.

Bird no.	Plasma oestradiol-17β concn. (ng./ml.)	Plasma oestrone concn. (ng./ml.)
1	10·7	28·5
2	21·4	31·6
3	15·6	15·2
4	15·9	25·4
5	8·3	17·3
6	12·6	18·9

Table 10. *Recovery of added non-radioactive oestradiol during the determination of the oestrogens in plasma of the laying hen*

Identical samples of plasma of a laying hen (1ml.) were augmented with various amounts of non-radioactive oestradiol-17β (10–500ng.). The total sample oestrone and oestradiol concentrations were determined as described in the Methods section. The total oestradiol recovery was the ^{3}H count of the purified [6,7-$^3H_2$]oestradiol-17β [^{35}S,^{131}I]pipsyl ester expressed as a percentage of the count of the added [6,7-$^3H_2$]oestradiol-17β. The recovery of the added oestradiol was calculated as the percentage of added non-radioactive oestradiol remaining after subtraction of the untreated plasma sample content from the treated sample content.

Plasma volume (ml.)	Added non-radioactive oestradiol (ng.)	Total sample of oestradiol (ng.)	Calc. recovery of added oestradiol (%)	Total oestradiol recovery during assay (%)	Plasma oestrone concn. (ng./ml.)
1	—	40·5	—	7·1	24·0
1	—	43·1	—	8·2	25·8
1	10	51·1	92·6	11·7	26·7
1	100	135·0	94·2	15·4	25·4
1	100	142·9	101·1	16·3	28·0
1	500	488·8	89·3	5·1	29·6
1	500	531·8	98·2	9·1	27·5
Mean			97·1	10·4	26·7

determinations. The quadruplicate determinations (Table 9) have a satisfactory reproducibility, even though the plasma volumes used in these assays were different, a factor that might have been expected to reduce the degree of precision.

Accuracy. The recovery of oestradiol when added to identical plasma volumes was employed as a measure of the accuracy of the technique. The results obtained indicate that the method has a high degree of accuracy. The oestradiol recovery was in the range 89·3–101·1% (mean 97·1%) for five determinations (Table 10). Consequently, this procedure may be used to obtain absolute and quantitative values for plasma oestrogen concentration without recourse to the use of recovery factors calculated from previously performed assays. This high and constant recovery was maintained even when oestradiol concentrations well in excess of those normally encountered in 10ml. plasma samples were examined.

Specificity. The most conclusive evidence of the specificity of a technique of this kind is obtained from the determination of plasma known to be free of oestrogens. Plasma that could with certainty be said to be free from oestrogen was not available for assay. Individual samples of plasmas of cockerel and non-laying hen have been examined, but all these certainly contained some oestrogenic material. However, the specificity of the method is ensured by the repeated chromatography of the esterified oestrogens, which ensures satisfactory separation from non-oestrogenic compounds. An analysis was only accepted as sufficiently specific if the $^{3}H/^{35}S$ and $^{131}I/^{35}S$ ratios of the upper and lower halves of the final ester spots were identical. In 2 years of continuous operation, only about 5% of all assays performed by this technique have been discarded for failure to satisfy this criterion.

Sensitivity. Blank determinations were used to calculate the sensitivity of the technique. A series of eight determinations for each oestrogen carried out over a 4-week period were examined, and the smallest detectable amounts of oestrogen differing significantly from zero were calculated. The sensitivity for oestrone was 3·0ng. and that for oestradiol was 2·1ng. These values are higher than those obtained by Svendsen (1960) in the examination of human plasma, but the higher concentrations found in hen plasma make this difference relatively unimportant. Oestrogen determinations on plasma from the laying hen in unusual physiological states or from the non-laying or cockerel may be limited by this degree of sensitivity.

A disadvantage of the technique is its time-consuming nature. Thus only six duplicate assays could be carried out in a 5-day week, a number too low for use as a routine assay.

There has been little previous work on the concentration of oestrogens present in avian plasma. In this present study, both the oestradiol concentration and oestrone concentration were in the range 10–30ng./ml. of plasma.

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