Selective Accumulation of Tritium-Labelled Hexoestrol by the Reproductive Organs of Immature Female Goats and Sheep

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(*Received* 14 *January* 1959)

A considerable amount of work has been done on the metabolism by living animals of oestrogens, both natural and synthetic, but, as pointed out by Dodds, Folley, Glascock & Lawson (1958), the doses given have nearly always been very large compared with the physiological dose. This has been necessary because the methods available for the detection of the oestrogens or their derivatives were relatively insensitive even when labelling with ^{14}C was used (Twombly, 1951; Twombly & Schoenewaldt, 1951; Hanahan, Daskalakis, Edwards & Dauben, 1953; Budy, 1955). Apart from the work of Dodds *et al.* (1958) (see below) the smallest doses have been used by Hanahan *et al.*, who gave [^{14}C]-stilboestrol to rats in doses of 5 μg., which is still more than ten times the physiological dose. It has been generally concluded that these oestrogens do not accumulate in organs such as uterus, vagina, mammary gland and pituitary gland, which respond to them physiologically. If this is so it would be necessary to attribute the response of these organs to some such mechanism as a selective sensitivity to the circulating oestrogen.

Glascock (1956) pointed out that by using tritium as a labelling agent very high specific activities are attainable and that this is extremely important when substances biologically as potent as the oestrogens are studied. In the rat, for example, 0·2 μg. of hexoestrol administered subcutaneously has a detectable action on the vagina (Dodds *et al.* 1944), whereas about $\frac{1}{200}$ of the subcutaneous dose is effective when administered locally to the vagina (Emmens, 1940–41). This indicates that perhaps only a very small portion of a subcutaneous dose of oestrogen need reach the responding organs and that extremely sensitive methods of detection, and the use of the smallest effective doses, are probably necessary if any selective localization of the oestrogen in these organs is to be demonstrated.

Dodds *et al.* (1958) traced the excretion of microgram doses of tritium-labelled hexoestrol (specific activity about 10^7 counts/min./μg.) in rats and rabbits, but did not measure the radioactivity of body tissues. Subsequent studies with rats (M. Gabr & R. F. Glascock, unpublished work) indicated that a fleeting accumulation of hexoestrol, which was maximal about 7 hr. after a subcutaneous injection in oil, occurred in uterus, liver and, to a lesser extent, in kidney.

The purpose of the present study was to follow the distribution in organs and excreta of small doses of labelled hexoestrol at various times after injection into immature female goats and sheep. Distribution studies in ruminants were of additional interest because of the use of synthetic oestrogens in livestock production to increase rate of gain in body weight. By using physiological doses of hexoestrol it has been possible to show a selective distribution in those organs most sensitive to oestrogens as well as in those organs involved in their excretion. A preliminary account of some of this work has been published (Glascock & Hoekstra, 1958).

METHODS

Tritium-labelled hexoestrol. This was prepared by hydrogenation of dienoestrol with tritium–hydrogen on the micro scale over a palladium catalyst, after which it was purified and separated chromatographically from the less oestrogenically potent isomer *iso*hexoestrol (R. F. Glascock & G. S. Pope, unpublished work; for a preliminary account of the method see Glascock, 1954*a*). The hexoestrol is believed to be generally labelled in stable positions. The labelled hexoestrol was stored in the dark as a dilute solution in benzene (283 μg./ml.) to minimize radiolysis. A suitable portion of the benzene solution was placed in a small flask, the benzene removed under a stream of nitrogen and the labelled hexoestrol dissolved in arachis oil to give a concentration of 50 μg. of hexoestrol/ml. This solution was injected subcutaneously into the experimental animals. The original benzene solution of hexoestrol was assayed for radioactivity by the butane gas method of Glascock (1954*b*) after suitable dilutions with unlabelled hexoestrol. The arachis oil solution of hexoestrol was also assayed for radioactivity to verify the assay result and to make sure that the oily solution used for injection had been thoroughly mixed. The tritium-labelled hexoestrol was shown by both assays to have a specific activity of $0{\cdot}97 \times 10^7$ counts/min./μg.

Treatment of experimental animals. In the experiment with kids five immature crossbred females were used. They were 87–100 days old and weighed 14·3–22·8 kg. On separate days each kid received a subcutaneous injection (at a site on the left side of the neck) of 25 μg. of tritium-labelled hexoestrol contained in 0·5 ml. of arachis oil. This is about

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the physiological dose of hexoestrol (see Discussion). Each kid was maintained in a metabolism cage for separate quantitative collection of urine and faeces. The kids were killed at 2, 5·5, 8, 24 and 48 hr. after the injection by cutting the blood vessels of the neck and bleeding to death under pentabarbitone sodium anaesthesia. Blood samples were taken from the last three animals at approximately the same intervals until death. The various organs and tissues were removed promptly with care to prevent any contamination with bile, urine or intestinal contents. Blood was rinsed from the surface of each organ, but no attempt was made to remove residual circulating blood.

A similar experiment was done on lambs to confirm the results of the experiment on kids and to extend observations on the distribution of hexoestrol to certain additional organs. Four crossbred female lambs about 4 months old and weighing 23·9–30·4 kg. were used. Each of the first two lambs received a single subcutaneous injection of 24 μg. of tritium-labelled hexoestrol in 0·48 ml. of arachis oil. One was killed 4 hr. and the other 8 hr. afterwards. Each of the other two lambs received the same total dose by eight injections at hourly intervals of 3 μg. of hexoestrol in 0·06 ml. of arachis oil. They were killed 8 hr. after the first injection. The divided dose was given in an attempt to simulate physiological conditions more nearly, since the previous experiment with kids had demonstrated a rapid absorption of hexoestrol from the injection site. Blood samples were taken at 20 or 30 min. intervals up to the time of killing.

Preparation of excreta and tissues for assay of radioactivity

In both experiments, faeces, intestinal contents (including abomasum contents), bile (in gall bladder), urine (passed) and urine (in bladder) were assayed separately. Faeces and intestinal contents were weighed and thoroughly mixed in a Waring Blendor with known amounts of water. A small portion representing 1–2 g. of dry matter was removed and weighed, and 1–2 mg. of unlabelled hexoestrol was added as carrier to trap the small quantities of radioactive hexoestrol which might otherwise be lost by volatilization. The sample was freeze-dried under vacuum, weighed and assayed for radioactivity. Urine and bile samples were freeze-dried after addition of carrier hexoestrol and assayed. To avoid inconveniently high counting rates it was necessary to dilute some of the samples of bile and urine solids before assay by adding a known weight of dextrin to the measured samples before freeze-drying.

The organs and tissue collected from the kids for assay of tritium were: blood, uterus, vagina, mammary glands, ovaries, pituitary gland, liver, kidneys, lungs, intestine, skeletal muscle (thigh), perinephric fat, pancreas, adrenal glands, salivary glands and skin. The discrete organs were weighed. Most of the organs were cut into small pieces (or minced in a domestic mixer) and thoroughly disrupted and mixed with about 2 vol. of water in a Waring Blendor or small laboratory homogenizer (depending on sample size) and the mince was strained through muslin to remove any uncomminuted connective tissue; finally a representative portion was stored in the deep-freeze. For assay the samples were thawed, a portion of about 10 ml. of homogenate was taken, 1–2 mg. of carrier hexoestrol was added and the sample was freeze-dried to provide the dried sample for combustion and assay of tritium. Blood samples were freeze-dried directly after addition of carrier. The uteri were divided into the endometrium and the myometrium for separate assay. This was done as well as possible by scraping the inner surface of the uterus with a microtome blade, but the separation was not complete and considerable endometrial tissue was included with the myometrium. Fat was melted in an oven. The skin samples from the abdomen, which could not be prepared in the manner described, were digested in 10% (w/v) NaOH in aqueous 50% ethanol for 24 hr. on a steam bath. The digest was then acidified to pH 2–3 and extracted five times with diethyl ether; the ether extract was dried with anhydrous Na_2SO_4, the solvent was removed and the extract assayed. Radioactivity was calculated per unit original dry weight. Thus for skin only those metabolites of hexoestrol which were soluble in diethyl ether after alkaline hydrolysis were measured. The relationship of this amount to the total is not known, but it is believed that the major portion of the radioactivity was extracted by this process.

The experiment with lambs was similar except that samples of oviducts, brain, mesenteric lymph nodes, thymus, heart, spleen, and bone (femur) were also taken, and samples of salivary glands and pancreas were not. The site of injection was also assayed. The femur was cut and the marrow and spongy bone scraped free with a knife and treated as the other tissues. Sections of the bone shaft were frozen in liquid air, crushed in a cooled block-and-piston arrangement, passed several times through a mincer, dried and assayed. The injection site included nearby skin and muscle and weighed 150–200 g. (fresh wt.). These tissues were cut into small pieces and digested in excess of ethanolic NaOH for 48 hr. on a steam bath. Measured portions of the digest were neutralized, a weighed amount of dextrin was added to dilute the radioactivity of the dry matter, carrier hexoestrol was added and the samples were freeze-dried, weighed and assayed.

Measurement of radioactivity. The water obtained by combustion of 15–30 mg. of dried samples was converted into butane and counted in a gas counter as described by Glascock (1954*b*). The yields of combustion water, required for calculation of specific activities of the dry tissue itself, were ascertained by condensing the combustion water into a small tube under high vacuum. The tube was sealed and removed from the high-vacuum line, marked with a glass knife and weighed. The weighed tube was broken at the mark, returned to a chamber on the high-vacuum line, frozen with liquid air and evacuated. The water was transferred to the tube containing butylmagnesium bromide for conversion into butane, and the empty tube which had contained water was removed from the vacuum line and weighed. This procedure was shown to be reliable by subjecting pure compounds (glucose and stearic acid), of known water yield and radioactivity, to combustion. Butane samples of total activity of several hundred counts/min. were counted for 10 000 counts or more, whereas fewer counts were made on samples of lower total activity down to a total of not less than 1000 counts for total activities of the same order as background (30 counts/min.). The combined accuracy of water-determination and counting of the samples with higher activity appeared to be within ± 3% and was somewhat worse for those with lower activities. Nevertheless, specific activities as low as 2 counts/min./mg. dry matter could be reproducibly

Table 1. *Gross distribution of hexoestrol in female kids and lambs*

The kids each received a single subcutaneous injection of 25 μg. of tritium-labelled hexoestrol in arachis oil and were killed at the indicated times afterwards. Lambs 1 and 2 each received a single subcutaneous injection of 24 μg. of hexoestrol and were killed 4 and 8 hr. later. Lambs 3 and 4 each received eight injections of 3 μg. at intervals of 1 hr. and were killed 8 hr. after the first injection.

	Estimated percentage of total dose in sample								
	Kids					Lambs			
Animal no.	1	2	3	4	5	1	2	3	4
Body wt. (kg.)	14·3	17·7	22·8	14·0	14·2	30·4	25·0	23·9	25·9
Time after injection (hr.) ...	2	5·5	8	24	48	4	8	8	8
Dose	Single	Single	Single	Single	Single	Single	Single	Divided	Divided
Site of injection (skin and muscle)	—	—	—	—	—	21·6	1·8	7·1	12·8
Other body tissues*	28·7	20·7	10·5	1·5	0·8	30·7	16·6	26·3	26·7
Bile	1·3	5·4	3·9	0·4	0·04	4·1	0·6	2·0	3·0
Intestinal contents	7·8	31·8	40·9	17·4	1·1	18·0	49·8	43·8	36·2
Faeces	0·0	1·0	0·5	18·4	36·1	0·04	0·07	0·2	0·0
Urine	9·0	26·5	29·8	43·2	39·8	6·7	29·3	14·1	13·8
Total	56·8	85·4	85·6	81·0	77·8	81·1	98·2	93·5	92·5
Total hexoestrol in urine/total hexoestrol in bile, intestinal contents and faeces	0·47	0·69	0·66	1·19	1·07	0·31	0·58	0·31	0·35

* See Table 4.

detected. For the assay of such samples any contamination of the vacuum line by a previous sample was allowed for by applying a correction derived from the assay of unlabelled glucose. The correction provided by this blank was always less than 1 count/min./mg. dry matter. Samples of organs and tissues counted at rates from 0 to 205 counts/min./mg. dry matter (0–350 counts/min./mg. of combustion water), whereas those from certain of the excretory products, some of which were diluted with dextrin, counted at considerably higher rates (up to 700 counts/min./mg. dry matter).

RESULTS

In the calculations the radioactive compound in the samples is assumed to be hexoestrol itself and will be referred to as such, although in fact its identity has not yet been established.

General distribution of hexoestrol. In Table 1 is shown the gross distribution of hexoestrol (expressed as a percentage of the dose) at intervals after its injection into the kids and lambs. An exact radioactivity balance could not be made because the total weight of every organ assayed (e.g. muscle, skin, intestine) was not known, and the total radioactivities of the residual carcasses were not measured. However, an approximate measure of total radioactivity in the tissues was made from dry-matter content and their estimated contribution to the total body weight (muscle 40 %, bone and skull 20 %, skin 10 %, intestine 2 %). Total recoveries of injected radioactivity thus calculated for the kids (Table 1) were 56·8 % from the kid which was killed 2 hr. after injection and 79–86 % for the other four killed 5·5–48 hr. after injection. The low recovery for the first animal would be consistent with incomplete absorption of the hexoestrol from the injection site, which was not assayed in this experiment. However, even after 24 and 48 hr., when tissue levels of radioactivity were very low and nearly all the injected radioactivity was in urine, faeces and intestinal contents, total recoveries were only about 80 %. The reason for incomplete recoveries is not known. All samples of body water, as represented by the water obtained from blood samples during freeze-drying, had less than 1 count/min./mg., indicating little or no complete oxidation of hexoestrol (a counting rate of 1 count/min./mg. of body water would be equal to about 4 % of the total dose). Also no detectable radioactivity (<1 count/min./mg. of water) followed the volatile fraction during freeze-drying of urine and faeces. In the experiment with lambs, in which the sites of injection were assayed and all lambs were killed within 8 hr. of the injection, total recoveries were 81, 98, 94 and 92 %. Twombly & Schoenewaldt (1951) reported that dried tissues from animals injected with stilboestrol labelled with ^{14}C in the β-ethyl position lost radioactivity even when stored in the refrigerator. In this work the samples from the lambs were in general counted after shorter periods of storage than those from the kids but it is not known whether or not this is significant.

It is evident (Table 1) that hexoestrol injected in arachis oil was quite rapidly absorbed from the site of injection. Thus after 2 hr. (kid 1) at least 57 % and after 5·5 hr. (kid 2) at least 85 % of the dose was distributed in the body tissues and excretory pathways. It was shown by direct assay

that in the lambs (Table 1) 21·6 % of the injected hexoestrol remained at the injection site 4 hr. after a single dose whereas only 1·8 % was found there after 8 hr.

The injected hexoestrol rapidly passed into excretory pathways (urine, bile and intestinal contents). The percentages of the total dose in these pathways were 28, 65 and 75 % respectively in kids killed 2, 5·5 and 8 hr. after injection and 29 and 80 % in lambs killed 4 and 8 hr. after injection. One route of excretion was the bile, in which as much as 5·4 % of the dose was found 5·5 hr. after injection. The radioactivity from the bile undoubtedly contributed the major portion, if not all, of the radioactivity found in the intestinal contents. The urine was the only other major route of hexoestrol excretion. Table 1 also gives the ratios of the amounts of radioactivity in urine (including bladder urine) to the combined amount in bile, intestinal contents and faeces. With increasing time after injection of the labelled oestrogen a greater relative portion of the radioactivity was found in the urine.

Consistent with the rapid excretion of hexoestrol was a rapid decrease in the amount found in the body tissues: 24 hr. after injection into the kids (Tables 1 and 4) only about 1·5 % of the dose remained in the body tissue, whereas 2, 5·5 and 8 hr. after injection about 29, 21 and 11 % respectively of the dose remained in the tissues.

Circulating levels of hexoestrol. In Fig. 1 the concentration of circulating hexoestrol in kids 3, 4 and 5 is plotted against time after injection. A figure of 70 ml. of blood/kg. body weight (Courtice, 1943) was used in making these calculations. In the kids the concentration of hexoestrol in the blood reached a maximum 2 hr. after the injection. Owing to steepness of the curves about the maximum and the infrequency of taking blood samples it is possible that the true maxima were considerably higher than indicated. Nevertheless, for kid 5 the maximum corresponds to the circulation of 22 % of the dose. These blood curves also confirm the rapid absorption of the labelled hexoestrol from its site of injection and its nearly complete clearance from the blood within 24 hr. Unfortunately the 0·9 μg. of circulating hexoestrol (3·6 % of dose; see Table 2) in the kid killed 2 hr. after injection does not correspond with the blood hexoestrol levels found for the other kids at that time; thus it is not likely that any animal was killed at the time of the maximum level of circulating hexoestrol. Of the five kids, that in which 2·0 μg. of hexoestrol (8·2 % of the dose; see Table 2) was circulating when it was killed 5·5 hr. after injection had the highest circulating level at the time when samples of body tissues were taken.

In the lambs, from which blood samples were taken more frequently, the increase in concentration of circulating hexoestrol was not as rapid as in the kids (Fig. 1). It was still increasing in the first lamb when killed 4 hr. after injection, and in the second lamb the maximum concentration occurred 3 hr. after injection. Thus there appears to be considerable variation in the rate of absorption of the injected hexoestrol, or in its rate of removal from the blood stream or in the rate of both these processes. In the two lambs which received the divided dose the concentration of hexoestrol in the blood did not reach a steady value as expected but increased up to the time of slaughter, 8 hr. after

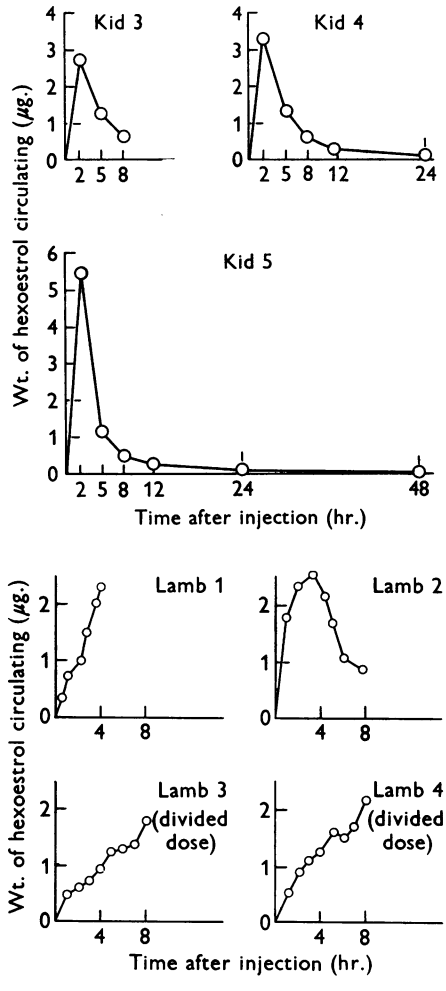

Fig. 1. Circulating amounts of hexoestrol in female kids and lambs at various intervals after its subcutaneous injection; there was assumed to be 70 ml. of blood/kg. body wt. See text and Table 1 for details of treatment of the animals.

the first dose. At this time they had approximately the same amount of the hexoestrol circulating as did the first lamb killed 4 hr. after a single dose.

Concentration of hexoestrol in specific organs and tissues. Table 2 shows the concentration of hexoestrol in the organs and tissues studied. It was impossible to dissect the immature mammary glands free from the fat in which they lay embedded and which accumulated virtually no hexoestrol (Table 2). To obtain a value for the concentration of hexoestrol in the fat-free gland, nitrogen determinations were therefore made on each of the samples and the hexoestrol concentrations corrected to 11 % of nitrogen, which was the average nitrogen content of the dry matter of other tissues studied.

Table 2 shows that, apart from differences attributable to times of killing, the general pattern of distribution of hexoestrol was similar in all animals, both kids and lambs. The concentration in the intestine of lamb 3 is anomalously high, however, especially as the concentrations in other corresponding organs of lambs 3 and 4, on which identical experiments were done, are about the same. After a divided dose (lambs 3 and 4) the concentration in most organs was 1·5–2·5 times as high as that after a single dose (lamb 2).

Although many more animals would have to be used for a detailed study of the changes with time in the concentration of hexoestrol in various organs, the experiment on the kids does give some idea of the results which would then be obtained. In the animal killed 2 hr. after the injection the whole uterus and vagina contained the highest concentration, followed by kidney, liver, intestine and ovaries. In the animal killed 5·5 hr. after injection the kidney had the highest concentration, closely followed by whole uterus and then ovaries and vagina. The high concentration in kidney tissue at this time is probably the result of a high rate of excretion (see below), because in the animal killed 3 hr. later, when excretion had passed its maximum, the concentration of hexoestrol in the kidney was only one-quarter of that of the vagina (which in this animal was the organ with the highest concentration) and occupied sixth place in the descending order of concentration.

Table 2. *Concentration of hexoestrol in various organs and tissues*

See text and Table 1 for details of treatment of the animals.

	Hexoestrol (μmg./g. of dry matter)								
	Kids					Lambs			
Animal no.	1	2	3	4	5	1	2	3	4
Time after injection (hr.)	2	5·5	8	24	48	4	8	8	8
Dose	Single	Single	Single	Single	Single	Single	Single	Divided	Divided
Organ or tissue									
Uterus (endometrium)	14·1	21·1	7·3	1·9	0·3	9·4	7·8	13·8	13·6
Vagina	13·8	12·3	12·0	2·3	0·4	9·5	7·4	10·7	10·8
Uterus (myometrium)	17·7	19·7	6·8	1·4	0·3	5·8	6·0	13·0	12·6
Kidneys	8·6	21·8	3·0	1·2	0·2	10·5	6·2	12·3	17·7
Oviducts	—	—	—	—	—	5·4	4·9	7·0	6·3
Ovaries	4·7	14·1	4·5	1·5	0·4	3·8	4·6	4·8	4·4
Mammary glands*	4·6	9·1	3·7	0·9	0	6·7	3·7	6·5	7·0
Intestine	5·0	7·3	2·2	0·3	0	4·7	3·3	17·5	5·9
Liver	7·0	5·2	2·8	0·6	0·3	6·8	2·9	3·9	5·1
Pituitary gland	3·6	2·6	0·9	0·6	0·1	4·2	1·7	2·8	2·9
Lungs	3·2	3·2	0·8	0·2	0·1	1·8	0·9	1·4	2·5
Lymph nodes	—	—	—	—	—	1·4	0·8	1·4	1·2
Adrenal glands	0·9	1·9	0·4	0·2	0	1·9	0·7	0·9	1·4
Skin	0·8	0·9	0·6	0·4	0·3	0·5	0·4	0·7	0·8
Salivary glands	1·2	1·6	0·5	0·1	0·1	—	—	—	—
Thymus	—	—	—	—	—	1·0	0·4	0·8	0·7
Heart	—	—	—	—	—	1·0	0·4	0·9	1·0
Spleen	—	—	—	—	—	0·6	0·4	0·7	0·6
Pancreas	1·0	0·9	0·4	0·1	0·1	—	—	—	—
Skeletal muscle	2·8	0·5	0·3	0	0	0·5	0·5	0·3	0·4
Brain	—	—	—	—	—	0·3	0·2	0·4	0·4
Bone	—	—	—	—	—	0·2	0·1	0·2	0·2
Fat (perinephric)	0·4	0·1	0·1	0	0	0·2	0·2	0·2	0·2
Blood solids	4·7	8·7	2·2	0·5	0·1	5·6	2·7	5·6	6·5

* Since samples of mammary glands were heavily contaminated with fat, the figures were corrected to 11·0 % nitrogen, the average nitrogen content of the other organs studied.

To compare their capacity for accumulating hexoestrol the mean concentrations in various organs for all the animals except kid 5 have been calculated, as shown in Table 3, the concentration in uterine endometrium being set arbitrarily at 100. Kid 5 was omitted because the hexoestrol concentration in the organs was too low for differences to be significant (see Table 2). The highest concentration is in the organs which respond physiologically to hexoestrol (uterus and vagina) and in the kidneys, which excrete it. In the oviducts, ovaries, mammary glands, intestine and liver the concentration was about half that in endometrium. In the pituitary gland, which is also an organ which responds to oestrogens, the concentration was about one-quarter of that in the uterine endometrium, but was still considerably higher than in many other tissues. The lungs also appeared to have a slight ability to concentrate hexoestrol, whereas in lymph nodes, adrenal glands, skin, salivary glands, thymus and heart the concentration was only 7–10 % of that in uterine endometrium. The concentration in other organs and tissues including spleen, pancreas, skeletal muscle, brain, bone and perinephric fat was only 1–5 % of that in uterine endometrium. The radioactivity of some of these organs may have been due to residual blood solids whose mean specific activity was about 40 % of that of uterine endometrium. It is obvious, however, that there was a true selective accumulation in reproductive organs, since only a small fraction of their radioactivity could have arisen from residual blood. As the concentration in the pituitary glands of the lambs, though relatively low, was on the average nine times that in the portion of the brain to which it was attached, it was probably also due to a selective accumulation.

Table 3. *Relative concentration of hexoestrol in dry matter of various organs and tissues*

Mean concentrations in all tissues assayed (except those of kid 5 see text) were calculated on the basis of 100 for the concentration in uterine endometrium.

Organ	No. of animals	Mean relative concentration (±S.E.)
Uterus (endometrium)	8	100
Vagina	8	99·3±11·4
Uterus (myometrium)	8	88·9±6·7
Kidneys	8	84·9±10·4
Oviducts	4	54·3±3·6
Ovaries	8	50·9±6·3
Mammary glands	8	48·9±3·8
Intestine	8	47·3±11·9
Liver	8	39·9±5·3
Pituitary gland	8	23·7±3·8
Lungs	8	14·8±1·7
Lymph nodes	4	11·0±1·3
Adrenal glands	8	9·7±1·6
Skin	8	7·6±2·0
Salivary glands	4	7·0±0·7
Thymus	4	6·6±1·3
Heart	4	7·4±1·2
Spleen	4	5·2±0·4
Pancreas	4	5·6±0·6
Skeletal muscle	8	5·4±2·2
Brain	4	2·9±0·1
Bone	4	1·6±0·2
Perinephric fat	8	1·5±0·3
Blood solids	8	39·2±3·8

The concentration–time curves for selected organs, and for bile and bladder urine of the kids are shown in Fig. 2. Each point as plotted represents a single animal and the exact shapes of the curves would therefore have to be verified by using more animals at each time interval. The maximum concentration of hexoestrol in muscle and liver was found in the animal killed 2 hr. after the injection. This was true also of pituitary gland, perinephric fat and possibly vagina, although the difference in concentration in vaginal tissue from the first two kids killed (2·0 and 5·5 hr. after injection) was very small (Table 2). The maximum concentration in endometrium and kidney was found in the animal killed 5·5 hr. after injection, as it was also in ovary, mammary gland, intestine and adrenal glands. The remaining organs assayed (lungs, skin, salivary glands and pancreas) had about the same concentration in the two animals first killed, although it was higher than in those killed later. As the concentration in blood solids was not highest in the animal killed 2 hr. after injection, as would be expected from concentration–time curves of kids 3, 4 and 5 (Fig. 1), the significance of the times of these maxima is doubtful. It may well be, however, that the organs showing most true accumulation reach their maximum concentration later than others.

Fig. 2 also shows that most of the radioactivity of the kidney and the liver could have been caused by the residual urine and bile. The specific activity of the bladder-urine solids, which may have been somewhat greater or less than that of the urine actually in the kidney at the time of killing, was in all the kids from 20 to 80 times that of the dry matter of the kidney. Similarly the specific activity of bile solids from the gall bladder was 40–120 times that of the liver dry matter. These graphs show that although the reproductive organs and kidneys consistently showed the greatest accumulation of hexoestrol it was only transient and had disappeared within 24 hr. There was, however, a tendency for the ratio of uterine concentration to blood concentration to increase as the blood concentration itself decreased.

In Table 4 are expressed the estimated percentages of the total dose of hexoestrol which occurred in certain organs and tissues at various times after injection. Some of the values are approximations

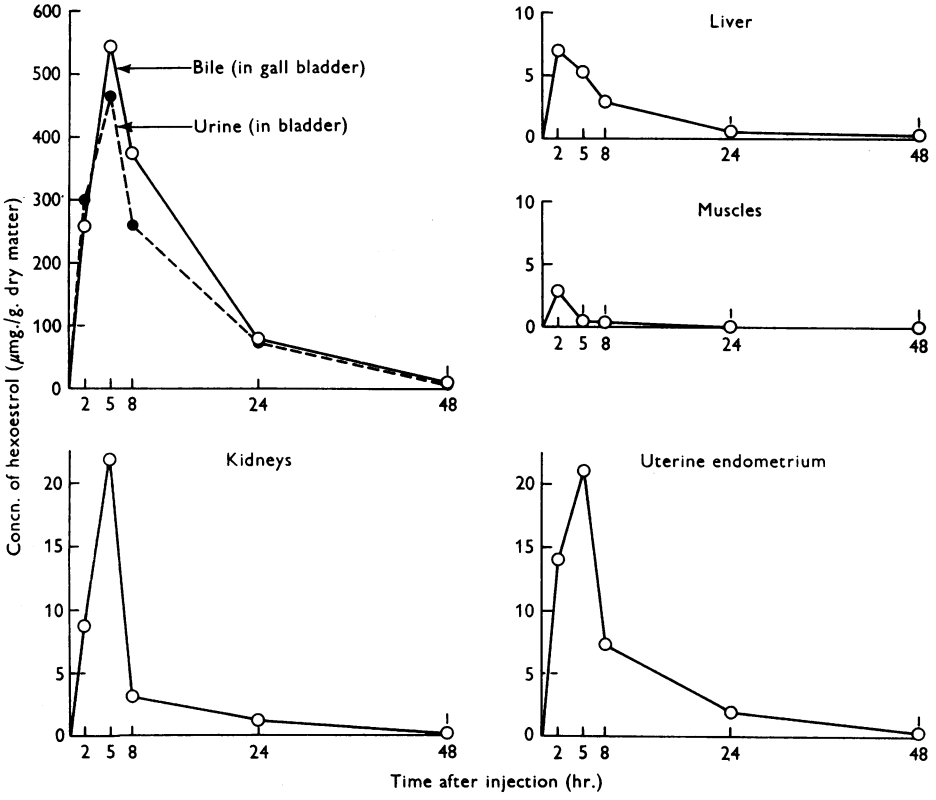

Fig. 2. Hexoestrol concentration–time curves for bile, urine and selected organs from the kids. Each point represents a single animal killed at that time. Each kid received a single subcutaneous injection of 25 μg. of tritium-labelled hexoestrol.

Table 4. *Distribution of hexoestrol in whole organs*

See text and Table 1 for details of treatment of the animals.

	Estimated percentage of total dose in organ								
	Kids					Lambs			
Animal no.	1	2	3	4	5	1	2	3	4
Body wt. (kg.)	14·3	17·7	22·8	14·0	14·2	30·4	25·0	23·9	25·9
Time after injection (hr.)	2	5·5	8	24	48	4	8	8	8
Dose	Single	Single	Single	Single	Single	Single	Single	Divided	Divided
Organ or tissue									
Uterus	0·06	0·17	0·05	0·01	0·001	0·05	0·05	0·09	0·07
Vagina	0·05	0·04	0·04	0·01	0·001	0·03	0·04	0·05	0·05
Kidneys	0·5	1·2	0·25	0·1	0·01	1·2	0·6	1·4	2·1
Liver	2·2	1·9	1·4	0·2	0·08	4·2	1·8	2·6	3·1
Intestine	1·1	2·1	0·8	0·1	—	2·4	1·4	6·9	2·5
Lungs	0·4	0·4	0·2	0·02	0·01	0·4	0·2	0·4	0·5
Heart	—	—	—	—	—	0·2	0·06	0·2	0·2
Skeletal muscle	19·4	4·5	3·4	—	—	7·8	6·4	3·7	5·3
Skin	1·4	2·2	1·9	0·7	0·6	1·7	1·2	1·8	2·1
Bone	—	—	—	—	—	2·5	1·1	1·7	1·8
Blood	3·6	8·2	2·5	0·4	0·07	10·2	3·7	7·5	9·0
Total body tissues	28·7	20·7	10·5	1·5	0·8	30·7	16·6	26·3	26·7

only. For example, considerable portions of uterine and vaginal tissues were retained as uncomminuted connective tissue (see Methods section). Assay of some samples of the washed uncomminuted tissues showed that they had about half the specific activity of the homogenizable portion. This may be of significance and deserves further study in relation to oestrogenic effects on connective tissues (Joseph, Engel & Catchpole, 1954; Asboe-Hansen, 1958). To estimate total concentrations of hexoestrol the assumption was made that 50 % of uterine and vaginal tissues were homogenizable and that the dry matter of the remaining tissues had half the specific activity of the homogenizable portion. Only a small portion of other organs was not homogenizable. The methods of calculating the total radioactivities of such organs as intestine, skeletal muscle, skin, bone and blood and their possible inaccuracies have already been discussed. It is felt, however, that the data in Table 4 are reasonably accurate and show that even though the organs such as uterus and vagina, which respond to oestrogens, accumulated the hexoestrol, the maximum amount accumulated in the whole organ was only a small fraction of the total dose (less than 0·2 %). At the same time, because of their much greater size, organs such as liver, kidney and intestine accumulated as much as 1–7 % of the total dose. There appeared to be an early (2 hr.) accumulation of hexoestrol in skeletal muscle which, however, is unconfirmed, since it was observed only in a single animal. Also a slower turnover of the oestrogen in skin than in most other organs is indicated.

DISCUSSION

That the dose used in this work was physiological is supported by calculations made from body weights and known values for rats (Dodds *et al.* 1944) and cows (F. Glover, personal communication), and also by the work of Robinson, Moore & Binet (1956), who found that the physiological dose of oestradiol benzoate for a ewe was 20 μg. Oestradiol benzoate and hexoestrol have about the same oestrogenic activity when administered subcutaneously (Emmens, 1940–41). Unfortunately, whether or not a physiological response had been produced was not observed in this work. Immature animals were used because it was supposed that, as in the rat (Wiberg & Stephenson, 1957), the uterus of goats and sheep is especially sensitive to oestrogens at this stage of growth.

The results on the excretion of physiological doses of hexoestrol by kids and lambs are in general agreement with those obtained in previous work on rats and rabbits (Dodds *et al.* 1958). The injected hexoestrol was rapidly excreted in both urine and faeces and only a small portion of the dose remained in the body tissues after 24 hr. Also in agreement with the work on rats (Dodds *et al.* 1958), little or none was completely oxidized. The importance of the biliary system as an excretory route for oestrogens has been stressed by many workers, as has the significance of an enterohepatic circulation of oestrogens (Cantarow, Rakoff, Paschkis, Hansen & Walkling, 1943; Sandberg & Slaunwhite, 1957). The observation in the present work that the ratio of total hexoestrol in urine to that in the bile, intestinal contents and faeces (Table 1) increased with time after injection, coupled with the finding of significant amounts in intestinal tissues, is in agreement with the supposition that a hepatic–biliary–enteric circulation of hexoestrol occurs in kids and lambs. It could be argued that such a circulation is of importance in maintaining the level of oestrogen in the blood. However, even at physiological doses the rapid excretion of hexoestrol is evidently the predominant metabolic process. Alternatively it could therefore be argued that the rapid excretion of oestrogens is essential to the efficient regulation of processes which respond to them.

Undoubtedly the most important contribution of this work is the discovery that when small doses of a synthetic oestrogen are administered to young female goats and sheep the highest concentration, either of the oestrogen itself or of a non-volatile derivative, is found in the organs which respond physiologically to it or excrete it. Although long suspected, this, as far as the authors know, is the first time that it has been demonstrated. Also of interest is the accumulation in the pituitary gland, which, though less than that in uterus and vagina, was about nine times that in the immediately adjacent brain. This suggests that oestrogens exert their effect on the pituitary gland direct. The concentration in the mammary glands, though again less than in uterus and vagina, is consistent with the fact that the glands respond to oestrogens (see, for example, Folley, 1956). The reason for accumulation in ovaries is not clear. As the ovaries secrete oestrogen one would not expect them to accumulate it. The accumulation in lungs, though low, is nevertheless greater than that in many other organs, and may be associated with the large proportion of epithelial tissue they contain. Turner (1956) found that the lungs of steers which had been fed with stilboestrol contained more oestrogenic activity than organs such as liver, muscle and kidney. The low concentration in the salivary glands was of interest in view of work on the possible relationship of the sex hormones in the saliva of pregnant women to the sex of the foetus. Rapp & Richardson (1952) obtained evidence that circulating androgens but not oestrogens are

secreted in saliva. Green (1954), however, found that after a dose of 1 g. of stilboestrol the saliva of an old man contained 24·4 μg./100 ml. and that of an old woman 5·8 μg./100 ml. Our work suggests that at physiological doses virtually no hexoestrol passes into the saliva of kids.

At the highest observed concentration the total amount accumulated in the uterus was only 0·2 % of the dose, and in the vagina only 0·05 %. This, however, is not wholly surprising in view of the much smaller doses which are effective when applied locally. The very brief accumulation in these organs does not conflict with observations made by other workers on the changes which occur in the uterus even within 6 hr. of giving oestrogen. Examples are increased water imbibition, electrolyte shifts and increased use of carbohydrate (Szego, 1957); increased uptake of serum albumin (Kalman, 1955); increased incorporation *in vitro* of precursors into proteins and nucleic acids and increased activity of amino acid-activating enzymes (McCorquodale & Mueller, 1958). Our work appears to support the protoplasmic-receptor theory of oestrogen action of Szego (1957).

Similar studies to ours have previously been made on rodents but not on goats or sheep. The failure of other workers to demonstrate a localization of oestrogen in the organs which respond to it is, however, probably due to the use of massive doses, coupled with relatively insensitive methods of detection, rather than to the use of a different species or a different oestrogen. In our work the maximum counting rate in the uterus was about 200 counts/min./mg. dry matter; if the hexoestrol had had only one-thousandth of its actual specific activity (that is about the same as that of the [^{14}C]oestrogens used by previous workers), then the radioactivity accumulated in the various organs could not have been detected. It further appears from the work of Budy (1955) and of Twombly & Schoenewaldt (1951) that if the dose had been increased 1000-fold the pattern of distribution would have been greatly altered and more hexoestrol would have been found in tissues such as bone, adrenal glands, lymph nodes and heart than in those organs most sensitive to it. It seems likely that selective localization on the organs which respond occurs only when small amounts are administered. This suggests that there is a limited amount which can be selectively accumulated. However, such a statement could be verified by comparable experiments with larger doses.

The radioactive compound in the samples has been assumed to be hexoestrol for the purposes of calculation although in fact it has not been identified. Workers who have used massive doses of synthetic oestrogens (1 g. or more to a rabbit; e.g. Smith & Williams, 1948; Dodgson, Garton, Stubbs & Williams, 1948) found that up to 70 % of the dose became conjugated with glucuronic acid, much of which was excreted in the urine. They also found some evidence of sulphate formation. Smith & Williams (1948) considered that there was further metabolism to oestrogenically inactive products. Hanahan *et al.* (1953) obtained evidence of conjugation of stilboestrol at much lower doses (5 μg. to a rat). Many workers have observed both conjugation and conversion into chemically different compounds in the metabolism of the natural oestrogens.

SUMMARY

1. Hexoestrol generally labelled in stable positions with tritium was injected subcutaneously into immature female goats and sheep in physiological doses. The excreta and certain body organs and tissues were assayed for radioactivity at various intervals after injection of the animals.

2. The radioactive hexoestrol was rapidly absorbed from the site of injection and excreted into the urine and into the intestinal contents and faeces by way of the bile. Little or none was completely oxidized to water. Only small amounts (<2 %) remained in the body tissues 24 hr. after injection. The existence of a hepatic–biliary–enteric circulation of hexoestrol or its derivatives was indicated.

3. There occurred marked selective localization of hexoestrol in the organs known to respond to oestrogens (uterus, vagina, mammary glands and pituitary gland), and in the organs of excretion and re-absorption of hexoestrol (kidney, liver, intestine) and also in ovaries and oviducts. Although these organs contained the highest concentration for at least 24 hr., the accumulation was transient with maximum concentrations occurring from 2 to 5·5 hr. after injection, and dropping rapidly to barely detectable levels 48 hr. after injection. The relatively high concentrations in kidney and liver could be attributed to the very high concentration in urine and bile solids. Lungs appeared to have a slight ability to concentrate the hexoestrol, and the other 12 organs and tissues studied, including adrenal glands, lymph nodes, skin, muscle, bone and perinephric fat, had only from 1 to 10 % of the concentration in the uterus and vagina.

4. Although the uterus and vagina had higher concentrations than any other organs, the maximum percentage of the total dose accumulated by them at any time was less than 0·2.

We wish to thank Dr A. T. Cowie for permitting the use of the post-mortem room and for catheterizing some of the animals; and Dr S. J. Rowland for the determination of nitrogen in tissue samples. The skilled assistance of Mr B. W. E. Peaple and Mr G. Jones in the gas counting is also gratefully acknowledged. The work was done while

one of us (W.G.H.) was on leave from the Department of Biochemistry, University of Wisconsin, Madison, Wisconsin, U.S.A. as a Merck Senior Postdoctoral Fellow of the National Academy of Sciences, National Research Council, Washington D.C.

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A Ribonucleoprotein of Skeletal Muscle and its Relation to the Myofibril

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(*Received* 12 *February* 1959)

From the work of a number of investigators there is evidence for the association of nucleic acid with preparations of certain myofibrillar proteins. This property is particularly marked in tropomyosin, which can be obtained from a variety of species and muscle types as a crystallizable complex, nucleotropomyosin, containing up to 20% of nucleic acid (Hamoir, 1951*a*, *b*; Sheng & Tsao, 1954). Smaller amounts of ribonucleic acid have been reported to be present in purified myosin preparations (Mihalyi, Laki & Knoller, 1957; Mihalyi, Brodley & Knoller, 1957). The significance of the ribonucleic acid associated with these proteins has been a matter for speculation, but the recent finding (Perry & Zydowo, 1958, 1959) that a ribonucleoprotein is one of the components of the extra protein (Szent-Györgyi, Mazia & Szent-Györgyi, 1955) suggests that the myofibrillar fraction of the cell may be the origin of the ribonucleic acid. This paper is concerned with the characterization of the ribonucleoprotein extracted from the myofibril and its relation to the distribution of ribonucleic acid in the muscle cell.

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METHODS

Preparation of myofibrils and muscle granules. Myofibrils were prepared from the back and leg muscles of the rabbit and the breast muscles of the hen by the method of Perry & Grey (1956), modified as described by Perry & Zydowo (1959). Washed granular preparations were prepared from the same sources by the method of Perry & Zydowo (1959).

Preparation of ribonucleoprotein. The dilute extra-protein fraction, prepared as described previously (Perry & Zydowo, 1959), in 0·04 M-KCl containing 6·7 mM-potassium phosphate buffer, pH 7·2, was adjusted to 0·1 M-KCl by the addition of solid KCl and to pH 7·6 with M-2-amino-2-hydroxymethylpropane-1:3-diol (tris). This solution was run into a column of diethylaminoethylcellulose equilibrated with 0·1 M-KCl containing 0·02 M-tris chloride