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The Excretion of Microgram Doses of Hexoestrol by Rabbits and Rats

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Extremely small doses of all the closely related synthetic female sex hormones are capable of producing a physiological response in laboratory animals. The effective subcutaneous dose of meso-hexoestrol for a rat is 0.2 µg. (Dodds *et al.* 1944), and from work on mice it appears that about a two-hundredth of the subcutaneous dose is effective when administered locally to the vagina (Emmens, 1940-41). The metabolism of such extremely potent substances is clearly of considerable interest, and this interest is heightened by their well-known use in cancer, particularly carcinoma of the prostate. Treatment with oestrogens causes a temporary regression of the malignancy, which, however, recurs after varying intervals of time when they are no longer effective. This was noted by Smith & Williams (1948), who suggested that the failure of continued treatment might be due to an alteration in the metabolism of the drug. Metabolism of female sex hormones is also of interest from the point of view of the induction of artificial lactation and of the fattening of cattle.

As pointed out by Hanahan, Daskalakis, Edwards & Dauben (1953) studies on the metabolism of oestrogens have been carried out in the main through the massive doses which were necessitated by the relatively insensitive methods

available for their detection in the excreta and tissues of the experimental animals. The isotopic tracer method is particularly suited to the elucidation of this problem because of the very small amounts of radioactive substances which can be detected. Such a method was applied by Albert, Heard, Leblond & Saffran (1949), who studied the metabolism of [¹³¹I]iodo-α-oestradiol after injection into mice at doses ranging from 1.7 µg to 2 mg. The iodo-compound, unlike the parent oestradiol, however, is not physiologically active even at doses as high as 100 µg. and this, coupled with the fact that the actual doses used were mostly very much higher than was the effective dose of oestradiol itself, somewhat detracts from the value of the work. However, these workers note that the outstanding feature of the metabolism of their compound was accumulation in the gastrointestinal tract, which agrees with what has been found for physiologically active oestrogens.

Twombly (1951) and Twombly & Schoenewaldt (1951) have studied the metabolism in female mice of ¹⁴C-labelled stilboestrol administered in doses of about 2 mg., which is more than a thousand times the effective physiological dose. They found that most of the dose was excreted in 21 hr., 70-85% by way of the faeces and 15-30% by way of the

urine. There was no selective absorption in any organ other than the liver and complete oxidation was negligible. Budy (1955) studied the metabolism of ^{14}C -labelled oestrone administered to female mice in doses of 0.1 mg. She found it to be more slowly excreted than stilboestrol, with some evidence of localization in liver, adrenals, lymph nodes, blood and bone, but none in reproductive organs.

Hanahan *et al.* (1953) have approached more nearly to effective physiological dose levels in their work with ^{14}C -labelled stilboestrol. Their compound was labelled in the ethyl side chain and was administered to rats in 5 μg . doses. This is about ten times the median-effective dose but was necessitated by the low specific activity (2.5×10^4 counts/min./ μg .) attainable by the use of ^{14}C -labelling. This specific activity was sufficient for gross-distribution studies, but would probably not be sufficient for a study of the more detailed metabolism that is ultimately required. Hanahan *et al.* found that a release of a conjugated derivative by way of the bile and excretion in the faeces is the major metabolic pathway, urinary excretion being negligible.

The present experiments have been carried out with tritium as a labelling agent. With tritium very much higher specific activities are attainable than with ^{14}C : the tritio-hexoestrol used in this work had a specific activity of more than 10^7 counts/min./ μg . compared with only 2.5×10^4 counts/min./ μg . of ^{14}C -labelled stilboestrol as used by Hanahan *et al.* (1953). And whereas the specific activity of the preparation used by those workers probably approached the maximum attainable, a tritium-labelled hexoestrol higher in specific activity by another factor of 10 should be possible and its preparation is being planned. Tritium, of course, is inferior to ^{14}C in that it does not label a specific atom in the carbon skeleton of the compound being studied and in that the possibility of exchange or labilization reactions must be eliminated before positive conclusions can be safely drawn. But it has the overriding and important advantage of conferring very high specific activities which make possible the use of physiological doses and less.

EXPERIMENTAL

Tritium-labelled hexoestrol. This was prepared by hydrogenation of dienoestrol with tritium-hydrogen (nominally 10% of tritium) on a micro scale. A preliminary account of the method used has been published (Glascok, 1954*a*). From the method of preparation and purification it is believed to be generally labelled in stable positions. In the preliminary experiments, which were carried out before a technique for the chromatographic purification of the labelled material had been worked out, the hexoestrol was

used after twice recrystallizing from benzene-light petroleum (b.p. 90–110°). It had m.p. 176–178°, i.e. 7° lower than that of the authentic material. It was stored in ethanol solution at a concentration of 270 μg /ml. In the main experiments the chromatographically purified material, m.p. 183–184°, was used. As this had a much higher specific activity than the earlier specimen it was stored in the dark at 4° in benzene solution at a low concentration (55 μg /ml.) to minimize chemical attack by any possible products of radiolysis of the solvent.

Assay of labelled hexoestrol. Both preparations were diluted with unlabelled carrier hexoestrol so that the dilution had a specific activity of the order of 2000 counts/min./mg. For the first preparation 0.095 ml. of the stock solution was diluted to 100 ml. with ethanol containing 94.1 mg. of unlabelled hexoestrol. This was added to avoid the manipulation of very dilute solutions, from which solute might be lost by adsorption or other means. In 1 ml. of this solution was dissolved 131.5 mg. of hexoestrol, the solvent was removed by evaporation under a jet of hydrogen and the residue dried to constant weight. The product had a specific activity of 1350 counts/min./mg. of combustion water, from which it was calculated that the hexoestrol in the stock ethanol solution had a specific activity of 5.4×10^6 counts/min./ μg . By a similar procedure the specific activity of the second preparation was found to be 1.136×10^7 counts/min./ μg .

Measurement of radioactivity. All the samples were burnt and the combustion water was converted into butane and counted in a gas counter as described by Glascok (1954*b*). Except for samples of very low specific activity, all samples of butane were divided into at least two portions, which were counted separately for at least 10 000 counts. The several values for specific activity so obtained usually agreed to within $\pm 1.5\%$ of their mean. The whole of the butane from samples of very low specific activity (e.g. blood water, blood solids, skin) was put into the counter at once and counted for as long as possible, usually at least 1000 counts. The probable error of counting of these samples is therefore likely to be about $\pm 3\%$. From the specific activity of the butane that of the combustion water can be calculated. When this is known the specific activity of a pure compound such as hexoestrol can be calculated from its chemical formula, but that of a mixed substance such as faeces, urine or blood solids can be calculated only if its yield of water on combustion is known. This was ascertained where necessary by carrying out a combustion, condensing the water into a tube and weighing.

Administration to animals. In the preliminary experiments two virgin female rabbits R1/54 and R1/53 were used, of weights 2.24 kg. and 3.19 kg. R1/54 received 1 μg . (5.4×10^6 counts/min.) and R1/53 received 100 μg . (5.4×10^7 counts/min.) of labelled hexoestrol subcutaneously in 0.5 ml. of aqueous solution. The animals were kept in metabolism cages and allowed to feed freely on their normal diet. Urine and faeces were collected for the periods 0–48 hr. and 48–96 hr. The faeces were dried at 100°, ground and assayed for tritium. Since experiments had shown that freeze-drying of aqueous solutions of hexoestrol containing less than 0.1 μg /ml. resulted in substantial losses, presumably by sublimation, 1 mg. of carrier hexoestrol was added to the urine samples before freeze-drying. Samples of from 2 to 4 ml. were then freeze-

dried in 20 ml. tubes on a vacuum system to constant weight. The residue in the tube was scraped off the walls as completely as possible, well mixed and assayed for tritium.

Two rat experiments were set up, each with five virgin females. Each animal received $1\mu\text{g.}$ (1.136×10^7 counts/min.) of chromatographically purified labelled hexoestrol subcutaneously in 0.2 ml. of arachis oil. They were kept in metabolism cages for the separate collection of urine and faeces and killed at 8, 24, 24, 32 and 48 hr. respectively after being injected. At death, samples of blood were withdrawn and freeze-dried, both residual blood solids and blood water, which is assumed to be representative of body water (see Tables 2 and 3), being assayed for tritium. Urine and faeces were treated and assayed for tritium as in the rabbit experiments.

RESULTS

Rabbit experiments. In Table 1 are shown the analyses and radioactivity measurements of urine and faeces from the two rabbits. Although the volume of urine passed by each rabbit during the two periods of collection is variable, the total of solids excreted was fairly constant at 12–15 g. (Table 1, column 5). These solids also showed little variation in composition, at least so far as is indicated by their water yield on combustion (column 6). The specific activity of this combustion water was low in the samples collected during the second 48 hr. period from the rabbit which had received only $1\mu\text{g.}$, but in all determinations it was high enough for the counting rate of the butane to be reliably measured. For both rabbits the total amount of radioactivity excreted in the urine solids was much lower during the second 48 hr. than during the first, although this difference is greater for the rabbit which had received $100\mu\text{g.}$ than for that which had received only $1\mu\text{g.}$ Whereas the first rabbit secreted more than one-fifth as much during the second 48 hr. as it did during the first 48 hr., the second rabbit secreted only about one-thirtieth as much. This difference may be merely biological variation or it may be due to the difference in dose.

Both animals excreted a considerable amount of radioactivity in the faeces although the specific activity of that obtained during the second 48 hr. from the rabbit which had received $1\mu\text{g.}$ was too low to measure. Whereas 32.6% of the total dose was excreted in the faeces of the rabbit which had received $1\mu\text{g.}$, only 22% was excreted in the faeces of the one which had received $100\mu\text{g.}$

Table 1 also shows that 81.5% of the $1\mu\text{g.}$ dose and 63.26% of the $100\mu\text{g.}$ dose was recovered in the excreta, which leaves a substantial fraction in each experiment unaccounted for. Clearly much of this might still be in the animal, either in the form of hexoestrol or its major metabolites, or in the form of metabolic water. The urine water of the

Table 1. *Recovery of tritium-labelled hexoestrol from rabbits*

Rabbit R 1/54 received $1\mu\text{g.}$ (5.4×10^5 counts/min.) and rabbit R 1/53 $100\mu\text{g.}$ (5.4×10^7 counts/min.) in aqueous solution subcutaneously.

		Combustion water from total solids										
		Period of collection (hr.)	Quantity	Total solids (g.)	Yield (%)	Total (g.)	Specific activity (counts/min./mg.)	Total activity (counts/min./mg.)	Equivalent as hexoestrol (μ g.)			
Rabbit R1/54	Urine	0-48	290 ml.	12.8	39.3	5.02	43.4	2.18×10^5	0.403	Total	0.815	
		48-96	415 ml.	14.8	41.5	6.1	7.6	4.64×10^4	0.086			
	Faeces	0-48	27.9 g.	—	52.0	14.5	12.15	1.76×10^5	0.326			
		48-96	21.6 g.	—	52.0	11.0	0	0	0			
											Total	0.815
R1/53	Urine	0-48	360 ml.	14.0	39.0	5.46	3895	2.16×10^7	40.0	Total	63.26	
		48-96	605 ml.	12.8	41.0	5.25	125	6.55×10^5	1.22			
	Faeces	0-48	50.8 g.	—	52.0	26.4	422	1.11×10^7	20.6			
		48-96	28.3 g.	—	52.0	14.7	53	7.77×10^5	1.44			
											Total	63.26

animal which had received 100 μ g. was assayed for tritium and found to have a specific activity too low to measure accurately, though of the order of 1 count/min./mg. Such a low specific activity was to be expected: if the whole dose had been oxidized the body water, of which the urine water may be assumed to be representative, would have acquired a specific activity of only 27 counts/min./mg. For the detection and accurate measurement of complete oxidation a much greater specific activity in the hexoestrol administered would clearly be necessary.

Rat experiments. The hexoestrol of higher specific activity was used in these experiments in the hope of detecting complete oxidation, if any. The rats were killed at different times during 48 hr. for the purpose of studying the variations, if any, in the amount of labelled hexoestrol attached to circulating blood solids and in the rate of appearance of tritium in body water. Table 2 shows the results obtained. The most important observation is the rapid rate of excretion of the drug. Even within 8 hr. (rat P1) 8.7% of the dose was excreted. Unfortunately, agreement between the only two replicates (L1 and R1, which were killed at 24 hr.) is very bad: whereas 73.5% of the dose was excreted by L1 only 47.7% was excreted by R1. The figures suggest that this low value could be due to loss of some urine from the cage in which rat R1 was kept, although there is no record of any accident to confirm that this actually happened. If the result of rat R1 is discarded, however, the recovery figures indicate that the bulk of the dose is excreted and that this excretion is virtually complete within 24 hr.

Table 2 also shows that the amount completely oxidized was low, although the specific activity of the body water would again be low (about 50 counts/min./mg.) even if the whole dose had been oxidized. However, the specific activity figures, which are all about 1 count/min./mg. or less, indicate that not more than 2% of the dose was in fact completely oxidized. The specific activity of the circulating blood solids was also low, showing that less than 1% of the dose was circulating at any time.

A second experiment (Table 3) gave results substantially in agreement with the first. It will be seen that the amount of labelled material excreted by the rat killed after 8 hr. was considerably greater (0.34 against 0.087 μ g.) than was observed in the first experiment, but with one exception the recoveries at other time intervals were similar. The exception (rat R1) showed a 110% recovery, a result which must be wrong. It might, of course, be due to a mistake in injecting. Although every care was taken to inject exactly 0.2 ml. of arachis oil solution of labelled hexoestrol it is possible that

some animals received slightly more or less than the correct amount. This could account both for the exceptionally high recovery in rat R1 of the second experiment and for the less than quantitative recoveries in all the others. However as the amounts recovered were predominantly less than quantitative, an assay of the skin at the site of injection was carried out on all the animals of the second experiment to find out whether absorption was complete. Samples of the skin were excised, weighed, dried and assayed for tritium. As shown in Table 3 the specific activities of the combustion water from these samples ranged from 16 to 278 counts/min./mg., but corresponded to negligible quantities of labelled hexoestrol.

DISCUSSION

This work shows that even at physiological dose levels the main metabolic pathway of hexoestrol administered subcutaneously is rapid excretion in both urine and faeces. Both rabbits and two out of the ten rats excreted more in the urine than in the faeces. The rabbit which was given only 1 μ g. excreted nearly equal amounts of radioactivity by these two routes, whereas that which was given 100 μ g. excreted twice as much in its faeces as in its urine. This cannot, however, be interpreted as the result of differing dose levels because there was considerable variation in the relative amounts excreted by the two routes in the rat experiments, where each animal received the same dose. Leaving rat R1 (Table 2) out of consideration for reasons already stated, it can be seen that the ratio of urinary to faecal excretion varies from 16:1 (rat P1, Table 2) to 0.25:1 (rat R1, Table 3). The high value of 16:1 might be due to the sample being an early one when excretion was clearly not yet complete, because in the others the scatter is not so wide, ranging from 1.6:1 (rat L2, Table 2) to 0.25:1. No explanation of this variable excretion by the two routes is immediately obvious.

The results are, however, in general agreement with those of Twombly (1951) and of Twombly & Schoenewaldt (1951). They are also in agreement with the results of Smith & Williams (1948), who found that urinary excretion of synthetic oestrogens by rabbits was 20–40% of the total dose after subcutaneous injections of up to 1 g. spread over several weeks. They differ, however, from the results of Hanahan *et al.* (1953) who, unlike earlier workers, found no urinary excretion of [14 C]-stilboestrol. These workers suggest that the apparent anomaly might be due to their having used a much smaller dose (5 μ g./rat). That this is not the explanation is suggested by the present work: even at 1 μ g. doses there is a substantial urinary excretion of hexoestrol or its metabolites.

It is true that Hanahan *et al.* were working with stilboestrol and not with hexoestrol but, as the excretory route is the same for both substances at high doses (Smith & Williams, 1948), one would expect it to be so at low doses, and the failure of Hanahan *et al.* to detect urinary excretion is therefore inexplicable.

Also of interest in the present work is the negligible amount of total oxidation that occurs. The amount of tritium in the body water does not amount to more than 2% of the initial dose and is probably less. This confirms that the hexoestrol was labelled in stable positions, since any labile tritium would have appeared in the body water. It agrees with the findings of Twombly (1951), Twombly & Schoenewaldt (1951) and of Hanahan *et al.* (1953), who detected no oxidation of [^{14}C]-stilboestrol. Also in agreement with the findings of these workers is ours that a negligible amount of hexoestrol is attached to circulating blood solids.

A problem that remains unresolved is the whereabouts of the 10–20% of the initial dose which is unaccounted for in the urine and faeces. Clearly it might still be in the carcasses of the animals but no analyses of tissues were made in these experiments. However, in other work (Glascock & Harris, unpublished work) it has been found that there is no accumulation of labelled hexoestrol in muscle or in any part of the nervous system, reproductive system, pituitary gland or mammary gland of spayed female rabbits killed 24 hr. after dosing with 100 μg . subcutaneously. It is of interest therefore to note that Twombly & Schoenewaldt (1951) found that, of a total recovery of 94.9% of 1.57 mg. of [^{14}C]stilboestrol injected into a mouse, only 0.17% remained in the body after 7 days. Further work is therefore necessary to discover whether or not some 10–20% of 1 μg . of hexoestrol really does remain in the body. When so large a fraction of the dose is excreted so rapidly one would not expect it to exert so profound and persistent a physiological effect unless the dose given were very much too big, the excreted fraction representing the excess. In these experiments, however, this cannot have been so: the dose of 1 μg . was at, or only slightly above, that required to produce a response. On the other hand, oestrogens of the hexoestrol type are some 200 times more active when administered locally to the vagina. Hence only a very small fraction of the dose need be retained, say in the reproductive system, for the persistence of the response after the bulk of the dose had been excreted to be more comprehensible.

SUMMARY

1. Hexoestrol generally labelled in stable positions with tritium has been injected subcutaneously in arachis oil solution at microgram dose levels into two rabbits and ten rats. One rabbit received 100 μg .; the other rabbit and the rats each received 1 μg .

2. The outstanding feature of the metabolism of the oestrogen is rapid excretion, either unchanged or as an unidentified non-volatile metabolite. Up to 90% of the initial dose was recovered in urine and faeces.

3. Excretion by both routes was substantial although the amount of radioactive material in the faeces was up to four times as much as in the urine. In some experiments, however, more was excreted in the urine than in the faeces.

4. Total oxidation as revealed by the specific activity of the body water was negligible and certainly less than 2% of the initial dose. The amount of labelled material circulating attached to the blood solids and remaining unabsorbed at the site of injection after 8 hr. or more was also negligible.

5. The results are broadly in agreement with earlier work carried out on the same and other synthetic oestrogens with very much larger doses.

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