of α -melanocyte-stimulating hormone in *N*-acetyl-corticotrophin A_2 is inhibited by other structural features of its molecule.

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The Preparation and Purification of Tritium-Labelled Hexoestrol of Very High Specific Activity on the 5 mg. Scale

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Tritium can be introduced into organic compounds by catalyst-induced exchange with ionic tritium, which is usually contained in the solvent (water, acetic acid, sulphuric acid, etc.) in which the reaction takes place, by radiation-induced exchange with tritium gas (Wilzbach, 1957), or by

catalytic addition of tritium to unsaturated compounds. Exchange with ionic tritium suffers from the disadvantages that the high temperatures required may cause decomposition (Bloch & Rittenberg, 1943), that specific activities are limited by that obtainable in the solvent, which

must be used in large excess, and that exchange may not be complete within a reasonable time. Exposure of organic compounds to tritium gas results in exchange with little decomposition even of very labile compounds but yields products of only low specific activities. Provided that a suitable unsaturated parent compound can be obtained, catalytic addition of tritium is undoubtedly the best method, both because of the high specific activities which are theoretically obtainable and because of the relatively mild conditions of the reaction.

Because the specific activities obtainable with tritium-labelled compounds are always high compared with those of ^{14}C -labelled compounds and because of its low cost the use of tritium is becoming more common, and some tritium-labelled compounds are available commercially. Many of the methods of preparation (e.g. those of Done & Payne, 1956; Crawhall & Smyth, 1958; Bernstein, Bennett & Fields, 1952) are carried out on the conventional scale for hydrogenation (5–10 m-moles). This almost always involves the dilution of the tritium with many times its own volume of hydrogen since, when the weight of compound being hydrogenated is equivalent to 100 ml. or more of hydrogen, it is scarcely feasible to use tritium at its maximum specific activity ($97\% \text{ }^3\text{H}_2 \equiv 2.7\text{ c/ml. at s.t.p.}$). In work with compounds of high specific activity the weight of compound required is usually small and hence its preparation on a micro-scale is suitable.

The highest specific activities so far reported seem to be those of the labelled oestrogens prepared by O'Donnell & Pearlman (1958), who hydrogenated 26.8 mg. of Δ^6 -dehydro-oestrone acetate with 3 c of tritium and obtained oestrone acetate of specific activity 17.5 mc/mg. in 16% radiochemical yield. The apparatus is not described in this preliminary communication but was clearly not on the conventional scale.

Dodds, Golberg, Lawson & Robinson (1939) reported the hydrogenation of 3:4-di-(4-hydroxyphenyl)hexa-2:4-diene (dienoestrol) to *meso*-3:4-di-(4-hydroxyphenyl)hexane (hexoestrol) in good yield over a palladium-black catalyst. Williams & Ronzio (1950), working on the conventional scale with tritium-hydrogen of low specific activity, used the same reaction in their preparation of tritium-labelled hexoestrol. They isolated both hexoestrol and *isohexoestrol* (the racemic compound) from their reaction mixture by crystallization procedures, but in all experiments obtained a yield of considerably more than 100%, which they thought might be due to solvent of crystallization.

For several years we have had in use in our laboratories a hydrogenation apparatus which works on the 50 μmoles scale ($\equiv 1\text{ ml. of hydrogen}$

at s.t.p.), in which it is therefore feasible to use tritium of maximum specific activity. It has been used for the preparation, from a nominal 10% tritium-hydrogen mixture, of labelled hexoestrol and stearic acid. Biochemical experiments with these compounds have been reported (Dodds, Folley, Glascock & Lawson, 1958; Glascock & Hoekstra, 1959; Glascock & Reinius, 1956; Glascock, Duncombe & Reinius, 1956). Labelled hexoestrol prepared from 97% tritium has also been prepared and is now being used.

A preliminary description of an early version of the apparatus has been published (Glascock & Reinius, 1954) and has been quoted (Glascock, 1954). Because of improvements in the apparatus and in the technique of purifying the tritiated hexoestrol a full description of the method is now presented.

It was to be expected that, when preparations on the 5 mg. scale were carried out, the separation of the isomers by crystallization as used by Williams & Ronzio (1950) would be difficult; indeed such attempts to isolate hexoestrol from the products of the hydrogenation of diennoestrol on this scale were unsuccessful. A paper-chromatographic method for the quantitative separation of hexoestrol from *isohexoestrol* was therefore developed with artificial mixtures of the isomers, and was then successfully applied to the products of hydrogenation of diennoestrol with hydrogen or tritium.

As the reaction may not always go quite to completion a small amount of unchanged diennoestrol may be present in the reduction products and cannot be separated from hexoestrol by the chromatographic method. A spectrophotometric method was therefore devised which enabled diennoestrol in mixtures of hexoestrol and *isohexoestrol* to be detected (to a lower limit of 2%) and reaction products were tested for diennoestrol before isolation of the hexoestrol was begun.

MATERIALS AND ANALYTICAL METHODS

Materials

Dienoestrol was obtained from L. Light and Co. Ltd., Colnbrook, Bucks, and recrystallized from aq. ethanol shortly before use, m.p. 227–228°. Dioxan was purified by treatment with HCl and then with KOH solution, followed by drying and redistillation as described by Vogel (1957). Palladium-black catalyst was prepared according to the method of Heilbron, Sexton & Spring (1929). Hydrogen gas, as supplied in cylinders by British Oxygen Gases Ltd., was purified by passage through a U-tube containing granular charcoal, previously heated to expel dissolved gas in a stream of H_2 , and cooled in liquid air. Commercial 95% ethanol (James Burrough Ltd.) was used in the spectrophotometric work. Benzene was the pure, crystallizable grade; light petroleum, ethyl acetate and methanol were laboratory-reagent grades and diethyl ether was the

anaesthetic grade (all supplied by May and Baker Ltd.). The B (3) solvent of Bush (1952) used for the column-partition chromatography was first made from these solvents and the upper phase was then redistilled to remove traces of non-volatile matter. Inorganic salts were of laboratory-reagent grade and the compound 4-aminophenyl 2-diethylaminoethyl sulphone was supplied by L. Light and Co. Ltd. The diazonium-salt reagent was prepared by diazotizing this compound as described by Boscott (1951).

Celite 545, used for partition chromatography, was obtained from Johns-Manville and Co. Ltd. It was prepared for use by heating in air at 600° for 16 hr. and then extracting in a Soxhlet apparatus first with benzene and then with methanol.

Alumina-impregnated paper. This was prepared by a method based on the work of Datta & Overell (1949), Datta, Overell & Stack-Dunne (1949) and Bush (1953). Eight Whatman 3 MM papers (46 cm. × 18 cm.) were dipped in filtered aqueous 6.5% (w/v) $\text{Al}_2(\text{SO}_4)_3 \cdot 12\text{H}_2\text{O}$, removed immediately and hung for 10 min. to drain in chromatography tanks, four papers to each tank. Sufficient aqueous NH_3 soln. (sp.gr. 0.880) was then added by means of a long-stemmed polythene funnel to cover the floor of each tank to a depth of about 2 cm. The tanks were kept closed for 24 hr., after which the NH_3 was sucked out and the papers were washed by passing tap water (filtered through glass wool to remove suspended matter) to the bottom of each tank and allowing it to overflow. Washing was continued for 24 hr. and the papers were dried in a well-ventilated room maintained at 28°. They were dry to the touch in 30 min. In order to prepare papers of constant adsorptive properties it was found to be particularly important to standardize the drying conditions: papers hung in the laboratory at 20–25° took more than 2 hr. to dry and these had an inconveniently high adsorptive power. The properties of the impregnated papers could be tested by chromatographing, as described later, 10 μg . of a mixture of hexoestrol and isohexoestrol on a test strip 2 cm. wide cut from the long edge of each. It was found that papers, when stored at 28° in the dark, retained their satisfactory properties for at least 1 year.

Analytical methods

Radioautographs. The positions of radioactive spots could be found by placing the paper in contact with Ilford Industrial G X-ray film. Times of exposure varied from 15 min. (when tritium of 97% purity had been used in the preparation) to several hours.

Measurement of radioactivity. The specific activity of the tritiated hexoestrol was determined either by liquid-scintillation counting or by gas counting. For scintillation counting, a portion of a suitable subdilution in toluene was added to a solution in toluene of 2:5-diphenyloxazole (4 g./l.) and counted in an Ekco N 612 scintillation counter cooled to -18°. For gas counting a portion of a suitable subdilution was added to a weighed amount of hexoestrol dissolved in ether. The ether was distilled off, and the residue dried to a constant weight and submitted to combustion and gas counting as described by Glascock (1954).

Spectrophotometric estimation of dienioestrol in mixtures with hexoestrol and isohexoestrol. Solutions were made up in 95% ethanol and measurements of extinction were made

with a Unicam SP. 500 spectrophotometer with 1 cm. silica cells.

The light-absorption curves for hexoestrol and isohexoestrol each at a concentration of 50 μg ./ml. were determined over the range 235–265 $m\mu$ and found to be almost identical, each with a very low minimum at 247 $m\mu$, whereas dienioestrol at a concentration of only 5 μg ./ml. had much greater absorption, decreasing steadily in the range 235–265 $m\mu$. The extinctions at 247 $m\mu$ for these hexoestrol, isohexoestrol and dienioestrol solutions were respectively 0.035, 0.02 and 0.27. When E at 247 $m\mu$ for six concentrations of dienioestrol in the range 1–10 μg ./ml. was plotted against concentration a straight line was obtained, indicating that Beer's law was obeyed. It thus seemed probable that measurement of E at 247 $m\mu$ could provide a simple method for the estimation of dienioestrol when mixed with hexoestrol and isohexoestrol, and that in a total concentration of 50 μg ./ml. the presence of as little as 1 μg . of dienioestrol/ml. (2% of total solute) could be detected.

To test this, the absorption curve of a solution containing hexoestrol (50 μg ./ml.) and dienioestrol (2.5 μg ./ml.; dienioestrol 4.8% of total solute) was compared with that of a hexoestrol solution (50 μg ./ml.) over the range 235–265 $m\mu$. The difference in E at 247 $m\mu$ of the two solutions indicated, from the extinction-concentration line for dienioestrol, the presence of 2.4 μg . of dienioestrol/ml. (4.6% of total solute). This and similar tests with dienioestrol, hexoestrol and isohexoestrol mixtures confirmed that the method was capable of estimating unchanged dienioestrol in reduction products in proportions as low as 5%, with an error not greater than $\pm 10\%$, and of reliably detecting as little as 2%. It was considered that unchanged dienioestrol in a proportion of 5% or less in reduction products would not interfere with the efficient isolation of the hexoestrol.

Melting point of hexoestrol. All melting points were determined on a Kofler heating-stage microscope RCH, the crystals being crushed beneath a cover slip. This is particularly important for hexoestrol, which sublimes rapidly above 150°.

We noticed that specimens of hexoestrol prepared in our apparatus from dienioestrol with either hydrogen or hydrogen containing 10% of tritium did not melt completely at 186°. A proportion estimated at 5–10%, visible through the analyser and polarizer in the crossed position, did not melt below a temperature of about 190°. It was feared that this indicated an impurity, and to examine this possibility several reference specimens of hexoestrol were tested. These comprised several commercial specimens as received, a specimen recrystallized from benzene, another recovered after slow sublimation at 110–115° and a specimen which had been recrystallized from ethanol containing a little HCl to remove any traces of alkali-metal salts present (this specimen was kindly supplied by Dr D. A. Peake of Boots Pure Drug Co. Ltd.).

All specimens showed identical behaviour at their melting points and it is concluded that this behaviour is not due to an impurity but is characteristic of pure hexoestrol, especially since it is known that most of the specimens of commercial origin had been synthesized by a method other than the reduction of dienioestrol (Dr D. A. Peake, personal communication).

Chromatography (analytical). It was found that the isomers of hexoestrol could not be separated on Whatman no. 2 paper by the solvent systems of Bush (1952). They

could be separated, however, on alumina-impregnated paper by the method which he described for the separation of steroid oestrogens.

Spots containing 5 and 10 $\mu\text{g.}$ of the hydrogenation product and 5 and 10 $\mu\text{g.}$ of a reference mixture (1:1) of hexoestrol and isohexoestrol were deposited at well-separated points along a base line 8 cm. from the end of a sheet of alumina-impregnated paper. It was important to use benzene for applying these compounds to the paper because polar solvents caused some irreversible adsorption. The chromatogram was then developed with 8% (v/v) of ethyl acetate in benzene in a chromatography tank, on the floor of which was a layer of the same solvent mixture to maintain the atmosphere saturated with vapour. The solvent front took 2–3 hr. to reach the end of the paper, which was then air-dried and sprayed with aqueous 5% K_2CO_3 and then with diazonium-salt reagent. If the papers had been properly prepared the position of the isomers in the reference mixture was revealed as pink spots at R_F 0.6 (hexoestrol) and 0.35 (isohexoestrol). Hydrogenation products, known from spectrophotometric analysis to be free from dienoestrol, gave two spots of the same R_F and colour as the spots of the reference mixture. Proof of the identity of the faster-running compound followed from its isolation described below. One would, of course, expect the other compound to be isohexoestrol, and although this was not proved by isolation the fact that the light-absorption curves for hydrogenation products and the standard hexoestrol–isohexoestrol mixture were the same was good evidence for its identity.

By visual comparison of the azo-dye spots given by the reference mixture and by samples of hydrogenation products it was estimated that the products contained 50–70% of hexoestrol and 30–50% of isohexoestrol. Although a closer estimate than is represented by this range was not possible visually we are convinced that the content of isohexoestrol was never less than 30%.

Other experiments showed that the minimum quantity of either isomer detectable by chromatography and the diazonium-salt reagent was about 1 $\mu\text{g.}$; much smaller quantities of labelled compounds could be detected by long exposure to X-ray film.

METHOD OF PREPARATION

Hydrogenation apparatus

The high-vacuum apparatus used is shown in Fig. 1. It was evacuated to a pressure of 10^{-6} mm. Hg or less by means of a mercury-vapour pump suitably backed by a mechanical pump. T_1 and T_2 were Toepler pumps with bulbs of capacity about 300 ml. The tubes below stopcocks 10 and 24 were calibrated as for a McLeod gauge (Glascock, 1954) and were of such a size that they were capable of measuring up to about 1.25 ml. of gas at s.t.p.

Hydrogenations took place in small flasks F_1 and F_2 , of capacity 0.4–0.5 ml., attached to the apparatus by means of a standard B5 ground-glass joint. The part of the apparatus to which the flask F_1 was attached is shown in greater detail in Fig. 2. The corresponding part of the apparatus to which F_2 was attached was exactly similar. The

manifold bounded by stopcocks 8 and 10, by the valve V_1 (see below) and F_1 was made of capillary tubing (internal diam. 2 mm.). The capacity of the manifold + flask F_1 was thus about 1.5 ml.

The bulbs of the device R_1 were of about 10 ml. capacity. It was charged with mercury so that by evacuating, or admitting air to, the bulb beneath stopcock 6 the other bulb was either emptied or filled (under the control of stopcock 7), the small glass valve V_1 preventing accidental overshooting of mercury into the manifold above it. The device was used to compress the charge of tritium-hydrogen to about 1 atm., as will be described later.

S was a tube containing purified dioxan in contact with several pieces of bright sodium. When the dioxan was first put into the tube it was frozen in liquid air and the space above it evacuated through stopcocks 31, 32 and 33. This process removed all the dissolved gas. The dioxan was then thawed and refrozen and the gas pumped off; this cycle was repeated several times. The bulb above the tube S had a capacity of 250 ml. and when filled with dioxan vapour in equilibrium with liquid at laboratory temperature (21°) it therefore contained an amount equivalent to about 50 $\mu\text{l.}$ of liquid, the volume used as a solvent for each hydrogenation.

The ampoule of tritium G was sealed to the apparatus below stopcock 19, a side arm being provided to hold the mild-steel weight with which the break-seal was opened. The space between stopcocks 18 and 19 had a capacity of about one-twentieth of the total space beneath stopcock 19 and could thus be used as a gas pipette for the withdrawal of small amounts of tritium.

P was a measuring device for taking any desired volume of gas up to the capacity of the Toepler pumps and was used, for example, when the tritium already withdrawn from G measured and transferred to the reaction vessel (F_1 or F_2) had to be diluted with H_2 to the volume required for the hydrogenation. The capacity of space x between stopcocks 16 and 17 was 0.25–0.35 ml. and that of space y beneath stopcock 17 was 1.0–2.0 ml. Gas was allowed to enter P and the manifold above it to a pressure p recorded on the manometer M . Stopcocks 16 and 17 were closed and the gas remaining in the apparatus was pumped away. The gas in space x was allowed to expand into either Toepler pump and the gas trapped by the rising mercury when the pump was operated in the usual way was measured. The gas in y was treated in a similar way. These volumes were proportional to the pressure p at which the gas was originally admitted to P and, after determining the calibration constants for both x and y with reference to both Toepler pumps, any volume of gas could be

Fig. 1. High-vacuum apparatus for hydrogenation on the scale of $50 \mu\text{moles}$. 1–33, Stopcocks; T_1 and T_2 , Toepler pumps; M , mercury manometer; R_1 and R_2 , devices for temporarily increasing the volume of reaction-flask manifold (see text); F_1 and F_2 , reaction flasks; P , gas-measuring device (see text); G , ampoule of tritium; S , solvent reservoir; H , reservoir of purified hydrogen.

Fig. 2. Device R_1 of Fig. 1 in greater detail. F_1 , Reaction flask; V_1 , glass valve to prevent overshooting of mercury into manifold above.

taken simply by adjusting the pressure at which gas was admitted to P , which could be done very accurately. In our apparatus the constants were 2.5 and $17.5 \mu\text{l./cm. Hg}$ pressure for T_1 and of similar values for the other Toepler pump T_2 .

Hydrogenation procedure

About 5 mg. of dienolestrol and 2 mg. of palladium black were accurately weighed into a reaction flask containing a piece of soft-iron wire (about 2 mm. long) encased in glass, which served as a magnetic stirrer. The flask was attached to the manifold at F_2 , the joint being lubricated with silicone grease. It was evacuated through stopcock 25, care being taken to avoid withdrawing the air too quickly otherwise rapid desorption from the catalyst might scatter it about the apparatus. The bulb above the solvent reservoir S was evacuated, stopcock 32 closed and stopcock 33 left open for a few minutes to allow the bulb to fill with vapour. The dioxan vapour in the bulb was then condensed into the flask F_2 , which was chilled in liquid air, by way of stopcock 25. Tritium was withdrawn from the ampoule and pumped past stopcock 24 into the reaction flask and manifold. In order for this to be possible the mercury had to be withdrawn from the upper bulb of R_2 , which was refilled with mercury after the gas has been transferred. This was always necessary when all but the smallest volumes of gas were compressed past stopcock 24.

The volume of tritium transferred at each stroke was noted and, if necessary, the volume was made up to the calculated value with H_2 measured in the device P as already described. The calculated volume depended on the

weight of dienooestrol and the blank value for catalyst and solvent. This blank was the volume of H_2 taken up irreversibly by a weighed amount of catalyst in 50 μ l. of dioxan. It amounted to 20 μ l./mg. of catalyst in our apparatus and with our materials. It was best to use about a 5% excess of total H_2 for the hydrogenation of dienooestrol in order to drive the reaction as near to completion as possible.

The initial volume of tritium-hydrogen was checked by allowing it to expand past stopcock 24 into the bulb of the Toepler pump with the mercury set at the level *A-B*, and remeasuring in the usual way. It was returned to the reaction flask and manifold, the liquid air was removed from around the reaction flask and the magnetic stirrer was started. The course of the reaction could be followed at timed intervals by removing the stirrer motor, chilling the reaction flask in liquid air and measuring the unreacted gas as described above. Uptake ceased in from 3 to 5 hr. when, allowing for catalyst blank, 98–102% of the theoretical volume of gas had been taken up.

At the end of the reaction the residual gas was either discarded or, by means of the other Toepler pump, transferred to a break-seal tube attached in the place of F_1 . The solvent dioxan was distilled into a tube below stopcock 28 and the small amount of gas which was released was measured in the Toepler pump and taken into account when calculating net uptake.

Isolation procedure

The contents of the reaction flask were extracted with 95% ethanol and the solution was filtered. A solution of concentration 50 μ g./ml. in 95% ethanol was then prepared on the assumption that the theoretical weight of hydrogenation products had been formed and that all had been extracted. From the extinction of this solution the dienooestrol content was determined and always found to be less than 5% when the theoretical volume of hydrogen had been taken up.

The specific activity of the product was also determined at this stage. To 100 mg. of carrier hexoestrol dissolved in ether a measured volume of a subdivision of the extract was added such that the carrier would acquire a convenient specific activity. The solution was then evaporated to dryness, the residue dissolved in dilute alkali (to displace any tritium on the phenolic groups), precipitated with acid, extracted with ether and isolated again. Its specific activity was then measured and the specific activity of the original mixed product, free from tritium in the phenolic groups, was calculated. This value was needed at a later stage in the purification when the concentration of hexoestrol in a benzene solution was calculated from the specific activity of the solution.

The main ethanolic solution was then reduced in volume to about 1 ml. by evaporation in a stream of N_2 , 0.1 ml. of N -KOH was added and the solution was diluted to 10 ml. with water. The pH was brought to 7.0 by the addition of dilute HCl and the solution was extracted with 3×10 ml. of benzene-ether (4:1). The combined extracts were washed with 3×5 ml. of water and then distilled until the volume of the solution was 2 ml. This removed both water and ether and left the hydrogenated product dissolved only in benzene.

The chromatographic separation was extended to the preparative scale. Half of the solution in 2 ml. of benzene was applied to each of two impregnated papers along the

base line. By applying the solution to the paper very slowly in a current of warm air from a pipette with its delivery end bent at a right angle the solute was deposited in a band about 1 cm. wide. The two sheets of paper were then eluted with 8% (v/v) of ethyl acetate in benzene by the descending method until the solvent front reached nearly to the end of the paper. The papers were dried at room temperature and radioautographs were prepared. The time of exposure depended, of course, upon the total radioactivity on the paper but with hexoestrol prepared from 10% tritium about 4 hr. was required to give sufficient blackening of the film. The position of the two isomers was then revealed as two bands across the film each about 4 cm. wide separated by a white zone of about the same width. The part of each chromatogram corresponding to the leading band was then cut out and the two rectangular strips (each about 5 cm. $\times$ 15 cm.) were pinned to a piece of unused alumina-impregnated paper and eluted overnight with the ethyl acetate-benzene solvent mixture. About 10 ml. of solvent passed through each of the strips, thus extracting all the hexoestrol, and was collected in a vessel standing on the floor of the tank. Because chemical attack ultimately due to radiation is least extensive in benzene solution the solvent mixture used for elution was distilled off and the residue dissolved in pure dry benzene. When tritium of the highest available specific activity had been used in the preparation the labelled hexoestrol was never isolated in the solid state at all because of radiolysis (see below). In these preparations the two strips of paper were eluted with pure benzene. Hexoestrol moves very slowly in this solvent but nevertheless about 90% was extracted overnight and most of the remainder within a further 24 hr. About one-third of the benzene was then distilled off to remove traces of water, the residual solution assayed for radioactivity and then made up to a volume corresponding to a convenient hexoestrol concentration (usually 50 μ g./ml.).

When a spot containing 60 μ g. of labelled hexoestrol separated in this way was chromatographed on alumina-impregnated paper no *isohexoestrol* was detected either by the diazonium reagent or by radioautography. And from the appearance of the spots produced by 2 and 5 μ g. samples of authentic *isohexoestrol* run at the same time it was concluded that the labelled hexoestrol contained less than 2% of *isohexoestrol*.

Even so, recrystallization, after removal of the benzene, did not give the hexoestrol in good yield with the correct sharp melting point. This was attributed to the high losses involved in recrystallization on the 2–3 mg. scale, to traces of foreign matter extracted from the chromatography paper and to traces of radiation-induced decomposition products. Further purification on a partition column was therefore used. A tube 1 cm. in diameter was packed as described by Martin (1949) with 10 g. of Celite 545 mixed with 5 g. of the lower phase of the solvent system of Bush (1952) (light petroleum-benzene-methanol-water; 10:5:12:3, by vol.). The column was first washed with 200 ml. of redistilled upper phase to remove soluble impurities. The benzene solution of labelled hexoestrol was evaporated to dryness and the residue taken up in a combination of 3 ml. of upper phase with 1 ml. of lower phase. It was then added to the top of the column, which was operated in the usual way at a flow rate of about 15 ml./hr., and 15 ml. fractions were collected. In an actual run with

hexoestrol, prepared from tritium, of nominal specific activity 330 mc/ml., and 5.5 mg. of dienestrol, fractions 8, 9, 10 and 11 all gave crystalline residues together weighing 2.8 mg., m.p. 186–188° undepressed on admixture with authentic hexoestrol. Yield 51%. This preparation had a specific activity of 13.7 mc/mg. After being stored in a desiccator for 2 weeks its melting point diminished by 10° because of radiolysis. Another preparation, made from tritium of nominal specific activity 2.5 mc/ μ l. (97% tritium) had a specific activity of 118 mc/mg. It was not further purified on the partition column because that would have involved evaporation of the benzene solution to dryness and we have not demonstrated that the column would remove the small quantities of radiolytic products that might form before the dry residue could be taken up into solution again. The impurities derived from the paper in no way impair the usefulness of the product for biological purposes as they are not labelled and are most unlikely to be oestrogenic.

The specific activity of these preparations was only about 25% of that expected if the tritium used had its nominal specific activity.

DISCUSSION

The preparation of tritium-labelled hexoestrol on this scale proceeded much as was expected from previously published work although the yield was less than was expected. Dodds *et al.* (1939) reported a nearly quantitative yield of hexoestrol when dienestrol was hydrogenated over a palladium-black catalyst, whereas substantial amounts of *isohexoestrol* are formed under the conditions of our work. This is shown not only by the amount of hexoestrol actually isolated but also by the amount of *isohexoestrol* revealed by chromatography. Williams & Ronzio (1950) reported a yield of at least 25% of *isohexoestrol* in their preparations but their observations may not be quite relevant because they used a different catalyst (palladium on charcoal). Moreover they separated the isomers by recrystallization, which is less nearly quantitative than chromatography and they had difficulty, as already noted, with total yields of greater than 100%.

Williams & Ronzio (1950) also reported that their reaction went more quickly with hydrogen containing tritium than with hydrogen alone. Their reaction was carried out in ethanol or acetone with activities of tritium which gave a product of specific activity 1 mc/mg. With levels of radioactivity from 10 to 100 times as great as theirs we have not observed that tritium increases the rate of the reaction in dioxan solution. Indeed with high activities of tritium (10% or more) we have observed the reverse effect: unless the reaction goes to completion within a few hours it may come to a standstill. The magnetic stirrer, which was not originally used (Glascock & Reinius, 1954), was introduced into the technique for this reason. We at first thought that the apparent inhibitory effect

of tritium might be due to some catalyst poisons contained in it but since careful purification over charcoal chilled in liquid air made no difference we now provisionally attribute the inhibitory effect to the formation of catalyst poisons by radiolysis during the course of the reaction.

Our method also differs from those carried out on the conventional scale in the relatively large amount of catalyst used. The weight of palladium is about 40% of that of the dienestrol instead of 1–2% as would normally be used. With smaller amounts of catalyst the reaction goes too slowly even with pure hydrogen. This is probably connected with the fact that the pressure of the hydrogen is not constant but diminishes as the reaction proceeds and finishes at only about 5% of the initial pressure.

Martin (1949), in his theoretical treatment of partition chromatography, predicted that it would in general be less successful than the adsorption method in separating isomers and our results are in accord with this theory. Alumina-impregnated paper is thought to be of possible value in dealing with some separation problems where paper-partition chromatography has failed; it has an advantage over adsorption columns in that the same paper may be used for microgram-scale analysis and thus an evaluation of its possible usefulness quickly and easily made. When it is used on a preparative scale the introduction of traces of foreign matter into the compound being isolated is probably unavoidable and the use of a Celite partition column for final purification is particularly to be recommended as the Celite and solvents are capable of being very highly purified.

SUMMARY

1. An apparatus for the catalytic hydrogenation of unsaturated organic compounds on the scale of 50 μ moles is described. It is used for the hydrogenation of dienestrol with hydrogen containing up to 97% of tritium, when a product of specific activity 118 mc/mg. is obtained.

2. A method for the separation of the isomeric products hexoestrol and *isohexoestrol* on alumina-impregnated paper is given together with a column-partition method for the further purification of the separated hexoestrol from unlabelled impurities derived from the paper.

3. A spectrophotometric method for the detection of unchanged dienestrol in the reduction products to a lower limit of 2% is also described.

4. Hydrogen uptake usually ceases within a few hours, when it is found that the theoretical volume of hydrogen ($\pm 2\%$) has been taken up and less than 2% of unchanged dienestrol remains in the reaction mixture.

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Fluorescence-Polarization Spectrum and Electronic-Energy Transfer in Tyrosine, Tryptophan and Related Compounds

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The polarization spectrum of the fluorescence is the set of values of the polarization of the fluorescence emitted upon excitation with light of different wavelengths. Simple symmetry relations exist among the polarizations of the fluorescence emitted in different directions, and by excitation with light of different states of polarization (Perrin, 1929; Soleillet, 1929), so that the values for a single direction of observation and a given state of polarization of the exciting radiation contain all the relevant information. The data recorded in this paper are for the fluorescence emitted at right angles to the direction of the exciting, unpolarized beam.

The absolute value of the polarization of the fluorescence emitted by molecules in solution can decrease as a result of the Brownian molecular rotations (Perrin, 1926), as well as by intermolecular transfer of the excited state (Sveshnikoff & Feofilov, 1940; Weber, 1954). In a dilute solution in which the average distance between molecules is much greater than the distance at which transfer can readily take place (of the order of 30 Å), and in a medium of high viscosity in which the rotational relaxation time of the molecules is much longer

than the lifetime of the excited state, both these causes of depolarization disappear and the polarization reaches a maximum absolute value, the principal polarization. In some well-studied cases this appears still inferior to the value that would be expected from a knowledge of the relative orientation of the absorption and emission oscillators, due to the molecular-torsional vibrations (Jablonski, 1950), and perhaps to other causes at present imperfectly understood. A plot of the principal polarization against wavelength, the principal polarization spectrum, reflects to a first approximation the relative orientations of the transition moments associated with the absorption and emission of light (Jablonski, 1935). These directions of the transition moments are expected to remain constant for each electronic transition. Ideally, the principal polarization spectrum should consist of regions of constant polarization where the excitation falls upon one electronic band alone, joined by regions of rapidly changing polarization where there is overlap of contiguous electronic bands. A study of the principal polarization spectrum should lead to the enumeration of the existing electronic bands, and to the distinction between