4. A mechanism is proposed for the reaction, and its relation to enzymic transphosphorylation is discussed.

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The Biosynthetic Preparation of [16-^{3}H]Aldosterone and [16-^{3}H]Corticosterone

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For both metabolic *in vivo* and analytical *in vitro* uses of radioactive steroids it is an advantage to add amounts of the labelled compound much smaller than are already present in the animal or extract but sufficiently radioactive to be followed with ease through any process. [4-^{14}C]Cortisol of sufficiently high specific activity (about 0·004 μc/μg.) for most purposes is now available. However, [4-^{14}C]corticosterone of similar specific activity is not suitable for many applications owing to the low rate of secretion of this hormone in man. (Ayres *et al.* 1957*a*; Peterson, 1957). Radioactive aldosterone, of specific activity high enough to be useful in metabolic studies, has not previously been prepared.

The advantages in the preparation and use of tritiated steroids are the ease and cheapness of their synthesis at high specific activities and their relative radiation safety for metabolic studies in man. The disadvantages of their use are the difficulty of assay, which is overcome by the application of methods employing a flow counter (Banks, Crawhall & Smyth, 1956) or a liquid scintillator (Davidson & Feigelson, 1957), and also the possibility of unstable labelling. This latter consideration has been discussed elsewhere (Pearlman, 1957*b*) and found to be unlikely for most metabolic applications of [16-^{3}H]progesterone. The same remarks apply to the use of 16-^{3}H-labelled corticosteroids.

The preparation of [16-^{3}H]progesterone of high specific activity has been described previously (Pearlman, 1957*a*). Progesterone has been shown to be an intermediate in the biosynthesis of aldosterone and corticosterone by capsule strippings of

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ox adrenal gland (Ayres, Hechter, Saba, Simpson & Tait, 1956*b*). This tissue has been shown to consist mainly of zona glomerulosa which, on incubation, produces much more aldosterone per gram of tissue than does whole adrenal cortex (Ayres, Gould, Simpson & Tait, 1956*a*). [16-^{3}H]Aldosterone and [16-^{3}H]corticosterone of high specific activity (about 2 μc/μg.) can therefore be prepared by incubating [16-^{3}H]progesterone with such tissue. A preliminary account of some of this work has been published (Ayres *et al.* 1957*a*).

MATERIALS AND METHODS

General

Solvents, kieselguhr (Celite 545) and silica gel for column chromatography and general use were prepared as previously described (Ayres, Garrod, Simpson & Tait, 1957*b*). For crystallization, ethanol was purified by refluxing over magnesium ethoxide, followed by distillation through a short column; benzene and light petroleum were purified by shaking with sulphuric acid, followed by washing with water and a 10% (w/v) aqueous solution of sodium carbonate. They were then dried over anhydrous calcium chloride and distilled through a short column. The light petroleum was of boiling point 80–100° except where stated otherwise.

Samples of authentic crystalline corticosterone and cortisol for melting-point comparisons were kindly given by Glaxo Laboratories Ltd., and the Medical Research Council Steroid Reference Collection respectively.

Melting points were determined on a Kofler block and are corrected.

Duralumin planchets for the assay of tritium were obtained from A. and H. Hall, 67 Elmers End Road, Beckenham, Kent, and were thoroughly scrubbed and washed in chloroform before use.

The Perkin–Elmer double-beam instrument, Model 21, was used for infrared-absorption spectroscopy. This was carried out in carbon disulphide solution (3 mm. microcell) for aldosterone diacetate and corticosterone monoacetate, in chloroform solution (1 mm. microcell) for cortisol monoacetate, aldosterone diacetate and corticosterone monoacetate and in a potassium bromide disk for cortisol monoacetate. Ultraviolet-absorption spectroscopy was carried out in ethanol (0·5 cm. cells) in the Hilger Uvispek H 700.

Acetylation was carried out by dissolving the steroid in 0·3 ml. of pyridine and adding 0·15 ml. of acetic anhydride. The resulting solution was left overnight at room temperature and then evaporated to dryness at 20°.

Assay of tritium

By flow-counter. The tritiated material was deposited from 0·2 ml. of a chloroform solution to cover completely a circular planchet, 2·5 cm. in diameter, which had a large number of circular grooves, about 0·05 mm. deep, scored on its surface. This ensured fairly uniform distribution of the material. The planchet was dried at 45° and assayed in a flow counter (Tracerlab Inc., Boston 10, Mass., U.S.A., Model SC18).

In preliminary experiments it was found that, if various weights of progesterone of equal total radioactivity were assayed in this way, the counting rate was greatly depressed from 0 to 60 μg. and then decreased more gradually for additional quantities. Fig. 1 shows the results obtained for different amounts of progesterone added to a constant quantity of [16-^{3}H]progesterone. Presumably the initial rapid drop in the count rate is due to the absorption of back-scattered β-rays of very low energy. The half-value thickness, calculated from the second part of the curve (approx. 60 μg./cm.^{2} solid) corresponds to what would be expected from the energy of the primary β-rays of tritium.

Ten samples were counted for each particular weight of material and the standard deviation calculated (Fig. 1). The coefficient of variation is about 12% for samples of negligible weight but decreases to 6% for weights above 100 μg. This large variation at negligible weight may be due to very small but variable amounts of grease which are impossible to remove by ordinary cleansing processes. This material would have a considerable effect on the count rate on the initial part of the curve of count rate against weight ($<$40 μg. of solid), but much less on the second part of the curve. It is therefore necessary, for accurate work, to add at least 100 μg. of progesterone to the specimen and we have used 160 μg. of the steroid for this purpose. When this is done, the extra addition of 10 μg. of all substances so far tested, including corticosterone, cortisol, progesterone, deoxycorticosterone and beeswax, has no significant effect on the counting rate.

Different amounts of wax added to a negligible but constant weight of [16-^{3}H]progesterone give rise to a similar curve to that shown in Fig. 1, but the extra addition of 10 μg. of various steroids to 160 μg. of wax significantly lowers the counting rate below that obtained by adding 10 μg. of wax. When stearic acid is used as the major component, the same effect can be demonstrated.

As a routine procedure, therefore, if the sample weighs less than 10 μg., it is dissolved in 0·3 ml. of a solution of progesterone (800 μg./ml.) in chloroform and 0·2 ml. of the mixture placed on the planchet. The counting efficiency of this procedure is 16% and the coefficient of variation 6·5%, if random statistical counting errors are negligible.

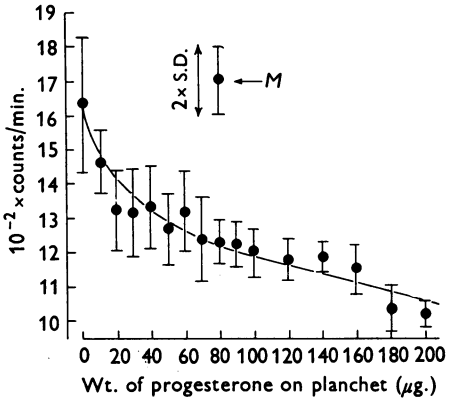

Fig. 1. Counting rate plotted against weight of progesterone on planchet. A negligible but constant weight of [16-^{3}H]progesterone was added to all planchets. The mean (*M*) and standard deviation at a particular weight were obtained by counting ten samples.

If the sample is of greater weight, it is made up to at least 160 μg. with progesterone and counted on the planchet. An equal quantity of this mixture plus a negligible weight of radioactive [16-^{3}H]progesterone standard, giving a high counting rate at least five times that of the sample, is also counted, from which a correction for self-absorption can be applied to the original count. The coefficient of variation of this method, which has successfully been applied to the assay of various crude extracts, is about 9% if random statistical counting errors are negligible. To avoid possible charging effects (Banks *et al.* 1956) samples less than 2 mg. ($<300\,\mu$g./cm.^{2}) were used.

It was also possible to assay after paper chromatography and development of the soda fluorescence of the tritiated steroids. The appropriate area of the developed paper was counted in the flow counter with an overall efficiency of 0·24% for Whatman No. 2 paper before spraying with sodium hydroxide solution and 0·22% after spraying and heating.

By bremsstrahlung *emission.* It was convenient, when dealing with large amounts of tritium, to assay samples by their *bremsstrahlung* emission. The planchet, prepared as described above, was counted by a G.E.C. type E.H.M. 2 mica-window counter giving 1·3 counts/min./μc of tritium. Other sources, such as the steroid deposited on a glass slide, gave similar counting efficiences. No gain in efficiency was obtained by using a Mullard MX 118 soft X-ray Geiger tube. The half-value absorption thickness for the *bremsstrahlung* radiation was about 6 mg./cm.^{2} of cellophan, and hence self-absorption effects were usually negligible.

Standardization. A combustion method (Avivi, Simpson, Tait & Whitehead, 1954) was used to obtain the overall efficiency of the counting procedures and also the absolute activity of the steroids. The activity of a sample of [16-^{3}H]progesterone was thus compared with tritiated water, supplied by A.E.R.E., Harwell, and calibrated there by mass spectrometry. Therefore all values given in this paper for efficiencies and activities are in terms of this standard water sample.

EXPERIMENTAL

Preparation of [16-^{3}H]*progesterone*

3β-Hydroxypregna-5:16-dien-20-one acetate (589·5 mg.) was hydrogenated with 20 c of tritium gas (7·9 ml. at s.t.p., 98·7% of H) as described by Pearlman (1957*a*). Partition chromatography instead of chromatography on alumina was used for isolating [16-^{3}H]progesterone from the products of the Oppenauer oxidation stage. The solvent system methanol–water (4:1, v/v)/light petroleum was used with 80 g. of Celite 545 and 40 ml. of stationary phase in a 3 cm. diameter column, $R_{\text{progesterone}}$ 0·25. Crystalline [16-^{3}H]progesterone (107 mg.), m.p. 121°, was obtained from light petroleum, $[\alpha]_D^{15} + 190 \pm 9°$ in chloroform (*c*, 0·1), light-absorption maximum 240 mμ (ϵ 16 300). The specific activity was 6·35 μc/μg.

Incubation of progesterone, extraction and preliminary purification of adrenocortical steroids

This preparation of [16-^{3}H]progesterone (96·5 mg.) was incubated with a total weight of 3·3 kg. of capsule strippings of ox adrenal gland.

Ox adrenal glands were obtained from the slaughterhouse approximately 40 min. after death and then placed at 0° until reaching the laboratory. The glands were then cleaned of all fat and connective tissue except the capsule. A slit was made along one side of the gland by passing a scalpel beneath the capsule, the blade being kept parallel with the capsule surface. The capsule was then peeled off with forceps. This tissue (8 g./flask) was incubated with 234 μg. of [16-^{3}H]progesterone and 80 ml. of Krebs–Ringer solution (Krebs, 1933) buffered at pH 7·4 with 0·04 M-2-amino-2-hydroxymethylpropane-1:3-diol. The solution also contained 3 mM-sodium fumarate, 3 mM-nicotinamide and 10 mM-glucose. Incubation was carried out in oxygen at 37° with shaking for 2 hr. The medium was then drained from the tissue and extracted three times with an equal volume of chloroform at room temperature, the emulsion being broken by centrifuging at 5°. The chloroform extract was then evaporated to dryness in an evaporator (Craig, 1950) and the solvent recovered with condensers maintained at −20°. The extract (from 72 g. of tissue at a time) was then dissolved in 100 ml. of ethyl acetate–light petroleum (1:1, v/v) and the solution put on a 2 cm. diameter column containing 15 g. of silica gel. The corticosteroids were eluted with 50 ml. of ethyl acetate–methanol (1:1, v/v), which was then evaporated to dryness (Bush & Sandberg, 1953). Of the added radioactivity 66% was found in this fraction. Less than 1% appeared in the ethyl acetate–light petroleum eluate or in methanol strippings of the column. The dried material from the ethyl acetate–methanol fraction was then extracted with ethanol at 0°. This removed over 99% of the radioactivity present and achieved considerable purification, particularly from material which tended to clog the subsequent partition columns.

Preliminary analysis

Values for the total corticosterone, aldosterone and cortisol were obtained in the following manner. A portion from the ethanol extract, equivalent to 17 g. of tissue, was chromatographed on several partition columns, each of which had a resolution of 1000 theoretical plates. The first column separated corticosterone ($R_{\text{corticosterone}}$ 0·9) from a mixture of cortisol and aldosterone (R 0·48), both of which ran at the same speed in this solvent system [methanol–water (1:1, v/v)/benzene–ethyl acetate (21:4, v/v), system *A*]. They were completely separated from one another on a second column system ($R_{\text{aldosterone}}$ 0·32, R_{cortisol} 0·18), with methanol–water (1:1, v/v)/benzene, system *B*. Aldosterone was further purified on another column ($R_{\text{aldosterone}}$ 0·10) with methanol–water (1:1, v/v)/benzene–light petroleum (7:3, v/v), system *C*. The corticosterone was also further purified on another column system ($R_{\text{corticosterone}}$ 0·32), with methanol–water (4:1, v/v)/benzene–light petroleum (17:3, v/v), system *D*. All columns were 60 cm. in length and 1 cm. in internal diameter and contained 24 g. of Celite 545 and 12 ml. of stationary phase.

Portions of the eluted fractions from the columns were chromatographed on paper in the Bush *C* system (Bush, 1952) for cortisol and aldosterone and a modified *B*2 system [methanol–water (4:1, v/v), stationary phase] for corticosterone. The amounts of steroid in the fractions of the final column of each particular compound were estimated by comparing their soda fluorescence with that of standards by a fluorimeter after paper chromatography (Ayres *et al.* 1957*b*). During the purification of the steroids

Table 1. *Amounts and specific activities of corticosterone, aldosterone and cortisol produced*

The amount produced is the total from 3·3 kg. of capsule strippings (corrected for recovery) and is according to preliminary analysis.

Compound produced	Amount produced (mg.)	Specific activities (μc/μg.) Column fractions of free compound before crystallization	Cryst. free compound	Column fractions of acetyl derivative
Corticosterone	59	2·77 ±0·1	$2{\cdot}6_2 \pm 0{\cdot}1$	$2{\cdot}7_7 \pm 0{\cdot}1$
Aldosterone	9·9	1·74 ±0·07	1·82±0·07	1·75±0·07
Cortisol	8·2	$0{\cdot}76_5 \pm 0{\cdot}03$	0·76±0·03	0·72±0·03

the recovery was kept as high as possible (all fraction tubes containing the appropriate steroid were pooled for the next column). From the extract of the incubating fluid to the final paper spot, the recovery was about 80% in all three cases. An estimate of aldosterone made by direct paper chromatography of the extract on the Bush *B*5 system [methanol–water (1:1, v/v)/benzene] was about 35% higher than that after purification, even after correction for recovery. This was due to the presence in the original extract of an unidentified compound giving soda fluorescence and moving only slightly slower than aldosterone in the Bush *B*5 system. This compound was completely separated from aldosterone by the same solvent system on a column (system *B* and also in system *C*). The incubation gave 2·5, 3·0 and 18 μg./g. of tissue for cortisol, aldosterone and corticosterone respectively, all values being corrected for recovery (Table 1).

Isolation of steroids

The ethanol extract from 3·3 kg. of tissue was purified on a partition column, 3 cm. in diameter and 60 cm. long, containing 80 g. of Celite 545 and 40 ml. of stationary phase. Solvent system *A* was used, which again separated corticosterone from a mixture of cortisol and aldosterone.

Corticosterone. The corticosterone was further purified on four columns by solvent system *D*. The first column contained 80 g. and the other three 24 g. of Celite 545, with corresponding column dimensions (60 cm. × 2 cm. or 1 cm. diameter) and volumes of stationary phase (always half the weight of Celite, v/w). The quantities of steroid in the fractions of the final column were estimated by u.v. absorption (taking ϵ 16 500 at u.v.-absorption maximum 240 mμ) and tetrazolium reaction in solution (Mader & Buck, 1952) compared with that of authentic corticosterone. The tritium was assayed by flow counter and from *bremsstrahlung* emission. For those fractions containing appreciable steroid (usually arranged to be a maximum of three, height of theoretical plate 0·7 cm. for these columns), the estimate by u.v. absorption and tetrazolium reaction agreed within the limits of experimental error. Paper chromatography in the modified Bush *B*2 system of a portion of these fractions revealed only one spot. This gave equivalent and symmetrical soda-fluorescence and tetrazolium reactions quantitatively in agreement with the measurements carried out in solution. The amount of steroid and radioactivity corresponded in all fractions and the mean specific activity from the two tubes containing most steroid was $2{\cdot}7_5 \pm 0{\cdot}1$ μc/μg. (Table 1).

These two fractions were pooled and a portion taken for crystallization, which was carried out from ethanol. The crystals were washed with ethanol at 0° and light petroleum (b.p. 40–60°) at room temperature and dried over silica gel *in vacuo* at 60° for 15 hr. Crystals (10·8 mg.) were obtained, m.p. 179–182·5°, u.v.-absorption maximum 240 mμ. Authentic corticosterone gave m.p. 180–182·5°; a mixed melting point with the radioactive crystals was 179–182°. A solution of the crystals gave equivalent u.v. absorption at 240 mμ and tetrazolium reaction and also soda-fluorescence and tetrazolium reaction after paper chromatography in the Bush *B*2 or Zaffaroni propylene glycol–toluene systems. Only one spot could be seen after both these reactions as a result of paper chromatography in the two systems, and this ran at the same speed as corticosterone. Over 97% of the radioactivity was coincident with the soda-fluorescence area. The specific activity of the crystalline material was $2{\cdot}6_2 \pm 0{\cdot}1$ μc/μg. (Table 1).

The washings from the crystallization were acetylated and a portion was chromatographed in the Bush *B*3 paper system. Only one symmetrical and coincident spot could be seen after soda-fluorescence and tetrazolium reaction and this ran at the same speed as corticosterone acetate. The remainder was chromatographed on a column containing 24 g. of Celite 545 and with solvent system methanol–water (4:1, v/v)/light petroleum–benzene (3:2, v/v), system *E* ($R_{\text{corticosterone}}$ 0·28). The radiochemical purity of the fractions containing corticosterone acetate was investigated in a similar manner to that described for the final column of the free compound and was satisfactory. The steroid in the peak fractions (containing 4·1 mg. of corticosterone acetate) was investigated by infrared-absorption spectroscopy; about 250 μg. gave spectra (including the carbonyl and fingerprint regions) identical with that of authentic corticosterone acetate. The mean specific activity of the two peak fractions was $2{\cdot}7 \pm 0{\cdot}1$ μc/μg. (Table 1). A portion of the crystalline free compound was also acetylated and gave one spot in the Bush *B*2 paper system. This had the same R_F as corticosterone acetate. Over 95% of the radioactivity was coincident with the soda-fluorescence area.

Aldosterone. The aldosterone was chromatographed on a second column with 80 g. of Celite 545 and solvent system *A* and then separated from cortisol and the other major impurity, previously mentioned in the description of the preliminary analysis, by two further 24 g. columns, with solvent system *B*. The final 24 g. column employed as a solvent system methanol–water (1:1, v/v)/benzene–ethyl acetate (19:1, v/v) ($R_{\text{aldosterone}}$ 0·40), system *F*. The purity of the steroid in the fractions of this column was examined as described for corticosterone except that the Bush *B*5 paper system was used. It was satisfactory according to the same criteria. The mean specific activity

from the two tubes containing most steroid was $1{\cdot}74 \pm 0{\cdot}07\ \mu c/\mu g$. (Table 1).

These two fractions were pooled and crystals obtained from 10% (v/v) aqueous ethanol. The crystals were washed with ethanol at $-5°$ and light petroleum (b.p. 40–60°) at room temperature, and dried over P_2O_5 *in vacuo* at 45° for 15 hr. This gave 1·87 mg. of crystals, m.p. 111–115°, remelting at 156–162° with some small remnants up to 165°, u.v.-absorption maximum 240 mμ. We have been unable to obtain for comparison synthetic (+)-aldosterone crystals of high melting point. The final melting point of crystals from natural sources as determined by Kofler block has been reported as 153–158° (Simpson *et al.* 1953); 157–163° (Simpson & Tait, 1955, carried out under conditions identical with those of the present study); 156–165° (Luetscher, Dowdy, Harvey, Neher & Wettstein, 1955). A solution of crystalline material gave equivalent u.v. absorption ($\lambda_{max.}$ 240 mμ, ϵ 16 500) and tetrazolium reaction as compared with authentic aldosterone. The estimate obtained from these two methods was in agreement with that from soda-fluorescence and tetrazolium reactions after paper chromatography in the Bush *B*5 or *C*, the E_2B (Eberlain & Bongiovanni, 1955) or Zaffaroni propylene glycol–toluene systems. After both reactions only one spot could be seen in all four systems and this ran at the same speed as aldosterone. Over 95% of the radioactivity was coincident with the soda-fluorescence area. The specific activity of the crystalline material was $1{\cdot}8_2 \pm 0{\cdot}07\ \mu c/\mu g$. (Table 1).

The washings from the crystallization were acetylated and a portion run in the Bush *B*3 paper system. After soda-fluorescence and tetrazolium development only one spot could be seen. This had the same R_F as had aldosterone diacetate. The remaining steroid was chromatographed on a 24 g. column, with solvent system *E* ($R_{\text{aldosterone diacetate}}$ 0·35). The radiochemical purity of the fractions containing aldosterone diacetate (total 900 μg.) was satisfactory by the usual criteria. About 250 μg. gave an absorption spectrum in the infrared, including the carbonyl and fingerprint region, identical with aldosterone diacetate. The mean specific activity of the two fractions containing most steroid was $1{\cdot}7 \pm 0{\cdot}07\ \mu c/\mu g$. (Table 1).

Some of the crystalline compound was acetylated and gave one spot running at the same speed as corticosterone acetate ran in the Bush *B*3 system. Over 97% of the radioactivity was coincident with the soda-fluorescent area. A portion of the acetylated material was mixed with aldosterone [*carboxy*-^{14}C]diacetate prepared from authentic aldosterone (Ayres *et al.* 1957*b*) and chromatographed on a column, with solvent system *E*. The tritium was assayed by flow counter (the counts being corrected for the small contribution from the ^{14}C β-rays) and the ^{14}C on glass slides by a mica-window counter (Ayres *et al.* 1957*b*). The ^{3}H/^{14}C ratio was the same in all fractions within the limits of experimental error and 99 ± 5% of the counts originally present as free [16-^{3}H]aldosterone appeared in the fractions containing ^{14}C. This figure corresponds to the product of the percentage purity of the original aldosterone multiplied by the percentage yield to diacetate on acetylation.

Cortisol. The cortisol was further chromatographed on a second 80 g. column, with solvent system *A* and another three 24 g. columns employing systems *B*, *F* and *A*, in that order. The radiochemical purity of the free compound, in the fractions of the final column and after crystallization, and that of the cortisol monoacetate, in the fractions of its column, were investigated as described for aldosterone and corticosterone and found to be satisfactory. The relevant specific activities obtained were $0{\cdot}76_5 \pm 0{\cdot}03$, $0{\cdot}76 \pm 0{\cdot}03$ and $0{\cdot}72 \pm 0{\cdot}03\ \mu c/\mu g$. respectively (Table 1). The paper-chromatographic systems for investigating the free compound were as for aldosterone. The modified Bush *B*2 paper system and a 24 g. column, with solvent system *D*, ($R_{\text{cortisol monoacetate}}$ 0·45) were used to isolate and investigate the acetyl derivative.

Crystals of [16-^{3}H]cortisol were obtained from 50% aqueous ethanol and were washed with ethanol at 0° and light petroleum (b.p. 40–60°) at room temperature, then dried over silica gel *in vacuo* at 60° for 30 hr. Crystals (2·29 mg.) were obtained, which gave m.p. 217–220°, $\lambda_{max.}$ 241 mμ. Authentic cortisol gave m.p. 216–219°, mixed m.p. 215–219°. A further 950 μg. of cortisol monoacetate was obtained by acetylating the washings from the crystallization.

No attempt was made to achieve good recovery during the isolation of the radioactive steroids, and only the peak fractions were taken for further chromatography, because it was thought that radiochemical purity of the final material was the important consideration.

Stability of the [16-^{3}H]*progesterone*

Non-radioactive progesterone (Sigma) was purified by column chromatography, as described for the isolation [16-^{3}H]progesterone, and crystallized from the mobile phase. The dried crystals gave m.p. 121°.

[16-^{3}H]Progesterone, prepared and characterized as described above, except that the final specific activity was $3{\cdot}04\ \mu c/\mu g.$, was dissolved in benzene (770 μg./ml., 2·35 mc/ml.) and kept at 0° for 3 months. From this solution 154 μg. was then added to 15·2 mg. of non-radioactive progesterone and the specific activity of the mixture determined by flow counter and u.v. absorption as $(3{\cdot}04 \pm 0{\cdot}08) \times 10^{-2}\ \mu c/\mu g$. A portion of the mixture was now chromatographed on the same column system and the specific activity of the steroid estimated in the various fractions. The distribution of steroid and radioactivity was that of pure progesterone and the specific activity was equal in all fractions within the limits of experimental error. The mean specific activity of the two peak fractions was $(3{\cdot}01 \pm 0{\cdot}08) \times 10^{-2}\ \mu c/\mu g.$, indicating (99 ± 4)% pure [16-^{3}H]progesterone after 3 months' storage. The peak fractions were pooled and the progesterone crystallized from the mobile phase. The crystals were washed with light petroleum (b.p. 40–60°) at room temperature and dried over silica gel *in vacuo* for 15 hr. at 60°. The crystals (4·91 mg.) had m.p. 120·5–121·5° and specific activity $(2{\cdot}9_3 \pm 0{\cdot}08) \times 10^{-2}\ \mu c/\mu g$. This indicated that $(96{\cdot}5 \pm 4)$% pure [16-^{3}H]progesterone had been obtained after storage.

DISCUSSION

The yield of radioactivity as [16-^{3}H]progesterone from tritium gas was 3·6%. The reasons for this low yield have been discussed by Pearlman (1957*a*). The radioactive yield of [16-^{3}H]aldosterone from [16-^{3}H]progesterone was, from the results of the preliminary analysis, 2·9%. The overall yield

of radioactivity in the form of [16-^{3}H]aldosterone from tritium gas was 0·1%. The cheapness of tritium gas makes this low figure not a serious disadvantage. The preparation of [4-^{14}C]aldosterone in a similar manner, by incubating with a [4-^{14}C]intermediate such as [4-^{14}C]corticosterone, would be very expensive and would give maximum specific activities of about 0·03 μc/μg. As the secretion rate of this hormone by man is about 200 μg./day and the peripheral blood concentrations are about 0·03 μg./100 ml. of plasma (Ayres *et al.* 1957*a*) this material would be of too low a specific activity for most purposes. The overall yield of radioactivity from tritium to [16-^{3}H]-corticosterone is nearly 1%, which is satisfactory. The production of radioactive cortisol during the incubation is probably due to contamination with zona fasciculata tissue, which is unavoidable in the large-scale preparation of tissue. The specific activity of this compound is much lower than that of either corticosterone or aldosterone. This phenomenon of lower specific activity, which has been consistently observed when capsule strippings are incubated with either [16-^{3}H]- or [4-^{14}C]progesterone, and at different steroid-substrate concentrations, is as yet unexplained. It seems unlikely that it is due to high concentrations of cortisol or of precursors in the tissue before incubation. The use of [16-^{3}H]cortisol may have some advantages on the grounds of radiation safety in the study of the metabolism of this hormone in such conditions as human pregnancy. It could probably be made more efficiently by incubating [16-^{3}H]progesterone with whole adrenocortical tissue or by a microbiological process from the same radioactive steroid.

The synthetic preparation of [16-^{3}H]corticosterone and aldosterone from appropriate starting products might be achieved by routes similar to those employed in the preparation of [16-^{3}H]progesterone (Pearlman, 1957*a*). The synthesis of aldosterone (Schmidlin, Anner, Billeter & Wettstein, 1955) increases this possibility, especially as one of the steps involves hydrogenating a C-16-unsaturated compound. However, the steps are numerous from this point and the yield is low, especially of the naturally occurring (+)-aldosterone, which would have to be obtained for metabolic studies rather than the racemic mixture. In addition, the labile tritium at C-17 would have to be eliminated. Tritium-labelled adrenocortical hormones have been prepared by Fukushima & Gallagher (1952) by isotope-exchange procedures; the amounts of stably bound tritium incorporated were small, but still useful for metabolic experiments. Another route to the preparation of tritiated compounds may be found in the Wilzbach (1957) technique, which is very simple, requiring merely exposure of the compound to tritium gas. The tritiated product requires rigorous purification, as the formation of a variety of radioactive by-products may be expected. The isotopic labelling is of a random nature. The specific activity of the final product, although high, does not approach the order of radioactivity obtainable by synthetic methods.

The results reported here confirm the earlier findings that the zona glomerulosa produces a high ratio of aldosterone to cortisol compared with whole adrenal cortex (Ayres *et al.* 1956*a*). The isolation of crystalline radioactive aldosterone makes the identification of the preferentially produced compound more rigorous and confirms that the hormone obtained after incubation is being biosynthesized rather than merely released from the tissue into the surrounding fluid. They also confirm that progesterone can be an intermediate in the biosynthesis of aldosterone (Ayres *et al.* 1956*b*), which supports the conclusion of the one group (Rosenberg, Rosenfeld, Ungar & Dorfman, 1956) but not that of another (Wettstein, Kahnt & Neher, 1955).

The results of the experiment on the stability of [16-^{3}H]progesterone indicates that there is no significant radioactive disintegration when a solution of 2 mc/ml. in benzene is kept for 3 months at 0° in air, the [16-^{3}H]progesterone being then (99 ± 4) and $[96{\cdot}5 \pm 4)$% pure as determined by two methods. However, the slight indication of impurity from the latter result, although not quite significant, suggests that it may be better to store this steroid at a lower concentration. The stability of the radioactive adrenocortical hormones remains to be seen.

SUMMARY

1. [16-^{3}H]Progesterone (6·35 μc/μg.), prepared by the method of Pearlman (1957*a*), was incubated with capsule strippings of ox adrenal gland.

2. Crystalline [16-^{3}H]corticosterone, [16-^{3}H]-aldosterone and [16-^{3}H]cortisol were prepared from an extract of the incubation. The specific activities were $2{\cdot}6_2$, $1{\cdot}8_2$ and 0·76 μc/μg respectively.

3. The radiochemical purity of these steroids was established by various criteria.

4. Methods of assaying tritiated steroids by a flow counter and by means of *bremsstrahlung* emission are described.

5. The stability of the [16-^{3}H]progesterone has been examined and the purity found to be satisfactory after three months' storage.

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The Effects of the Hyaluronic Acid Complex on the Distribution of Ions

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Manery (1954) and Meyer & Rapport (1951) have suggested that hyaluronic acid, which is believed to be widely distributed in connective tissue, may have important effects on the distribution of ions within the body fluids. The present work was undertaken primarily to test this suggestion. Since there is evidence (Ogston & Stanier, 1950, 1952; Blumberg & Ogston, 1957*a*, *b*) that hyaluronic acid exists in synovial fluid and connective tissue as a complex with protein, it was decided to use this material, isolated from synovial fluid.

Although this preparation is chemically more complex than the polysaccharide isolated by Meyer & Palmer (1934) and others, the results obtained have also thrown light on its properties as a polyelectrolyte. Theoretical consideration of this aspect of the work leads to certain criticisms of the commonly used concept of 'ion-binding' (Klotz, 1953).

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Ionic distributions have been commonly used for the estimation of extracellular volumes, and the general question of the steric exclusion of solutes from solutions of hyaluronic acid complex has been studied through the investigation of the distribution of the trisaccharide raffinose in solutions of the complex.

EXPERIMENTAL

Hyaluronic acid complex. This was prepared by the method of Ogston & Stanier (1950). The complex was washed on a sintered-glass ultrafilter of average pore diameter 1 μ by redispersion in wash fluid followed by refiltration. Six washes were carried out with 0·2N-NaCl solution, and six with water. Wash fluids were not buffered, so that ions extraneous to the experiments might not be introduced. Curtain (1955) showed that this variation in wash fluids was permissible.

Dialysis. Dialysis bags containing about 3 ml. of a solution of hyaluronic acid complex in water were dialysed at 4° in glass conical flasks against solutions containing the ions under study or raffinose. All solutions were saturated with chloroform as antiseptic. After 5–7 days the phases in