CCLIV. THE ISOLATION OF PROGESTERONE AND 3:20-*ALLO*PREGNANOLONE FROM OX ADRENALS

By D. BEALL[1]

British Postgraduate Medical School, London

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The preparation from the adrenal gland of lipoid extracts which produced oestrous and progestational effects in the uterus of the immature rabbit was reported by Engelhart [1930]. These findings were confirmed by Callow & Parkes [1936], who showed that the active principles could be separated by the method of Allen & Meyer [1933]. They suggested, in view of the structure of the substances already isolated from the adrenal cortex, that the material responsible for the progestational activity might be progesterone or a closely allied compound.

By the courtesy of Prof. T. Reichstein of Zurich a concentrate of ox adrenal gland, obtained from his work on the cortical hormone, was placed at the disposal of Dr A. S. Parkes and made available to the author. Bioassays, carried out by Dr A. S. Parkes, showed this material to have an activity equivalent to 0·5 mg. progesterone per g.

As a preliminary step in the isolation of the active principle the crude material was saponified by treatment with sodium methoxide in anhydrous ether at room temperature. While such a saponification was not necessarily complete it provided a convenient method for the removal of a large amount of inactive acidic material without the destruction of progestational activity.

The alkali-insoluble material was then separated into ketonic and non-ketonic fractions. The ketonic material, on partitioning between suitable solvents, gave a semicrystalline concentrate from which a small amount of 3:20-*allo*-pregnanolone was isolated. Purification of the remainder of the concentrate by alumina adsorption, followed by vacuum sublimation, gave a crystalline product "*X*", M.P. 165–166°, which was subsequently shown to be a complex of *allo*-pregnanolone and progesterone.

Fractionating the crude concentrate has thus resulted in the isolation of progesterone M.P. 121° (uncorr.), $[\alpha]_D^{23°}+193°$, together with somewhat larger amounts of 3:20-*allo*pregnanolone M.P. 191–192° (uncorr.). Similar results have been obtained by Reichstein [1938] using comparable material and a preliminary report of the work has already appeared [Beall & Reichstein, 1938].

Bioassays carried out throughout the work showed that the activity of the original concentrate could be accounted for by its "*X*" content so that the greater part, if not all of the activity was due to progesterone.

Experimental

Melting points are uncorrected.

Micro-analyses were by Dr A. Schoeller, Berlin.

[1] Beit Memorial Research Fellow.

Preparation of the initial concentrate

The original material was prepared by N. V. Organon, Oss., from 1800 kg. of whole ox adrenals, and the concentrate on partitioning between pentane and 30% methyl alcohol [Reichstein, 1936; 1937] gave a pentane-soluble fraction weighing 1300 g., part of which was used in the present investigation.

Cold saponification

150 ml. of 7·5% sodium methoxide in methyl alcohol were added to 300 g. of the crude pentane-soluble material in 2 l. of dry ether. After 4 hr. at room temperature the mixture was diluted with 1 l. of ether and washed once with 500 ml. of 15% ethyl alcohol, once with 500 ml. of *N* KOH containing 15% ethyl alcohol and then once with 400 ml. of water. The aqueous solutions were combined and extracted with ether, the combined ether extracts being washed and taken to dryness to give 142 g. of "non-sap." Acidification of the alkaline washings, followed by ether extraction, gave 143 g. of "sap." All the progestational activity of the original material was found in the "non-sap." fraction.

Separation of the ketones of the "non-sap."

In a preliminary experiment 9 g. of a "non-sap." fraction in 50 ml. methyl alcohol containing 1·5 ml. of acetic acid were allowed to react for 1·5 hr. with 3 g. of Girard reagent T at room temperature [Reichstein, 1936]. The solution was then poured into 150 g. of ice and water containing 90% of the theoretical amount of NaOH necessary to neutralize the acetic acid, so that the final concentration of alcohol was 25%. It was then extracted four times with ether, the ether being combined and extracted three times with 35 ml. of 25% methyl alcohol.

The aqueous phase was then acidified to Congo red with HCl and, after standing for 1 hr., was ether-extracted to give ketones (A 1) 413 mg. Further acidification of the residual aqueous phase with HCl (10% by volume) and subsequent ether-extraction after 1 hr. gave 32 mg. of additional ketones (A 2). These were combined with the (A 1) fraction to give ketones (A).

The ethereal solution containing the unreacted ketonic and non-ketonic material, after being washed and taken to dryness, was refluxed in 50 ml. methyl alcohol, containing 3 ml. of acetic acid, with 3 g. of Girard reagent T for 1 hr. and then the ketones and non-ketones were separated as before, giving 280 mg. of ketones (B).

Bioassays showed that while the ketones A possessed an activity comparable with that of the original concentrate, the ketones B were inactive when given at five times this dosage level. Therefore in subsequent work the total ketones of the "non-sap." were separated by refluxing with Girard reagent and subsequently fractionated by reaction in the cold with more of this reagent. In this way the 142 g. of "non-sap." from 300 g. of original concentrate gave 4·4 g. of ketones A.

Partition of ketones A

4·4 g. ketones A were dissolved in 140 ml. ethyl alcohol which was then diluted to 200 ml. with water (70% final alcohol conc.) and extracted five times with 100 ml. portions of light petroleum, the latter being extracted in turn three times with 45 ml. portions of 70% alcohol. The alcoholic solutions were combined and extracted five times with 75 ml. portions of benzene which, on being taken to dryness, gave a semicrystalline residue weighing 1·63 g. and containing 92% of active material.

Isolation of 3:20-allopregnanolone and X

The 1·63 g. of semicrystalline benzene-soluble material were dissolved in a minimal amount of ether, several volumes of light petroleum were added and, after standing in the ice box, 303 mg. of solid material were obtained. Crystallization from benzene-light petroleum gave crystals melting at 182–189° which on recrystallization from aqueous ethyl alcohol gave colourless leaflets melting at 191–192° subsequently identified as 3:20-*allo*pregnanolone.

The remainder of the benzene-soluble material (1·3 g.) was dissolved in 60 ml. of alcohol which was then diluted with 90 ml. of water (40% final alcohol conc.) and extracted five times with 100 ml. portions of light petroleum. A small amount of insoluble material was present and was kept in the aqueous phase. The combined light petroleum solutions, which contained all the activity, were taken to dryness and the residue was sublimed *in vacuo*. At 80°/0·05 mm. it gave a small amount of inactive light oil but at 120°/0·05 mm. a partially crystalline yellow sublimate, containing all the active material, was obtained. Attempted crystallization of this sublimate from aqueous alcohol gave 232 mg. of sticky crystals which, on washing with ether, gave 36 mg. of crystals, M.P. 164–165°, which were designated "*X*".

Attempts to obtain more "*X*" from the ether mother liquors by cooling to −80° were unsuccessful, so the residue on evaporation was dissolved in 20 ml. of a mixture of one part benzene and twenty parts light petroleum and run through a column containing 5 g. of active alumina. The column was washed repeatedly with light petroleum until the movement of the coloured zone ceased. The coloured and colourless portions of the column were separated and eluted with a mixture of boiling benzene and alcohol, both extracts being progestationally inactive. The residue from the filtrate, however, had an activity which was equivalent to one-fifth that of progesterone, i.e. was of the same order as that of crystalline "*X*".

In another experiment sublimation of the alumina filtrate (450 mg.) from 3·24 g. of benzene-soluble material, followed by crystallization from aqueous alcohol gave 132 mg. of "*X*", M.P. 162–166°.

Separation of "X" into progesterone and 3:20-allopregnanolone

"*X*", M.P. 164–165°, was recrystallized twice from aqueous alcohol without any change in M.P. Recrystallization of this material from light petroleum gave crystals, M.P. 167–169°, which, on recrystallization from aqueous acetone melted at 165–166°. "*X*" therefore appeared to be a single substance.

Acetylation (pyridine and acetic anhydride at 100°), of 20 mg. "*X*" gave 12 mg. of an acetate, which, recrystallized once from aqueous alcohol, gave crystals melting at 136–137°. These were saponified by refluxing for 15 min. with 10 ml. of 2% alcoholic KOH and on crystallizing from aqueous alcohol formed needles which, alone, or mixed with authentic 3:20:*allo*pregnanolone (M.P. 191–192°) melted at 191–192°.

155 mg. of "*X*" dissolved in 10 ml. of 90% alcohol were treated with a solution of 1·2 g. of digitonin in 10 ml. of 90% alcohol and, after refluxing the mixture for 1 hr. it was left at room temperature overnight and then filtered. The filtrate was concentrated *in vacuo* and ether-extracted, the ether being well washed with dilute acid, alkali and then water. The residue, on evaporation, was sublimed at 120°/0·05 mm. to give 46 mg. of solid. Crystallization of this from light petroleum gave 10 mg. of progesterone which had M.P. 121° alone, and

mixed with authentic progesterone (M.P. 127°) melted at 121–123°. $[\alpha]_D^{23°}+193°$ ($l=0{\cdot}5$, $c=1{\cdot}01\%$ in ethyl alcohol). Its activity, on bioassay, was similar to that of progesterone. (Found: C, 80·1; H, 9·64%. $C_{21}H_{30}O_2$ requires C, 80·2; H, 9·62%.)

Identification of 3:20-allopregnanolone

76 mg. of crude *allo*pregnanolone, M.P. 178–185°, were acetylated and the acetate (65 mg.) was recrystallized twice from aqueous alcohol to give 52 mg. of *allo*pregnanolone acetate (leaflets) which alone, or mixed with authentic *allo*pregnanolone acetate (M.P. 142–143°), melted at 142–143°.

Saponification of the acetate in ether with sodium methoxide for 1 hr. followed by recrystallization from aqueous alcohol gave 32 mg. of leaflets which, alone or mixed with an authentic specimen of *allo*pregnanolone (M.P. 191–192°), melted at 191–192°. (Found: C, 79·1; 79·1; H, 10·5; 10·5%. $C_{21}H_{34}O_2$ requires C, 79·2; H, 10·8%.)

30 mg. of this pure *allo*pregnanolone were dissolved in 2 ml. of 90% acetic acid and treated with a solution of 50 mg. of chromic acid in 2 ml. of 90% acetic acid [cf. Butenandt *et al.* 1934]. After 6 hr. at room temperature the mixture was diluted with water, filtered, washed and the precipitate, recrystallized from aqueous alcohol, gave 12 mg. of *allo*pregnandione (leaflets), which, alone or mixed with authentic *allo*pregnandione (M.P. 198–199°), melted at 198–199°.

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