Metabolism of 11-Oxygenated Steroids

METABOLISM *IN VITRO* BY PREPARATIONS OF LIVER

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1. The isolation and partial purification of 11 β -hydroxy steroid dehydrogenase from rat and guinea-pig liver microsomes has been achieved by conventional methods. 2. The efficiency of different 11-oxygenated steroids as substrates has been examined. The relative efficiencies confirm in the main the stereochemical theory of the enzyme-coenzyme-substrate complex that was proposed earlier on the basis of studies *in vivo*. Δ^4 -3-Ketones and 5 α -hydrogen steroids are readily metabolized by the enzyme. 5 β -Hydrogen steroids and Δ^4 -3-ketones with certain large α -substituents are metabolized to a limited extent or not at all. Halogen substitution in the 9 α -position enhances the rate of reduction of 11-ketones but blocks the oxidation of the related 11 β -ols. 3. 9 α -Fluorocortisol is a competitive inhibitor of the oxidation of cortisol, but 9 α -fluorocortisone is reduced at five to ten times the initial velocity of cortisone. 4. 11 β -Hydroxy steroid dehydrogenase activity has been found in liver microsomes of rat, guinea pig, rabbit and calf. 5. Relative substrate efficiencies and K_m values are similar in whole (debris-free) homogenates, washed microsomes and acetone-dried powders of washed microsomes. 6. A variety of conditions have been examined for the observation of 11 β -hydroxy steroid dehydrogenase activity. NADP(H) is an efficient and NAD(H) a very poor coenzyme for the reaction.

The earlier papers in this series (Bush & Mahesh, 1959*a*, *b*, 1964) have described the metabolism of various 11-oxygenated steroids after their oral administration to healthy volunteers, particular emphasis being given to the reversible oxidation-reduction of the 11-oxygen function of such steroids. The aim of these earlier studies was to determine the influence of the 11 β -hydroxy steroid dehydrogenase reaction (Fig. 1) on the biological activity of adrenocortical steroids and to try to decide whether or not the reaction itself was directly responsible for the biological activities of this class of steroid hormones (Bush, 1956, 1961*a*, 1962). In addition, it was thought to be useful to obtain information about the metabolism of cortisone and its synthetic analogues in man because of the therapeutic uses of such steroids. These earlier results with different types of steroids were

interpreted as being largely due to differences in the reactivity of the substrates with the 11 β -hydroxy steroid dehydrogenase rather than to secondary factors such as differences in the absorption and distribution of such steroids after oral administration (Bush & Mahesh, 1959*a*, *b*, 1964). Although many arguments were given that supported this interpretation, it was obviously desirable to extend these observations to the enzyme system *in vitro*. In addition, there were many considerations that suggested that the study of this enzyme system would be of interest from a purely biochemical point of view.

In this paper we describe the studies that have been carried out with homogenates of rat liver and various preparations derived from them by ultracentrifugation. Some reference will be made to experiments with acetone-dried powders of liver microsomes. Most of this work has been described in preliminary communications (Bush & Hunter, 1961, 1962; Bush, Meigs & Hunter, 1962).

The existence of an 11 β -hydroxy steroid dehydrogenase was first inferred from studies of metabolites of cortisone and cortisol in urine by Mason (1950) and Burton, Keutmann, Waterhouse

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Fig. 1. Reaction catalysed by 11β -hydroxy steroid dehydrogenase, with 11-oxoprogesterone as the example. The nuclear hydrogen atoms are not shown.

& Schuler (1953). It was demonstrated in isolated preparations of rat liver by Caspi, Levy & Hechter (1953), Fish, Hayano & Pincus (1953) and Hurlock & Talalay (1959). Mahesh & Ulrich (1959, 1960) showed that homogenates of various tissues of the rat carried out the reaction (Fig. 1) and that the oxidative direction was particularly effectively carried out by the heavier fractions of homogenates of kidney. Osinski (1960), Sweat & Bryson (1960), Dougherty, Berliner & Berliner (1960), Meigs & Engel (1961), Stahl & Tapley (1963) and Koerner (1966) have observed similar but slower reactions in tissue cultures and other preparations of extra-hepatic tissues *in vitro*. Koerner & Hellman (1964) have reported similar studies to our own, with homogenates of rat livers.

Hurlock & Talalay (1959) were the first to obtain a fairly highly purified 11β -hydroxy steroid dehydrogenase by using acetone-dried powders of microsomes from rat livers. Their system was approximately equally effective with NAD^+ or NADP^+ as coenzyme, but was not soluble, although it needed prolonged centrifuging at 105 000g to sediment the enzyme. In view of their failure to obtain a soluble preparation, and because we wanted to study preparations that would behave as nearly as possible to the enzyme *in vivo*, we have carried out extensive studies with whole microsomes and other fractions of homogenates of liver, before attempting to use more highly purified preparations.

METHODS

Materials and reagents

The principal materials and reagents were the same as described in the earlier papers of this series and were prepared in the same way. We particularly emphasize the danger of easy oxidation of steroid 11β -hydroxy groups by residual traces of chromic acid. To avoid this, all glassware was washed, when possible, only with detergents, water, acetic acid and alcohols. If chromic acid was used, the glassware was allowed to soak in methanol-acetic acid-water (approx. 7:1:2, by vol.) for at least 10 min. before a final rinse with glass-distilled water.

Chemicals. A.R. grade reagents were used and were

obtained from British Drug Houses Ltd. (Poole, Dorset) unless otherwise stated. Purification of solvents was carried out as previously described (Bush & Mahesh, 1959a,b, 1964).

Lactic acid, fumaric acid, NADH, NADPH, isocitrate dehydrogenase, alcohol dehydrogenase, glutamate dehydrogenase and glucose 6-phosphate dehydrogenase were supplied by C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. The glucose 6-phosphate dehydrogenase was swirled gently and diluted immediately before use 1:100 (v/v) in 0.02M-glycylglycine, pH 7.4. D-Glucose 6-phosphate dihydrate was obtained from L. Light and Co. Ltd., Colnbrook, Bucks. L-Glutamate, NAD^+ , NADP^+ and lactate dehydrogenase were supplied by Sigma Chemical Co., St Louis, Mo., U.S.A. (agents: G. T. Gurr Ltd., London, S.W. 6). Tris was obtained initially from British Drug Houses Ltd., and later from Sigma Chemical Co.; 25.0 ml. of 0.2M-tris and 42.5 ml. of 0.1N-HCl were diluted with glass-distilled water to 100 ml., to give a final pH 7.4. The potassium dihydrogen salt of isocitrate obtained from *Bryophyllum* leaves was kindly provided by Professor Sir Hans Krebs (University of Oxford) and gave 102% of the theoretical amount when assayed spectrophotometrically with isocitrate dehydrogenase and NADP^+ .

Steroids. These were nearly all supplied as gifts by generous donors. The amounts were generally too small for the determination of physical properties without risking considerable waste, and were often unique samples from able chemists. It was therefore thought sufficient to demonstrate that they were at least 98% pure by chromatography and that their chromatographic behaviour agreed with their structural formulae (Bush & Mahesh, 1959a,b).

In one or two cases it was necessary to hydrolyse a steroid 21-acetate or prepare an 11-ketone by gentle oxidation of the related 11β -ols with chromic acid. In most of these cases it was possible to obtain a satisfactory melting point in addition to a chromatographic analysis.

Standard media for the preparation and incubation of tissue

Sucrose-EDTA for the preparation and fractionation of homogenates. EDTA (2%, w/v) was mixed with sucrose (0.25M) in the proportions 1:40 (v/v) immediately before use.

Medium for incubations under reducing conditions. This was made by mixing the following to give a final volume of 4.0 ml.: 1.5 ml. of Krebs-Ringer phosphate solution, pH 7.4 (Umbreit, Burris & Stauffer, 1957), 0.1 ml. of 1.6M-nicotinamide (160 μ moles), 0.1 ml. of 3mM- NADP^+ (0.3 μ mole),

0.1 ml. of 100 mM-glucose 6-phosphate (10 μ moles), 0.1 ml. of glucose 6-phosphate dehydrogenase (8 units), 2.0 ml. of tissue suspension and 1.0 ml. (0.2–0.50 μ mole) of steroid in organic solvent.

Medium for incubations under oxidizing conditions. The following medium was mixed to give a final volume of 4.0 ml.: 1.7 ml. of Krebs–Ringer phosphate solution, pH 7.4, 0.1 ml. of 1.6 M-nicotinamide (160 μ moles), 0.1 ml. of 3 mM-NADP⁺ (0.3 μ mole), 2.0 ml. of tissue suspension and 0.1 ml. (0.02–0.50 μ mole) of steroid in organic solvent.

Medium 1 for the spectrophotometric assay of 11 β -hydroxy steroid dehydrogenase. The following were mixed to give a total volume of 3.0 ml. and a final pH 9.0–9.2: 1.5 ml. of 67 mM-glycine–NaOH buffer, pH 9.1 (100 μ moles), 0.1 ml. of 24 mM-EDTA (2.4 μ moles), 0.1 ml. of 100 mM-KCN (10 μ moles), 0.2 ml. of 3 mM-NADP⁺ (0.6 μ mole), 1.0 ml. (4–12 mg. of protein) of microsome suspension and 0.1 ml. (0.02–0.50 μ mole) of steroid in dioxan.

Medium 2 for the spectrophotometric assay of 11 β -hydroxy steroid dehydrogenase. The following were mixed to give a total volume 12.0 ml. and pH 7.35–7.45: 9.5 ml. of 0.1 M-Na₂HPO₄, pH 7.4, 0.1 ml. of 1.6 M-nicotinamide (160 μ moles), 0.3 ml. of 3 mM-NADP⁺ (0.9 μ mole), 2.0 ml. (8–24 mg. of protein) of microsome suspension and 0.1 ml. (0.02–0.25 μ mole) of steroid in dioxan.

Other media for preparation and incubation of tissue. Many exploratory and other experiments were carried out with media differing slightly from those above. They are not described in detail since they were all prepared by standard methods (e.g. Umbreit *et al.* 1957) that are easily repeatable. In each case 4 ml. of the final incubation medium contained at least 1.4 ml. of Krebs–Ringer phosphate buffer or a similar buffer, and 160 μ moles of nicotinamide, unless stated otherwise.

Animals

Albino rats of the Wistar strain weighing 200–250 g. were used. Animals were maintained on a standard pellet diet (Oxoid Ltd., London, E.C. 4) under conditions of only moderately good ventilation and control of temperature. Many of the later experiments used animals suffering from mild chronic respiratory infection.

All the results reported here were obtained with male animals, and in most cases the animals were not starved. Similar results were obtained in numerous experiments with animals starved for 16 hr. and in a small number of experiments with females. When fed animals were used, it was found that the final extracts of the incubation media were sufficiently free of lipids to allow the immediate application of the extract to paper chromatograms for analysis (see below).

It was not feasible to control the condition of our animals as closely as could be desired, but the variability that must have existed did not seriously affect the results that we obtained. Though they may have been responsible for variations in the activity of the enzyme preparations from week to week, these variations were no greater than seems common with this type of work in other fields (cf. Koerner & Hellman, 1964).

Preparation of homogenates and fractions thereof

A rat was rapidly stunned and decapitated, and the liver was removed and placed in ice-cold 0.154 M-KCl. After

5 min. the liver was removed, lightly blotted with filter paper and minced in a stainless-steel tissue press. The weighed mince was added to sucrose–EDTA solution (0.2 g. of tissue/ml.) and homogenized with a stainless-steel Potter–Elvehjem homogenizer by using eight complete strokes at 600 rev./min.

The homogenate was diluted if necessary to the desired volume (usually 5–10 ml./g. of original mince) with sucrose–EDTA solution and centrifuged at 1500 g for 15 min. to remove the debris. In exploratory experiments, these homogenates were used as such, or fractionated in a Spinco model L preparative ultracentrifuge by standard methods to provide so-called mitochondrial, microsomal and supernatant fractions (Schneider & Hogeboom, 1950). These fractions were checked with a phase-contrast microscope and found to contain less than 10% of particles as cross-contaminants. In later work, microsomes were obtained from the whole homogenates by one or two standard procedures as follows.

Standard microsome preparation. The whole homogenate was diluted to a volume of 5 ml./g. of original mince and centrifuged at 8700 g for 20 min. (10 000 rev./min., Spinco rotor no. 30). The supernatant was collected and the sediment resuspended in fresh sucrose–EDTA (final volume 5 ml./g. of mince). This suspension was centrifuged at 8700 g for 20 min. and the supernatant combined with the first one. The combined supernatants were centrifuged at 105 000 g for 60 min. (39 000 rev./min., Spinco rotor no. 40) and the resulting supernatant was carefully removed and discarded. The crude microsomal pellet was resuspended in fresh sucrose–EDTA and centrifuged again at 105 000 g for 60 min. The final supernatant was carefully removed and the microsome pellet either used immediately or stored in the centrifuge tube at -15° .

Acetone-treated microsomes. Pellets obtained by the standard microsome preparation were brought slowly to 4° and suspended in a minimal volume of 0.05 M-tris buffer, pH 7.4. This suspension was then added drop by drop to 10 vol. of acetone at -15° with stirring. The suspension was stirred for a further 5 min. and the precipitate was packed by centrifuging at -15° for 5 min. at 600 g (2000 rev./min.) in an MSE refrigerated centrifuge. After the acetone supernatant had been decanted, residual acetone was removed by spreading the precipitate on a glass plate, or by suction on a Buchner filter. The preparation was then dried over P₂O₅ *in vacuo* for 5 hr. at 4° and stored over anhydrous CaCl₂ at -15° . By this method 1 g. of original wet liver mince gave 20–30 mg. of acetone-dried powder.

Ultrasonic treatment. The acetone-dried powder was suspended in 100 mM-phosphate buffer, pH 7.4, and the suspension chilled in an ice-salt bath. It was then treated for 15 min. with a 1.88 cm. titanium probe on a 60 W MSE ultrasonic disintegrator. The temperature of the suspension at the end of such treatment was usually 5–8 $^{\circ}$. In later experiments treatment was carried out similarly but with a 50 W Raytheon magnetostrictive oscillator (model S-102A) at 9 kcyc./sec.

Assays

Determination of microsomal protein. This was achieved by turbidimetry with trichloroacetic acid (Hoagland, Keller & Zamecnik, 1956).

Spectrophotometric assay of enzyme activities. The methods were based on the extinction at 340 m μ of NADH and

NADPH (Warburg & Christian, 1936). All NADH- and NADPH-regenerating systems were checked for adequate activity by adding NAD⁺ or NADP⁺ to our standard incubation media lacking steroid substrate. Reduction of NAD⁺ or NADP⁺ was 95% complete within 4–5 min. at room temperature (18–20°) with the systems employed in these studies.

For the assay of 11 β -hydroxy steroid dehydrogenase activity itself, special measures were needed to eliminate side reactions, especially those of 20-hydroxy steroid dehydrogenase and Δ^4 -3-oxo steroid 5 α -reductase (Tomkins, 1959; Brown-Grant, Forchielli & Dorfman, 1960). The homogenate was prepared as above to the stage of being free of heavy debris and was then incubated for 1 hr. at 37° while being shaken in air, before sedimentation of mitochondrial and microsomal fractions. This seemingly destructive procedure was devised by R. A. Meigs and found to be extremely successful in eliminating 20-hydroxy steroid dehydrogenase activity. Similar incubations of washed microsomes themselves were far less effective. The 5 α -reductase was largely eliminated by using male rats and by freezing the microsome pellets to –15° before use (Brown-Grant *et al.* 1960). Oxidation of NADH and NADPH in the absence of steroid substrate was decreased very greatly, or eliminated, by adding KCN to the incubation medium to a final concentration of 1.0 mM.

The spectrophotometric incubations of whole microsomes were carried out in cuvettes of 1 cm. path-length at room temperature (18–20°) in the Unicam SP.500 instrument. The final concentration of nicotinamide was 0.04 M in these experiments when NAD⁺ was used, but this reagent was excluded from incubations with NADP⁺ after it had been found unnecessary for the protection of NADP⁺. NAD⁺, in contrast, was rapidly hydrolysed in the absence of nicotinamide by this type of microsome preparation.

Incubations were carried out as follows. The frozen microsome pellet was resuspended in the sucrose medium and the microsomes were washed by centrifuging at 105 000g for 60 min. (39 000 rev./min., Spinco rotor no. 40). The resulting pellet was then resuspended and made up to volume, the addition of NAD⁺ or NADP⁺ being made 10 min. after the addition of nicotinamide and KCN. The reaction was started by adding the steroid in 0.05–0.10 ml. of ethanol or dioxan. The incubations were carried out at room temperature (18–20°). When microsomes disrupted by ultrasonic treatment were used, the microsome pellet was not washed after resuspension, but was subjected to ultrasonic treatment immediately before the other reagents were added.

Acetone-dried powders of microsomes were incubated similarly, except that usually cuvettes of 4 cm. path-length were used, nicotinamide was always included in the medium (cf. above), and the temperature was maintained at 37° in the constant-temperature cell housing of the SP.500 instrument. In all cases the reference cuvette was filled with the incubation medium prepared at the same time as the test solution. Dioxan (0.05–0.10 ml.) or ethanol (0.05–0.10 ml.) was added at approximately the same time as the steroid was added to the main cuvette.

It was found that, even in the presence of KCN, slight loss of NADPH usually occurred with these preparations. In prolonged incubations, or in the presence of high concentrations of NADPH, this assay therefore slightly underestimated the extent of oxidation of the 11 β -hydroxy

group. However, in every experiment reported here in which the spectrophotometric assay indicated low or zero rates of oxidation of the steroid, the results were checked by the extraction and determination of the steroids remaining at the end of the incubation. In all such cases there was no discrepancy between the results of the two methods of assay. In many other experiments the amount of 11-ketone was estimated directly by extraction and chromatography, and corresponded closely with the final amount of NADH or NADPH estimated spectrophotometrically by using a molar extinction coefficient 6.22×10^3 l./mole-cm.

Enzyme assay by estimation of steroid products. Incubations were carried out in tubes or flasks on a shaking incubator in a manner otherwise similar to that described above, but were more usually done at 25° or 37°.

Description of enzyme preparations

Liver preparations are usually described below in the conventional 'percentage' fashion, i.e. the equivalent weight of original wet liver from which the fraction was prepared per volume of incubation medium, multiplied by 100.

Extraction of steroids

A variety of methods were used according to the nature of the steroids being studied and the type of liver preparation being used. In none of the methods did the recovery of related 11-ketones and 11 β -ols differ significantly. Recoveries were at least 80% for the pure substrates, and in almost all experiments with purified microsome preparations 80–100% of the substrate was recovered as identifiable or partially identified steroid products. Immediate extraction was used to stop enzyme reactions and the commonest procedure was as follows.

The sample of incubation medium (2–10 ml.) was extracted twice with 2.5 vol. of wet methylene chloride–ethyl acetate (1:1, v/v). A common alternative was one extraction with 8 vol. of solvent. The combined extract was washed once with 0.05 vol. of 0.5 N-NaOH and twice with 0.05 vol. of water. A drop of acetic acid was added and the extract was then filtered on Whatman no. 2 or 4 paper to remove some of the water present. The extract was then evaporated to near-dryness under reduced pressure at 40–45°, and transferred with methanol and methylene chloride (or ethyl acetate) to a small tube. This solution was then evaporated with a stream of filtered air or N₂ to a volume (0.05–0.20 ml.) convenient for transfer to a paper chromatogram. Further details are given in earlier papers of this series and by Bush (1961b, chapter 3).

Chromatographic methods

These were similar to or identical with those described previously (Bush & Mahesh, 1959a,b, 1964; Bush, 1961b).

The principal solvent systems used were: (1) benzene-methanol–water (2:1:1, by vol.); (2) toluene-methanol–water (4:3:1, by vol.); (3) light petroleum (b.p. 100–120°)–toluene-methanol–water (67:33:85:15, by vol.); (4) light petroleum (b.p. 100–120°)–methanol–water (20:17:3, by vol.) at either 17–20° or 25.5 $\pm$ 0.5°. Other systems are described separately.

Quantitative estimations of steroids were carried out by direct scanning of paper chromatograms as described in previous papers of this series. Identification of steroids was carried out by microchemical reactions, spectrophotometry

and chromatography. The conditions used for the reactions were the same as those of previous papers of this series (Bush, 1961b, Appendix 3) except where mentioned in the text. In nearly all cases the main steroids (at least 70% of the original substrate) detected in these experiments were the substrate, its oxidation or reduction product at C-11 and, in reductive experiments, one other reduction product. C₂₁ 11 β -ols with 20,21-ketol groups were identified by brief oxidation with CrO₃ to the 11-ketones, or oxidation with CrO₃ after quick acetylation (5 min. at 65°) in acetic anhydride-pyridine (4:1, v/v) to the 21-acetoxy-11-ketones. 17,20,21-Triols were identified by oxidation with periodate to the 17-ketones. The 17-ketones were then briefly oxidized with CrO₃ as described above to demonstrate the presence of an 11 β -hydroxy group (11 β -ols), or oxidized for 15–18 hr. to reveal or exclude the presence of other oxidizable groups (Lieberman, Katzenellenbogen, Schneider, Studer & Dobriner, 1953).

Steroids with 3-, 17- or 20-hydroxyl groups were oxidized with CrO₃ for 15–18 hr. as described above and the products run on paper chromatograms in system (3) or (4). The ΔR_M values, (Bush, 1961b, p. 84) for the oxidation and the characteristic changes in the colour reaction given by alkaline *m*-dinitrobenzene were sufficient to give an unambiguous identification of the original metabolite. Since the main concern of this paper is the redox reaction at C-11 the details of the identification of the metabolites of side reactions are not given here; they were described fully by Hunter (1964).

The extracts of some types of incubation (especially liver fractions from starved rats) contained too much lipid for immediate paper chromatography. This was obvious on inspection of the extract after evaporation to near-dryness. Such extracts were distributed between light petroleum (10 ml.) and aqueous 90% (v/v) methanol (5 ml.) and purified by chromatography on silica-gel columns (Bush & Sandberg, 1953), or pre-fractionated by paper chromatography on Whatman 3MM paper (Bush & Mahesh, 1959a) by using single-length runs in a convenient solvent system.

RESULTS

Preliminary experiments

These experiments led to the setting up of the routine procedures that were used for the main body of results in this paper. Since the main purpose of this work has been the comparison of different steroids as substrates of 11 β -hydroxy steroid dehydrogenase, our preliminary experiments are not described in detail, but simply as a guide to other workers who may be helped by these early observations.

Sex. Reduction and oxidation of steroid 11-oxygen functions occurred at similar rates with liver preparations from rats of either sex (cf. Koerner & Hellman, 1964). Reduction of structures in the A ring of Δ^4 -3-oxo steroids and of 20-ketone groups also occurred with preparations from both sexes. The use of male rats was advantageous in preparing microsomes since, as expected, the reduction of Δ^4 -3-oxo steroids to 5 α -hydrogen

saturated products was greatly decreased (see the Methods section).

Behaviour of different types of preparation of liver. Whole 10–20% homogenates or homogenates lacking cellular debris metabolized cortisone and cortisol rapidly as long as nicotinamide was present. After incubation for 0.5 hr. with cortisone under nitrogen or oxygen + carbon dioxide (95:5), less than 30% of the substrate remained unchanged and a variety of expected reduction products were found, the major proportion of which consisted of cortisol. At later times (1–1.5 hr.) saturated 3 α - and 3 β -hydroxy steroids predominated. Final supernatants ('cell sap') and washed mitochondrial fractions were weakly active, but washed microsomes reduced 11- and 20-ketone groups rapidly. This was true with homogenates prepared in 0.154 M-potassium chloride or in 0.25 M-sucrose, and was not seriously affected by the presence or absence of EDTA in the incubation medium.

Microsomes stored as frozen pellets (–15°) retained their ability to oxidize cortisone to cortisone with little loss in activity over several months. Their ability to reduce cortisone to cortisol declined fairly rapidly, often falling to 30% of their original activity within 30 days. The stability of acetone-dried powders of washed microsomes was a good deal better, oxidizing activity being maintained unchanged in some preparations for as long as 3 months and reducing activity for up to 2 months; at –15°.

The activity of 20-hydroxy steroid dehydrogenase was usually low in washed microsomes but showed a fair degree of variation. It was decreased by repeated freezing and thawing followed by washing, and almost completely eliminated by preincubation of the homogenate (minus debris) at 37° with shaking under air. In contrast, preincubation of the washed microsomes themselves caused little decrease in the activity of this enzyme.

Effects of varying the cofactors. (a) Reductive direction. In early experiments it was found that NADP⁺ with either isocitrate dehydrogenase or glucose 6-phosphate dehydrogenase and their substrates as NADPH-regenerating systems gave excellent reduction of cortisone to cortisol, whereas NAD⁺ and lactate dehydrogenase gave little or no reduction of cortisone. Later it was found that glucose 6-phosphate dehydrogenase was superior to a new preparation of isocitrate dehydrogenase, and glucose 6-phosphate dehydrogenase was used in all the later experiments described here.

Because of the results of Hurlock & Talalay (1959), we made many attempts to improve the reduction of cortisone with NAD⁺ and an NADH-regenerating system. These included varying the concentration of NAD⁺, trying alcohol dehydrogenase, lactate dehydrogenase and glutamate

dehydrogenase as NADH-regenerating systems, varying the pH, and carefully checking the efficacy of the regenerating systems and of the stability of NAD^+ in the incubation media. The best results were obtained with a succinate dehydrogenase-fumarate regenerating system. With this system, only 2% of cortisone was reduced to cortisol in 40 min. with a 5% microsomes suspension, compared with 19.2% reduction with an NADPH-isocitrate dehydrogenase regenerating system. The latter was in turn several times less effective in other experiments than the glucose 6-phosphate dehydrogenase regenerating system and NADPH or NADP^+ .

Later, experiments were carried out with microsomes obtained from preincubated homogenates with both the glutamate dehydrogenase NADH-regenerating system and NADH added to the incubation mixture. Although the concentration of NADH remained high, as measured by the extinction at $340\text{m}\mu$, no reduction of cortisone was observed. Rapid reduction was obtained with NADPH with the same batch of microsomes suspension.

(b) Oxidative direction. The efficacy of NAD^+ and NADP^+ as cofactors for oxidation of cortisone to cortisone was compared in a large number and variety of liver preparations. NAD^+ was always inferior to NADP^+ as cofactor. The most clear-cut comparisons were afforded by spectrophotometric assays of microsomes from preincubated homogenates, in which the reduction of NAD^+ and NADP^+ in the absence of cortisone ('endogenous' reduction) was negligible (less than 2% of that in the presence of cortisone). Some oxidation of cortisone was obtained with NAD^+ as cofactor, but the rate of reaction varied from preparation to preparation and was never greater than one-third of that obtained with NADP^+ as cofactor.

Effects of different conditions of incubation. No attempt was made to carry out exhaustive studies of aerobic and anaerobic conditions. As in other studies of hydroxy steroid dehydrogenases (e.g. Tomkins, 1959; Mahesh & Ulrich, 1960) neither the oxidative nor the reductive reaction was significantly affected by gross changes in the oxygen content of the gaseous phase of the incubation vessels. The effect of strictly anaerobic conditions was not investigated. The enzymic activity of microsomes preparations was not altered more than fivefold by considerable variations in the organic constituents of the medium used for isolating them from homogenates, and a variety of buffers gave good results as incubation media. Reductions were carried out eventually in a Krebs-Ringer phosphate buffer, which gave initial velocities twice to three times those obtained with citrate or tris buffers, and oxidations in glycine (pH 9.1) or phosphate

Fig. 2. Dependence of the reductive and oxidative reactions on pH. $\blacktriangle$, Reduction of cortisone in tris buffer; $\bullet$, oxidation of cortisone in phosphate buffer (see the Methods section). The preparation used was washed microsomes stored for 17 days in glycerol-tris buffer at -15° . The ordinate shows the percentage conversion of substrate.

buffers (pH 7.4). As expected, rates of reduction were increased at lower pH values (5.0–6.5) and rates of oxidation increased at higher pH (see Fig. 2).

No attempt was made to obtain pH optima because of the complexity of any interpretation of such values when obtained with whole microsomes. As a routine, pH 7.35–7.4 was adopted for incubation in the reductive direction, and pH 9.0–9.15 for the spectrophotometric assays in the oxidative direction. All the comparisons of different steroids as substrates that are made below are based on experiments carried out at these pH values and with the same buffers. The major differences between different steroids as substrates for 11β -hydroxy steroid dehydrogenase were, however, unaffected by the variations of the incubation media that we studied.

Experiments in the reductive direction

Cortisone and related steroids. The following naturally occurring 11-oxo steroids and derivatives were reduced to the related 11β -ols with comparable initial velocities: cortisone, 11-dehydrocorticosterone, 11-oxoprogesterone and adrenosterone (androst-4-ene-3,11,17-trione). Some typical progress curves are shown in Figs. 3 and 4. The 11-oxoprogesterone and its 20-ketonic metabolites were estimated by their atypical colour reaction with alkaline *m*-dinitrobenzene (see

Fig. 3. Typical progress curves of the reaction in the reductive direction with adrenosterone (●) and cortisone (■) as substrates. The experiments were run in the standard reducing medium for whole-microsomes preparation in parallel at 37°.

Fig. 4. Typical progress curves with the same microsome preparation for reduction of cortisone (■), 11-oxoandrosterone (○) and adrenosterone (▲).

3 α ,17 α -dihydroxy-5 β -pregnan-20-one in Bush, Swyer, Stern & Willoughby, 1957).

Minor products of reaction with these substrates

were identified as those expected from reduction of other ketone groups and are not described here in detail (see Hunter, 1964). With the exception of the saturated 3-ketones, the proportion of substrate converted into such minor products in 40 min. incubations varied both with the substrate and with different preparations of microsomes and was rarely greater than 15% and never greater than 25% of the substrate.

Steroids with special features in ring A. The following related steroids were tested as substrates to examine the effect of structures in ring A (Bush & Mahesh, 1959a):

Cortisone, prednisone (1,2 - dehydrocortisone), 5 β -4,5-dihydrocortisone (17 α ,21-dihydroxy-5 β -pregnane-3,11,20-trione), 3,3',4,5-tetrahydrocortisone (3 α ,17 α ,21-trihydroxy-5 β -pregnane-11,20-dione), 5 α -4,5-dihydrocortisone (17 α ,21-dihydroxy-5 α -pregnane-3,11,20-trione), 2 α -methylcortisone, adrenosterone (androst-4-ene-3,11,17-trione), 2 α -methyladrenosterone, 11-oxoacetiocholanolone (3 α -hydroxy-5 β -androstane-11,17-dione), 11-oxoandrosterone (3 α -hydroxy-5 α -androstane-11,17-dione).

It was found that the presence of a 2 α -methyl group prevented reduction of the 11-ketone group of 2 α -methylcortisone completely, and that of 2 α -methyladrenosterone almost completely. 11-Oxoandrosterone was reduced, whereas in six experiments neither its 5 β -hydrogen epimer nor the analogous C₂₁ 5 β -hydrogen 3 α -hydroxy steroid, 3,3',4,5-tetrahydrocortisone, was reduced at position 11. The initial velocity of the reduction of prednisone was from one-third to one-half of that of cortisone, both with whole microsomes and with acetone-dried powders (cf. Fig. 12b).

Reduction of dihydrocortisone (5 β -hydrogen) and allodihydrocortisone (5 α -hydrogen) at position 11 was complicated by rapid reduction of the 3-ketone group, 60-90% of the substrate being reduced to tetrahydrocortisone (5 β -hydrogen-3 α -hydroxy) and 3-epiallotetrahydrocortisone (5 α -hydrogen-3 β -hydroxy) respectively. The degradations used to identify these products were the same as in Bush & Mahesh (1959a, Fig. 1) and final identifications were based on the characteristic relationship of the R_F and R_M values of epimeric saturated 3-hydroxy steroids and their 3-acetates described by Bush & Willoughby (1957). With dihydrocortisone (5 β -hydrogen) no reducing steroids were detected on the chromatograms in the position corresponding to any of the possible 11 β -ols that might have resulted from reduction at position 11 combined with other known metabolic reactions, and oxidation of eluates of the more polar regions of the chromatograms with sodium bismuthate failed to reveal any 11 β -hydroxy steroids among the resultant 17-ketones detected on the chromatograms with

solvent system (3). This absence of reduction of the 11-ketone group was found with whole microsomes, and with acetone-dried powders (cf. oxidative experiments below). With alldihydrocortisone (5 α -hydrogen), one experiment yielded no detectable alldihydrocortisol, and three others gave yields of 4, 2 and 8% of substrate as the 11 β -ol in 45 min. with microsome preparations or acetone-dried powders yielding approx. 20% of cortisol from cortisone under the same conditions.

Steroids with substituents at C-9 and C-12. In three sets of analogous pairs of steroids it was found that the 9 α -fluoro-11-ketone had an initial velocity of reduction to the 11 β -ol that was from four to ten times that of unsubstituted 11-ketone. Typical progress curves are shown in Fig. 5, the steroids being cortisone, adrenosterone and 11-oxoprogesterone, and their 9 α -fluoro derivatives. In two pairs, cortisone and adrenosterone, the 9 α -chloro derivatives had mean initial velocities twice to five times those of the unsubstituted parent steroids, the initial velocity being clearly the greatest with 9 α -chloroadrenosterone.

9 α -Bromocortisone gave rise to 5% of unchanged substrate and 31% of a product with the R_F value and other properties of cortisone (u.v. absorption, reduction of alkaline blue tetrazolium, yellow fluorescence with sodium hydroxide). An artifact was suspected, but numerous experiments gave similar results despite all precautions, such as avoiding exposure to alkali (Fried & Sabo, 1957). In addition, 9 α -bromocortisone was subjected to extraction from buffer solution, and extraction

after incubation with heat-coagulated microsomes, both experiments giving good yields (at least 85%) of unchanged starting material. Degradation of the main product of enzymically active incubations was therefore carried out according to Chart 1 of Bush & Mahesh (1959b), each product confirming the original tentative identification as cortisone (see the Discussion section).

The reduction of 11-dehydrocorticosterone and of its 12 α -fluoro and 12 α -bromo derivatives is illustrated in Fig. 6. The initial rate of reduction of the 12 α -fluoro derivative was about twice that of the unsubstituted analogue, whereas no detectable reduction at C-11 occurred with the 12 α -bromo analogue. Similarly, no reduction of 12 α -methyl-11-oxoprogesterone was observed at C-11. 12 α -Bromo-11-oxoprogesterone was reduced slowly, at one-fifth to one-half of the initial rate of 11-oxoprogesterone itself.

Experiments in the oxidative direction

Cortisol and related steroids. With microsome suspensions or acetone-dried powders of microsomes containing 1.5–4.0 mg. of protein/ml. in either standard medium 1 or 2 for the spectrophotometric assay of 11 β -hydroxy steroid dehydrogenase, it was found that cortisol was almost completely oxidized to cortisone in 10 min. at 37°. The spectrophotometric assay was therefore usually carried out at room temperature (18–20°). Corticosterone was oxidized at the same rate as cortisol (Fig. 7). It should be noted that in the spectrophotometric method of assay the earliest reading of extinction after adding the steroid was subtracted from all

Fig. 5. Typical progress curves with the same microsome preparation to show the influence of 9 α -fluorine substitution on the reductive direction of the reaction with cortisone (●), 9 α -fluorocortisone (■), 11-oxoprogesterone (Δ) and 9 α -fluoro-11-oxoprogesterone (▲) as substrates.

Fig. 6. Typical progress curves showing the effects of substitutions in the 12 α -position on reduction of the 11-ketone group with 11-dehydrocorticosterone (▲) and its 12 α -fluoro (■) and 12 α -bromo (●) derivatives.

Fig. 7. Spectrophotometric measurement of the oxidative reaction with cortisol (two experiments, ■ and □), corticosterone (●), tetrahydrocortisol (○) and alldihydrocortisol (▲). The conditions and medium used are described in the Methods section.

Fig. 8. Assay of the oxidative reaction by direct estimation of steroids extracted from the incubation medium (37°): cortisol (■); 11β-hydroxyandrost-4-ene-3,17-dione (▲); 11β-hydroxyandrosterone (●); 11β-hydroxyaetiocholanolone (○). The same microsome preparation (10% suspension) was used. The experiments were in parallel at 37° in the standard oxidative medium 1 (see the Methods section).

subsequent readings and taken as zero for purposes of graphical plotting. The lag period in the progress curves of Figs. 7, 9 and 10(a) is thus only apparent and not real.

Steroids with special features in ring A. The progress curves in Figs. 7 and 8 include typical results with steroids having saturated 3-ketone or 3-hydroxy groups, a 1,4-diene-3-ketone structure or a 2α-methyl group. It was found that no

Fig. 9. Spectrophotometric assay of the 11-oxidation of cortisol (●), 16β-methylcortisol (○) and 16α-methylcortisol (▲). The standard oxidative medium 1 was used at 25°. All incubations were run in parallel, with portions of the same batch of microsomes.

detectable oxidation of the 11β-hydroxy group occurred with 11β-hydroxyaetiocholanolone and 3,3',4,5-tetrahydrocortisol (5β-hydrogen-3α-hydroxy); with 2α-methylcortisol and 2α-methyl-11β-hydroxyandrost-4-ene-3,17-dione (2α-methyl-4-ene-3-ketone). In contrast, 11β-hydroxyandrosterone (5α-hydrogen-3α-hydroxy) was rapidly oxidized. Prednisolone (1,2-dehydrocortisol) was oxidized to prednisone (1,2-dehydrocortisone) at about one-fifth of the initial rate of oxidation of cortisol to cortisone.

Steroids with substituents at C-9 and C-12. No oxidation of the 11β-hydroxy group (i.e. less than 2% in 45 min.) was observed with the following steroids: 9α-fluorocortisol, 9α-fluoro-11β-hydroxyprogesterone, 12α-bromo-11β-hydroxyprogesterone, 12α-fluorocorticosterone, 12α-bromocorticosterone.

In each case at least 85% of the substrate was recovered unchanged by solvent extraction and estimation on paper chromatograms. No 11-ketonic metabolites were found.

Steroids with substituents in ring D. Fig. 9 shows typical progress curves with 16α- and 16β-methylcortisol. Oxidation started at about one-fifth (16α-epimers) and two-fifths (16β-epimers) of the initial rate found with cortisol in the same experiment. In other experiments similar results were obtained by extraction and determination of the steroid products.

Polysubstituted and complex steroid analogues. The 11-oxidation of 16α-methylprednisolone (16α-methyl-1,2-dehydrocortisol), triamcinolone (9α-fluoro-16α-hydroxyl-1,2-dehydrocortisol) and 2α-methyl-9α-fluoro-cortisol was compared with that of cortisol. 2α-Methyl-9α-fluoro-11β-hydroxyprogesterone was also compared with 11β-hydroxyprogesterone. Although the unsubstituted natural

Table 1. *Comparison of 11 β -hydroxy steroid dehydrogenase from livers of various species*

The substrates used were cortisol (oxidation) and cortisone (reduction) at a concentration of 35 μ M. The enzyme preparation was a 10% equivalent suspension of washed microsomes (see the Methods section).

Species	Conversion into main product in 45 min. at 37° (%)		10 ⁴ × Sp. activity (μ moles converted/min./mg. of protein)	
	Oxidation	Reduction	Oxidation	Reduction
Rat	8.0	5.0	1.40	0.87
Cow	2.0	4.8	0.69	1.70
Rabbit	12.2	18.0	1.10	1.78
Guinea pig	10.4	22.0	2.10	4.50

steroids were rapidly oxidized at C-11, these poly-substituted or complex derivatives were not oxidized detectably. At least 85% of unchanged substrate was recovered in experiments with these analogues when the reaction was measured directly by extraction and determination of the steroids. No 11-ketonic metabolites were found.

Livers of different species

Microsomes were prepared in the usual way from the livers of the following species: guinea pig (Hartley strain, National Institutes of Health, Bethesda, Md., U.S.A.), rabbit (Dutch) and cow (slaughterhouse material). Suspensions (10–20%) were prepared and incubated in both oxidative and reductive directions with cortisol and cortisone as substrates. 11 β -Hydroxy steroid dehydrogenase activity was present in the preparations from all three species. The most active preparation was that from guinea-pig liver as judged by the products at the end of 45 min. incubation (see Table 1).

Studies with inhibitors and measurements of K_m

Common inhibitors. *p*-Chloromercuribenzoate (sodium salt) at 10 μ M inhibited the oxidation of cortisol almost completely when added to the simple oxidative incubation medium. On the other hand, 0.1 mM *o*-iodosobenzoate caused no observable diminution in the rate of oxidation of cortisol. Complete inhibition was obtained with 1 mM *o*-iodosobenzoate.

Competitive inhibitors. Bush & Mahesh (1964) found evidence that 9 α -fluorocortisol inhibited the oxidation of cortisol to cortisone *in vivo* in human subjects, and in the studies *in vitro* described here it was surprising to find that several 9 α -fluoro-11 β -hydroxy steroids, lacking other substituents known to prevent the reaction at C-11, were apparently quite inert to oxidation by the system *in vitro* whereas the related 9 α -fluoro-11-ketones were excellent substrates for reduction at C-11. We therefore sought evidence of competitive inhibition and to this end made measurements of the apparent

Fig. 10. (a) Progress curves with 200 μ g. of cortisol (●), 100 μ g. of cortisol (▲) and 50 μ g. of cortisol (■) (b) Lineweaver-Burk plot for the 11-oxidation of cortisol with an acetone-dried powder of rat liver microsomes: K_m 25.3 $\times 10^{-6}$ M. The line was calculated by unweighted linear regression.

Michaelis constant of the microsome preparation for the oxidation of cortisol. A number of determinations were made with different acetone-dried powders of microsomes and a typical progress curve and Lineweaver & Burk (1934) plot are shown in Figs. 10(a) and 10(b). The values for K_m obtained with microsome preparations are shown in Table 2. Although the variation was large, the low value for

Table 2. *K_m* values for 11-oxidation of cortisol by liver microsomes

Spectrophotometric assay conditions are described in the Methods section.

Preparation	Age of preparation (days)	10 ⁶ × <i>K_m</i> (M)	Species	Date
Acetone-dried powder	13	25.3	Rat	May 1963
Acetone-dried powder	2	11.9	Rat	May 1963
Acetone-dried powder	9	9.1	Rat	June 1963
Acetone-dried powder	25	22.5	Rat	June 1963
Acetone-dried powder	2	13.4	Rat	December 1963
Acetone-dried powder	11	27.7	Guinea pig	May 1965
Acetone-dried powder	2	25.0	Guinea pig	May 1965
Acetone-dried powder	6	28.6	Guinea pig	May 1965
Ultrasonically treated microsomes	13	22.1	Guinea pig	January 1966
Ultrasonically treated microsomes	1	40.8	Guinea pig	July 1966

Fig. 11. (a) Dixon plot of the 11-oxidation of cortisol in the presence of 9α-fluorocortisol: (●) cortisol at 0.092mM; (○) cortisol at 0.184mM. (b) Lineweaver-Burk plot of 11-oxidation of cortisol in the absence (○) (*K_m* 18.9 × 10⁻⁶M) and in the presence (●) of 9α-fluorocortisol at a concentration of 0.084mM. The lines were calculated by unweighted linear regression. All curves were obtained by spectrophotometric assays at 37° in standard oxidative medium 2 with rat liver microsomes.

K_m (mean 2 × 10⁻⁵M) was similar to those found with other types of steroid dehydrogenase (Talalay, 1957). In contrast with the results with other steroid dehydrogenases (Talalay, 1957) we found no evidence of inhibition by excess of substrate with cortisol at concentrations up to 8 × *K_m*.

The inhibitory effect of 9α-fluorocortisol was examined by using Dixon's (1953) method of using increasing quantities of inhibitor at two concentrations of substrate. 9α-Fluorocortisol was used at concentrations of 5.5, 11 and 22 μM in the presence of cortisol at 23 and 46 μM as substrate. The oxidation of cortisol at C-11 was measured by the spectrophotometric method of assay at 340mμ at 37°. A typical Dixon plot is shown in Fig. 11(a), and Lineweaver-Burk plots in Fig. 11(b). Though the linearity is poor in the Dixon plot, the results clearly suggest that 9α-fluorocortisol was a competitive inhibitor of the oxidation of cortisol in this preparation with *K_i* approx. 5 × 10⁻⁶M.

Absence of competition by 2α-methylcortisol. Our interpretation of many of the results reported in this series of papers is that the differences in the relative efficiency of different 11-oxygenated steroids as substrates for 11β-hydroxy steroid dehydrogenase were probably due largely to differences in the affinity of the substrate with the enzyme-coenzyme complex. In the particularly noteworthy case of 2α-methylcortisol we have tested this hypothesis by examining its behaviour as a possible competitive inhibitor of oxidation of cortisol by the enzyme system. With cortisol at concentrations of 23 and 46 μM no diminution of its initial velocity of oxidation was observed with the spectrophotometric assay with concentrations of 2α-methylcortisol in the range 5.5-22 μM. Higher concentrations of 2α-methylcortisol were not tested because of possible complications due to the low water-solubility of 2α-methylcortisol compared with

Table 3. Comparison of K_m values obtained with different 11β -hydroxy steroids with acetone-dried powders of guinea-pig liver microsomes

Assay conditions are described in the Methods section.

Microsome preparation	Concn. of protein (mg./ml.)	$10^6 \times K_m$ (M)					
		Cortisol	1,2-Dehydrocortisol	Corticosterone	11β -Hydroxyprogesterone	16α -Methylcortisol	16α -Methyl-1,2-dehydrocortisol
1	1.60	25.0	—	—	—	—	—
2	1.80	27.7	—	—	—	—	—
3	2.14	28.6	100.0	—	—	—	—
4	2.14	—	56.2	—	—	—	—
5	2.30	—	69.4	28.8	—	—	—
6	2.72	—	—	10.2	18.5	—	—
7	2.10	—	—	—	15.2	68.0	—
8	2.00	—	—	—	—	29.4	204.0

Fig. 12. (a) Progress curves of the 11-oxidation of cortisol: (●) 100 μg . of cortisol; (▲) 50 μg . of cortisol; (□) 25 μg . of cortisol; (○) 10 μg . of cortisol. (b) Lineweaver-Burk plots for the 11-oxidation of cortisol (●), corticosterone (■), and 1,2-dehydrocortisol (▲) by an acetone-dried powder of guinea-pig liver microsomes. K_m values: cortisol, $28.6 \times 10^{-6} \text{ M}$; corticosterone, $28.8 \times 10^{-6} \text{ M}$; 1,2-dehydrocortisol, $69.4 \times 10^{-6} \text{ M}$. The lines were calculated by unweighted linear regression.

cortisol. Similarly, 2α -methylcortisone caused no detectable inhibition of the reduction of cortisone.

Other measurements of K_m . The K_m values of the oxidative direction of the reaction were determined by the method of Lineweaver & Burk (1934) for a number of 11β -hydroxy steroids with an ultrasonically treated acetone-dried powder of microsomes from guinea-pig liver. The spectrophotometric assay was used (medium 1, pH 9.0–9.2). The values for cortisol were similar to those found in earlier experiments with acetone-dried powders of microsomes from rat livers. Some typical progress curves and Lineweaver-Burk plots are shown in Figs. 12(a) and 12(b). The results are summarized in Table 3.

DISCUSSION

Validity of the analytical methods

In at least one experiment with each substrate the major product and any minor products consistently appearing in chromatograms of the extracts of the incubation medium were identified by schemes similar to or identical with those described in earlier papers of this series. In some cases the products from several incubations were eluted from corresponding areas of paper chromatograms and pooled before being submitted to microchemical degradations and further chromatography. In each case sufficient reference substances were available to obtain the probable identity of the products by the use of ΔR_M values (Bush, 1961b, 1965). In every case the final product obtained by microchemical degradation was resistant to oxidation by chromic acid in the cold (method of Lieberman *et al.* 1953), and had an R_M value identical with that of a known reference substance both on single-length and overrun (1.5–3-fold) chromatograms. The validity of such methods has been discussed fully elsewhere (Bush & Mahesh, 1959a,b; Bush, 1961b, 1965).

Whenever a substrate was recovered unchanged in high yield by extracting the incubation medium,

or its derivative produced by oxidation or reduction at C-11 was not detected on chromatograms of the product, a careful search was made for products that might have escaped our routine colorimetric methods of detection and determination. This was necessary because of the presence of variable but significant amounts of other steroid dehydrogenases in our microsome preparations. Thus, for instance, it would be conceivable, though unlikely, that an 11-ketonic substrate would be reduced to the 11 β -ol, but that this product would be rapidly and completely converted by reduction at other positions into a steroid lacking the Δ^4 -3-ketone group, the saturated 3- and 17-ketone group or the 20,21-ketol group that were detectable by our routine methods. The only known reactions by which this might occur would give rise to hydroxyl or diol groups that are readily oxidized to detectable groups by chromic acid, sodium bismuthate or periodate. To guard against this possibility, therefore, the regions of chromatograms calculated to contain such hypothetical metabolites (and neighbouring regions to allow for error) were eluted with methanol-ethyl acetate, the solvent was evaporated, and the residues were oxidized with these reagents in the appropriate order. The oxidized extracts were worked up and run on single-length chromatograms in solvent systems (3) or (4) or both, and the chromatograms were treated with alkaline *m*-dinitrobenzene. In nearly all cases this did not give rise to detectable products.

Those products that were occasionally detected in this way were investigated as follows to detect any 11 β -hydroxy steroids. The product from oxidation by bismuthate or periodate was acetylated and the acetate run on a paper chromatogram with a system giving a useful R_F value (0.2–0.5). The R_M value was obtained and the acetate eluted. The eluate was evaporated to dryness and subjected to brief oxidation with chromic acid. The products were extracted and washed in the usual way and run again on a paper chromatogram with the same solvent system. In no case did the acetate treated with chromic acid in this way differ from the starting material. The oxidation of an 11 β -hydroxy group is easily characterized by the ΔR_M value of the reaction, its very high velocity (complete in 5–10 min. at 20–25°) and the failure of acetylation to protect the hydroxyl group (see, e.g. Fig. 1. of Bush & Mahesh, 1959a). All our 'negative' results have been checked in this way because of their importance to the interpretations to be suggested.

Validity of the biochemical methods

The exact physicochemical characterization of an enzyme reaction requires the isolation and use of a highly purified enzyme. The physiological relevance of such studies can, however, only be assessed by a

comparison of reactions observed *in vivo* with those observed in biochemical preparations of increasing purity and, usually, decreasing 'organization'. Our aim in these studies has been to study 11 β -hydroxy steroid dehydrogenase *in vitro* in a state that corresponds closely to its condition *in vivo*, while minimizing those factors that might complicate the interpretation of results obtained in the whole organism, organs or intact cells. In the oxidative direction our assays were apparently uncomplicated by the presence of other enzymes metabolizing steroids and in most cases reasonably linear Lineweaver-Burk plots were obtained. However, some Lineweaver-Burk plots were significantly non-linear. In these cases we have used the weighted linear regression method suggested by Dowd & Riggs (1965) for calculation, and compared the results with calculations by the method of Woolf (see Hofstee, 1959) in which v is plotted against v/s . Although this treatment confirmed the findings of the simpler method, we would not place too much weight on the significance of minor differences in K_m values reported here.

The apparent K_m for the oxidation of cortisol (approx. $2 \times 10^{-5} M$) was very similar for washed microsomes and for acetone-dried powders of washed microsomes under the conditions of the spectrophotometric assay. A similar value ($3 \times 10^{-5} M$) was obtained by Koerner & Hellman (1964) with acetone-dried powders of whole rat liver homogenates acting on cortisol.

Complicating reactions were far more obvious with reductive than with oxidative incubations and included reduction of 3-, 17- and 20-ketone groups. They were also more obvious with less polar 11-oxo steroids (e.g. 11-oxoprogesterone and 11-oxo-androstenedione) than with more polar compounds such as cortisone. Full details are given by Hunter (1964). Koerner & Hellman (1964) were unable to demonstrate reduction of cortisone to cortisol by homogenates of whole rat liver because of these interfering reactions, but were able to do so with acetone-dried powders. It appears from their paper, however, that erythrocytes, nuclei and other heavy debris were not removed by preliminary centrifugation. In our work whole homogenates freed of heavy debris could be used to obtain reduction of cortisone to cortisol, although the concentration of cortisol often failed to rise after the first 5 min. at 37° because of the metabolism of both substrate and product at other positions in the molecule. Washed microsomes, when prepared as described above, reduced cortisone to cortisol very satisfactorily and could be almost completely freed of other steroid-metabolizing enzymes by: (a) preincubation of whole homogenates for 1 hr. at 37°; (b) repeated freezing, thawing and washing; (c) the use of male rats.

In one strain of rat recently used (Sprague-Dawley; Charles River, Cambridge, Mass., U.S.A.) 20-hydroxy steroid dehydrogenase activity was so low that no evidence for its presence was found with cortisone as substrate, and only a small amount of activity was obtained with 11-oxoprogesterone as substrate. 17-Hydroxy steroid dehydrogenases were relatively weakly active in such microsomes.

The most serious obstacle was the high activity of 3-hydroxy steroid dehydrogenase in most of our preparations, especially 3 β -hydroxy steroid dehydrogenase. This, however, was only a complication in the incubations of saturated 3-oxo steroids because Δ^4 -3-oxo steroids are not significantly attacked by this class of enzymes (Tomkins, 1959; Ringold, Graves, Hayano & Lawrence, 1963). We conclude that our incubations used to compare the relative efficiency of different steroids as substrates for 11 β -hydroxy steroid dehydrogenase were negligibly complicated by side reactions in the oxidative direction, and were only slightly complicated by such reactions in the reductive direction, except with saturated 3-oxo steroids.

An important possible complication with our enzyme preparations and these rather hydrophobic substrates was the presence of lipid acting as a separate 'solvent' phase for the substrate, or of proteins and other substances of large molecular weight capable of associating strongly with ('binding') the substrates. We do not believe that these factors were serious for the following reasons. First, the volume of lipid capable of preferentially dissolving the steroid was extremely small, being approx. 1–2% of the volume of the aqueous phase, and the concentration of protein was likewise small, being in the range 1–4 mg./ml. Secondly, the relative efficiencies of 11-oxoprogesterone, 11-oxoandrostenedione and cortisone as substrates were not significantly different whether washed microsomes, acetone-dried powders of washed microsomes, or 2-methylpropan-1-ol-extracted (Hurlock & Talalay, 1959) acetone-dried powders of washed microsomes were used. These preparations differ considerably in the amount and type of lipid that is present. Finally, any complication of this sort would tend to decrease the active concentration of the less polar steroids in the aqueous phase more than that of the more polar steroids. In fact the less polar steroids (e.g. 11-oxoprogesterone) were usually more rapidly metabolized than the more polar steroids (e.g. cortisone). The failure to observe significant differences in relative efficiency of different steroids as substrates in the three different types of preparation described above also makes it very unlikely that the access to the active site of the enzyme in whole microsomes was significantly affected by the presence of a lipid barrier.

It therefore seems likely that our use of an impure

insoluble microsomal enzyme preparation does not prevent us from attempting an interpretation of our results in terms of the probable affinity and reactivity of the different steroid substrates with the active site of the enzyme.

Comparison with other results and with results in vivo in man

With few exceptions, our results can be correlated very closely and satisfactorily with the earlier results and interpretations of Bush (1956) and of Bush & Mahesh (1959*a,b*, 1964). They differ in one respect from those of Meigs & Engel (1961) with homogenates of human liver. Thus naturally occurring steroids with approximately planar A–B ring junctions (e.g. Δ^4 -3-oxo steroids and 5 α -hydrogen-3 α -hydroxy steroids) are readily metabolized reversibly by 11 β -hydroxy steroid dehydrogenase. In contrast, steroids with moderately angulated or 'bent' A–B ring junctions (e.g. $\Delta^{1,4}$ -3-oxo steroids) are less rapidly metabolized, and the metabolism of 11-oxygenated steroids with fully angulated A–B ring junctions (e.g. 5 β -hydrogen-3-oxo steroids and 5 β -hydrogen-3 α -hydroxy steroids) is not detectable.

In contrast, Meigs & Engel (1961) found that the 11 β -hydroxy groups of tetrahydrocortisol (5 β -hydrogen-3 α -hydroxy) and 11 β -hydroxyaetiocholanolone were oxidized, albeit slowly, by homogenates of human liver. Hubener, Fukushima & Gallagher (1956) found that whole homogenates of rat liver capable of reducing cortisone to cortisol failed to reduce 3,3',4,5-tetrahydrocortisone to 3,3',4,5-tetrahydrocortisol. However, these results, although agreeing with ours, are of doubtful interpretation in view of the failure of Koerner & Hellman (1964) to demonstrate the reduction of cortisone to cortisol in whole rat liver homogenates in the presence of nicotinamide, and the contrasting success of Hubener *et al.* (1956) in the absence of nicotinamide. The absence of nicotinamide in the incubations of the latter workers probably resulted in low and poorly controlled concentrations of NADPH and NADH.

It is certain, however, that in the livers of both humans and rats 11 β -hydroxy steroid dehydrogenase acts very slowly if at all on 5 β -hydrogen saturated steroids and that most reported studies with most substrates of this type have shown no measurable rate of reaction in circumstances in which 11-oxygenated 5 α -hydrogen and Δ^4 -3-oxo steroids have been rapidly metabolized at C-11. Similar relationships of substrates have recently been obtained by Koerner (1966) with 11 β -hydroxy steroid dehydrogenase from rat lung and testis.

It is possible, however, that our rat preparations and those of Koerner & Hellman (1964) were slightly more limited in the range of substrates that

they could attack than the similar enzyme or enzymes in human liver, and that the oxidation of tetrahydrocortisol to tetrahydrocortisone observed *in vitro* by Meigs & Engel (1961) either was due to an enzyme absent from our microsome preparations or was too slow *in vivo* to give rise to detectable amounts of tetrahydrocortisone in the experiments of Bush & Mahesh (1959a). We mention this possibility because of two fairly striking differences (the only ones) between our results with rat liver microsomes and those of Bush & Mahesh (1959a,b) *in vivo* in man. In man, these authors found that approx. 5% of orally administered 2 α -methylcortisone was reduced to 2 α -methylcortisol, and that a similar proportion of 2 α -methylcortisol was oxidized to 2 α -methylcortisone under the same conditions. In contrast, 2 α -methyl-11-oxoandrostenedione was appreciably reduced to 2 α -methyl-11 β -hydroxyandrostenedione (although much less completely than the naturally occurring 11-oxoandrostenedione), suggesting that the 17 β -C₂ side chain was an important source of steric hindrance to the formation or reactivity of the enzyme-substrate complex, whose influence is shown up by the added hindrance of the 2 α -methyl group. Repeated experiments with rat liver microsome preparations, however, have failed to reveal any reduction at C-11 of 2 α -methylcortisone (less than 1-2% in 1-2 hr.), or more than minimal reduction of 2 α -methyl-11-oxoandrostenedione, and have shown no oxidation of the related 11 β -ols. The almost complete failure of the 2 α -methyl C₁₉ substrates to react with our preparation is a definite difference from the results of Bush & Mahesh (1959b) in man, and may indicate a significant difference in the steric properties of the active site of the principal 11 β -hydroxy steroid dehydrogenase of human and rat liver. The other differences discussed here are few and of minor significance compared with the very large number of points of similarity between the properties of the enzyme from human and rat liver microsomes *in vitro*. These differences would all be explicable in terms of a greater steric restraint imposed by the active site of the rat enzyme. This is strongly suggested by the almost complete absence of the reaction with the 2 α -methyl C₁₉ substrates. Alternatively, it is possible that different enzymes are responsible for the reaction of C₁₉ and C₂₁ 11-oxygenated steroids.

Significance of variations in substrate efficiency with 11 β -hydroxy steroid dehydrogenase

It is reasonable to assume that liver 11 β -hydroxy steroid dehydrogenase is similar to other NAD⁺- and NADP⁺-linked dehydrogenases in its reaction mechanism. Thus we shall assume that: (a) the substrate associates strongly with the active site

only when the coenzyme is already associated with the enzyme (Dalziel, 1957; Theorell & McKinley-McKee, 1961); (b) the coenzyme transfers hydrogen directly to or from the substrate in the ternary enzyme-coenzyme-substrate complex by the stereo-specific mechanism of Westheimer, Fisher, Conn & Vennesland (1951).

As discussed previously (Bush & Mahesh, 1959a,b) such a mechanism requires an attachment of the steroid by its α -surface to the active site of the enzyme-coenzyme complex, and hydrogen transfer to or from the 11 α -position. C-11 is an extremely hindered region of the steroid molecule. The axial 11 β -hydroxy group can be acetylated only under forcing conditions and the resulting ester cannot be hydrolysed by ethanolic potassium hydroxide, although enzymic hydrolysis is possible (Charney, Weber & Oliveto, 1959). Even the semi-equatorial 11-ketone group is very inert to Wolff-Kishner reduction and carbonyl condensing reagents (e.g. Fieser & Fieser, 1959). The 11 β -hydroxy steroid dehydrogenase reaction is therefore likely to be extremely sensitive to the steric properties of the substrate.

A complete interpretation of the mechanism of this enzyme reaction must await a more detailed analysis of kinetic data with purer preparations. There are, however, good grounds for supposing that the differences we have found in the initial velocities of reaction of the non-halogenated steroids in these studies are due largely to steric factors. In view of the close parallelism between these results and the inferences drawn from the results *in vivo* of Bush & Mahesh (1959a,b) we shall confine this discussion to those features that bring further evidence to bear on the stereochemical theory of the reaction given by Bush & Mahesh (1959b), by using Fig. 8 of that paper which is reproduced as Fig. 13 here. The proposal that the main surface of contact in the enzyme-coenzyme-steroid complex lay around the plane BB' rather than AA' was based on the inferred relative rates of reduction of 2 α -methylcortisone, 1,2-dehydrocortisone and cortisone. The results *in vitro* have confirmed the inferred relative rates of reduction, and have also shown that the same relationship holds in the oxidative direction of the reaction.

Crucial confirmation of the hypothesis indicated by Fig. 13 has been obtained by the results *in vitro* with an additional range of steroids. Thus, in accord with earlier suggestions, the large 9 α -chlorine atom produced no significant hindrance to reduction in either 9 α -chlorocortisone or 9 α -chloroadrenosterone; both these steroids were in fact reduced at about five times the initial velocities of the unsubstituted parent steroids. Oxidation of the related 9 α -chloro-11 β -ols was much slower but this, and the greatly increased rate of reduction of the

Fig. 13. (a) Composite projection on a plane normal to a line joining C-8 and C-10 of cortisone, prednisone and 2α-methylcortisone. C-2, C-2 of cortisone and 2α-methylcortisone; C-2', C-2 of prednisone; 3-oxygen, as in cortisone and 2α-methylcortisone; 3'-oxygen, as in prednisone. The heavy continuous line is the α-side profile of C-2 and C-3 and of the 4-hydrogen atom. The heavy broken line is the profile of the 2α-methyl group. Because of conjugation the repulsion between the 2α-methyl and 3-oxo groups has been assumed to lead only to bending and slight lengthening of the 2α-methyl-C-2 bond. This repulsion was calculated with 'small' van der Waals radii; all other van der Waals radii are taken from Pauling (see Bush & Mahesh, 1959b). The conformation of the side chain is not necessarily the preferred one. (b) Hypothetical projection of possible association between cortisone and active region of the 11β-hydroxy steroid dehydrogenase. AA', Plane of enzyme 'surface' on simplified theory with direction of hydrogen attack on α-carbonyl bond, a; BB', more probable orientation of enzyme 'surface' and hydrogen attack, b, to account for relative hindrance of 2α-methyl group, the A ring of prednisone, the influence of the 17β-side chain and of the 9α-chloro and 12α-bromo groups (see the text); c, circumference within which a hydrogen atom or hydride ion must approach C-11 before hydrogenation is more likely than recoil.

9α-chloro-11-ketones are, explicable on other grounds (see below).

In contrast, large 12α-substituents have been found to produce considerable hindrance to the reaction in three different steroids and in both directions. Thus 12α-bromocorticosterone and 12α-methylcorticosterone are completely unreactive in the oxidative direction, and the related 11-ketones are not reduced. 12α-Bromo-11β-hydroxyprogesterone was not oxidized by a variety of microsome preparations. However, 12α-bromo-11-oxoprogesterone was reduced by the enzyme, but only at one-tenth to one-half of the rate with 11-oxoprogesterone. These results with the 12-substituted corticosterones and their related 11-ketones only admit a steric explanation, since these substances are inert to both directions of the reaction, the methyl group has a positive and the bromo group a negative inductive effect, and both groups are similar in size (Van der Waals radii 1.8-2.0Å). Because the negative inductive effect of bromine is almost equal to that of chlorine (Ketelaar, 1958), one would expect (see below) that the initial rate of reduction of a 12α-bromo-11-ketone would be comparable with that of a 9α-bromo- or 9α-chloro-11-ketone in the absence of steric hindrance,

namely two to four times that of the unsubstituted ketone. The 9α-halogen is tertiary and the 12α-halogen is secondary, but both are axial and adjacent to C-11. The slow reduction of 12α-bromo-11-oxoprogesterone by our microsome preparations, although unexpected, was therefore probably at only 5-10% of the rate expected in the absence of steric hindrance. This difference between the behaviour of the 11-oxoprogesterone analogues and the corticosterone analogues may well be due to significant hindrance by the 21-hydroxyl group of the latter, a speculation that is in accord with the earlier supposition that C-21 and its substituents will be a point of contact with the plane BB' but not with AA' (Figs. 13 and 15).

Further evidence supporting the postulate of a point of close association between the steroid and the enzyme-coenzyme complex at C-21 is the significant decrease of initial oxidation rates caused by introducing 16α- and 16β-methyl groups (Fig. 14). Uncertainty as to the conformation of the C-20,21 side chain, however, makes a detailed interpretation difficult (see, e.g., Wellman & Djerassi, 1965). Thus, if the 16-methyl group exerted its effect entirely by influencing the conformation of the side chain, one would expect a

Fig. 14. Two possible extreme conformations of the α -ketol side chain in 16β -methylcortisol considered by Taub *et al.* (1960). (a) The least strained position of C-21 towards the α -side; (b) the most strained position, showing the strong 1,3-interactions between the methyl groups at C-13, C-21 and C-16.

greater decrease in rate with a 16β -methyl group than with a 16α -methyl group, because the former almost certainly causes C-21 to take up an α -sided orientation by rotation around the C-20–C-21 bond (Taub, Hoffsommer, Slaters, Kuo & Wendler, 1960). In fact, 16α -methylcortisol is oxidized at about one-half of the rate of 16β -methylcortisol. It seems likely that the effect of the 16β -methyl group is an indirect one and due to the rotation of the side chain proposed by Taub *et al.* (1960), but that the 16α -methyl group exerts its effect by a repulsive interaction of its own with the active site. Though this interpretation is not incompatible with our original stereochemical hypothesis, it was unsuspected and requires further investigation.

Significance of the effects of halogen substituents

With the exception of the results with 9α -bromocortisone (see below) the behaviour of the 9α - and 12α -halogen-substituted 11-oxygenated steroids can be summarized as follows: (a) 9α -halogens increase the initial velocity of reduction of 11-ketones of three series of steroids, and totally block oxidation of the related 11β -ols or greatly diminish its velocity; (b) in all substrates examined, the relative effects of fluorine and chlorine were in the order of their electronegativities ($-F > -Cl$); (c) 12α -fluorine (external Van der Waals radius $1.35\text{\AA}$) also increased the initial velocity of reduction of 11-ketones, but only by one-third to one-half the amount found with 9α -fluorine; (d) 12α -bromine (external Van der Waals radius $1.95\text{\AA}$) blocked or greatly inhibited the reduction of 11-ketones and oxidation of 11β -ols, as did 12α -methyl (external Van der Waals radius $2.0\text{\AA}$).

Bush & Mahesh (1964) attributed the more efficient reduction of 9α -fluoro-11-ketones and the almost complete absence of oxidation of 9α -fluoro- 11β -ols *in vivo* in man to a greater stability of the 9α -fluoro- 11β -ol, and hence an increase in the free energy of reduction of the 9α -fluoro-11-ketones, on the basis of Eschenmoser's study of chemical oxidations of 11β -hydroxy steroids. A. Eschenmoser

& J. Schreiber (personal communication) supposed that this was mainly due to stabilization of the 11β -hydroxy group by the negative inductive effect of the 9α -halogen (see also Roček, Westheimer, Eschenmoser, Moldoványi & Schreiber, 1962). In a study of the influence of fluorine substitution on the redox equilibria of steroid 3-ketone–3 α -ol pairs catalysed by 3α -hydroxy steroid dehydrogenase, Ringold, Lawrence & Graves (1964a) found that (equatorial) 2α -fluorine substitution increased the free energy of reduction of saturated 5α -hydrogen-3-ketones from 5.8 to 8.5 kcal./mole, corresponding to 100-fold decrease in the equilibrium constant expressed as ketone/alcohol. They attributed this to destabilization of the ketone group on the basis of the well established bathochromic shift of the carbonyl stretching frequency in the infrared spectrum and other chemical findings.

The situation is rather different in the 9α - and 12α -halo-11-oxo steroids, because in both cases the halogen is an axial substituent and the reduction product is a *trans*-1,2-diaxial halohydrin. However, Ringold, Ramachandran & Forchielli (1964b) found that the substitution of (axial) 6β -fluorine produced a larger effect than the substitution of (equatorial) 6α -fluorine on the enzymic reduction of Δ^4 -3-oxo steroids, an effect complicated by two factors: (a) an inhibition of polarization of the C-4–C-5 double bond; (b) facilitation of hydride transfer from NADPH (Ringold *et al.* 1964a).

Though we now favour the interpretation by Ringold and co-workers (which they have already applied to our earlier findings *in vivo*; Ringold *et al.* 1964b), the main argument we wish to offer is independent of whether or not the relative destabilization of the α -halo-11-ketone with respect to the α -halo- 11β -ol is due to a destabilization of the α -halo-11-ketone with respect to its unsubstituted analogue. Our argument is simply that the effects of 9α -halogen substitution on the reduction rates of 11-ketones by 11β -hydroxy steroid dehydrogenase can for the moment be attributed to the influence of the halogen on the redox properties of the 11-ketone– 11β -ol couple itself, without invoking

any specific effects of the halogen on the enzyme-substrate complex. In other words, a thermodynamic explanation of these findings is adequate at present.

On the other hand, some specific interaction of 9 α -fluorine with the enzyme appears to be necessary for the 11 β -ols to explain our observations on oxidative reactions. Thus 9 α -fluorocortisol acted as a simple competitive inhibitor of the enzyme in the oxidation of cortisol, having K_i $5 \times 10^{-6} M$ by Dixon's (1953) method. This is approx. $0.2 \times K_m$ for cortisol itself. This phenomenon is striking and presumably underlies similar findings *in vivo* in man (Bush & Mahesh, 1964).

It was not found possible to devise a completely satisfactory system for the spectrophotometric measurement of reductions by this enzyme, so that we have not yet obtained K_m values for the various 11-ketones. Such values, together with values of $V_{max.}$, will be necessary for a complete understanding of these findings.

Effect of 12 α -fluorine. The enhancement of reduction rates of α -haloketones has now been observed in the steroids with 2 α -fluoro saturated 3-ketones, with 4-fluoro-, 6 β -fluoro-, and 6 α -fluoro- Δ^4 -3-ketones (Ringold *et al.* 1964*a*), with 16-fluoro-17-ketones (Offner, Ryan, Smith, Fried & Munson, 1962) and with a sizable range of 9 α -fluoro-11-ketones.

The increase in initial reduction velocity caused by substitution of 9 α -fluorine (axial, tertiary) in all members of the last class, covering three types of steroids (cortisone, 11-oxoprogesterone and adrenosterone), is in the range five- to ten-fold. It seems unlikely that the axial secondary 12 α -fluorine would differ significantly in its steric and electronic effects on the 11-ketone group (Fried, Kessler & Borman, 1958). The fact that 12 α -fluorine substitution caused only a twofold increase in the initial velocity of reduction of 11-oxoprogesterone suggests therefore that the enhancing effect of the 12 α -fluorine is partially opposed by some other effect. It seems unlikely that 12 α -fluorine would seriously affect the conformation of the 17 β -acetyl side chain. If anything, dipole-dipole interaction would tend to stabilize the preferred conformation suggested by Wellman & Djerassi (1965).

It is reasonable, however, to suppose that 12 α -fluorine is large enough (external Van der Waals radius 1.35Å) to produce a significant steric repulsion to the approach of the active site of the enzyme to the α -side of the region of C-12, an effect that produces complete or almost complete inhibition of the reaction with the larger 12 α -bromine (1.95Å); or 12 α -methyl (2.0Å) substituents.

Metabolism of 9 α -bromocortisone. We were surprised to observe that 9 α -bromocortisone gave no identifiable quantities of 9 α -bromocortisol on

Fig. 15. Diagram summarizing the principal points of close apposition of an 11-oxo steroid with the enzyme and co-enzyme in the ternary substrate-enzyme-coenzyme complex that have been postulated for 11 β -hydroxy steroid dehydrogenase.

incubation with this enzyme under reducing conditions. The main product in 15 small-scale (50–200 $\mu g.$) and two large-scale (0.5–2.0 mg.) incubations appeared to be cortisone. At first we suspected that exposure to alkali during extraction might have led to the formation of 8,9-dehydrocortisone (Fried & Sabo, 1957), and that our routine chromatographic procedure had failed to differentiate it from cortisone. This possibility was eliminated by adding 9 α -bromocortisone to cold incubation mixture and extracting it immediately, in parallel with incubations that had been run for the preceding 30–60 min., and by a complete degradation of the main reaction product according to Chart 1 of Bush & Mahesh (1959*b*). The product was distinguished from 9,11-dehydrocortisone and was apparently identical with cortisone.

This debromination to the saturated ketone was also puzzling in that the resulting cortisone was not reduced to cortisol. Our present hypothesis is that the 9 α -bromine atom of this substrate combines with a group in the active site of the enzyme, thus inactivating it. Unfortunately, there was insufficient material left at this stage of our investigations to test this hypothesis. Dr J. Fried (personal communication) has informed us that debromination without unsaturation of the C-8–C-9 bond, i.e. hydrogen-bromine exchange, is readily observed with this class of steroid, so that the hypothesis proposed is at least reasonable on chemical grounds.

These results *in vitro* therefore both confirm the earlier inferences of relative rates of reaction of different steroids with 11 β -hydroxy steroid dehydrogenase and add a number of crucial tests that support the hypothesis put forward by Bush & Mahesh (1959*b*). The active site of the enzyme-coenzyme complex appears to require close contact with the steroid around the upper half of its α -surface and parts of its 'top edge'; more specifically the region defined by the following atoms (see

Fig. 15): hydrogen atoms 1β , 2α , 12α , 12β , 21 ; carbon atoms 1 , 2 , 11 , 12 , 20 , 21 .

Without further analysis it is not possible to say whether 'poor fit' caused by steric hindrance in these regions diminishes the reaction velocity by lowering the affinity of the steroid for the enzyme-coenzyme complex, or by decreasing the rate of hydrogen transfer due to a less favourable mutual orientation of coenzyme and steroid in the ternary active complex. In one case, however, that of the 2α -methyl group, the loss of reactivity is clearly due to a lowering of the affinity of the steroid for the enzyme-coenzyme complex. Thus 2α -methylcortisone and 2α -methylcortisol have no detectable activity as competitive inhibitors of the reaction of cortisone and cortisol with our microsome preparations. Their unreactivity as substrates must therefore be due to a failure to combine with the active site of the enzyme-coenzyme complex.

A more detailed analysis of the kinetics of this enzyme reaction is necessary before anything more about its mechanism can be suggested. It is possible, for instance, that the theory of the stereochemistry of the mechanism of α -hydroxy steroid (i.e. 3α or 17α) dehydrogenase comprehensively worked out by Ringold and his co-workers (Ringold, 1967; Ringold, Graves & Clark, 1966) might be applicable to the 11β -hydroxy steroid dehydrogenase. However, it seems to us that the 11 -position is so much more hindered than positions 3 and 17 that the 11 -hydroxy steroid dehydrogenase will be unlikely to be amenable to such a generalized stereochemical theory as now seems feasible for other hydroxy steroid dehydrogenases.

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REFERENCES

- Brown-Grant, K., Forchielli, E. & Dorfman, R. I. (1960). *J. biol. Chem.* **235**, 1317.
- Burton, R. B., Keutmann, E. H., Waterhouse, C. & Schuler, E. A. (1953). *J. clin. Endocrin. Metab.* **13**, 48.
- Bush, I. E. (1956). *Experientia*, **12**, 325.
- Bush, I. E. (1961a). In *The Human Adrenal Cortex*, p. 138. Ed. by Currie, A. & Grant, J. K. Glasgow: E. and S. Livingstone Ltd.
- Bush, I. E. (1961b). *The Chromatography of Steroids*, pp. 83-132. Oxford: Pergamon Press Ltd.
- Bush, I. E. (1962). *Pharmacol. Rev.* **14**, 317.
- Bush, I. E. (1965). *Meth. biochem. Anal.* **13**, 357.
- Bush, I. E. & Hunter, S. A. (1961). *Biochem. Pharmacol.* **8**, 142.
- Bush, I. E. & Hunter, S. A. (1962). *Biochem. J.* **84**, 16P.
- Bush, I. E. & Mahesh, V. B. (1959a). *Biochem. J.* **71**, 705.
- Bush, I. E. & Mahesh, V. B. (1959b). *Biochem. J.* **71**, 718.
- Bush, I. E. & Mahesh, V. B. (1964). *Biochem. J.* **93**, 236.
- Bush, I. E., Meigs, R. A. & Hunter, S. A. (1962). *J. Endocrin.* **24**, ii.
- Bush, I. E. & Sandberg, A. A. (1953). *J. biol. Chem.* **205**, 283.
- Bush, I. E., Swyer, G. I. M., Stern, M. I. & Willoughby, M. L. N. (1957). *J. Endocrin.* **67**, 689.
- Bush, I. E. & Willoughby, M. L. N. (1957). *Biochem. J.* **67**, 689.
- Caspi, E. Y., Levy, H. & Hechter, O. M. (1953). *Arch. Biochem. Biophys.* **45**, 169.
- Charney, W., Weber, L. & Oliveto, E. (1959). *Arch. Biochem. Biophys.* **79**, 402.
- Dalziel, K. (1957). *Acta chem. scand.* **11**, 1706.
- Dixon, M. (1953). *Biochem. J.* **55**, 170.
- Dougherty, T. F., Berliner, M. L. & Berliner, D. L. (1960). *Ann. N.Y. Acad. Sci.* **88**, 78.
- Dowd, J. & Riggs, D. S. (1965). *J. biol. Chem.* **240**, 863.
- Fieser, L. F. & Fieser, M. (1959). *The Steroids*, pp. 636, 662. New York: Reinhold Publishing Corp.
- Fish, C. A., Hayano, M. & Pincus, G. (1953). *Arch. Biochem. Biophys.* **42**, 480.
- Fried, J., Kessler, W. B. & Borman, A. (1958). *Ann. N.Y. Acad. Sci.* **71**, 494.
- Fried, J. & Sabo, E. F. (1957). *J. Amer. chem. Soc.* **79**, 1130.
- Hoagland, M. B., Keller, E. B. & Zamecnik, P. (1956). *J. biol. Chem.* **218**, 345.
- Hofstee, B. H. (1959). *Nature, Lond.*, **184**, 1296.
- Hubener, H. J., Fukushima, D. K. & Gallagher, T. F. (1956). *J. biol. Chem.* **220**, 499.
- Hunter, S. A. (1964). M.Sc. Thesis: University of Birmingham.
- Hurlock, B. & Talalay, P. (1959). *Arch. Biochem. Biophys.* **80**, 467.
- Ketelaar, J. A. A. (1958). *Chemical Constitution*, p. 148. Amsterdam: Elsevier Publishing Co.
- Koerner, D. R. (1966). *Endocrinology*, **79**, 935.
- Koerner, D. R. & Hellman, L. (1964). *Endocrinology*, **75**, 592.
- Lieberman, S., Katzenellenbogen, E. R., Schneider, R., Studer, P. E. & Dobriner, K. (1953). *J. biol. Chem.* **205**, 87.
- Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
- Mahesh, V. B. & Ulrich, F. (1959). *Nature, Lond.*, **184**, 147.
- Mahesh, V. B. & Ulrich, F. (1960). *J. biol. Chem.* **235**, 356.
- Mason, H. L. (1950). *J. biol. Chem.* **182**, 131.
- Meigs, R. A. & Engel, L. L. (1961). *Endocrinology*, **60**, 152.

- Offner, P., Ryan, K. J., Smith, O. W., Fried, J. & Munson, P. L. (1962). *Cancer Chemother. Rep.* no. 16, p. 285.
- Osinski, P. A. (1960). *Nature, Lond.*, **187**, 777.
- Ringold, H. J. (1967). *Recent Progr. Hormone Res.* **22**.
- Ringold, H. J., Graves, J. M. H. & Clark, A. (1966). *Proc. nat. Acad. Sci., Wash.*, **56**, 255.
- Ringold, H. J., Graves, J. M. H., Hayano, M. & Lawrence, H. (1963). *Biochem. biophys. Res. Commun.* **13**, 162.
- Ringold, H. J., Lawrence, H. & Graves, J. M. H. (1964a). *J. Amer. chem. Soc.* **86**, 4510.
- Ringold, H. J., Ramachandran, S. & Forchielli, E. (1964b). *Biochim. biophys. Acta*, **82**, 143.
- Roček, J., Westheimer, F. H., Eschenmoser, A., Moldoványi, L. & Schreiber, J. (1962). *Helv. chim. Acta*, **45**, 2554.
- Schneider, W. C. & Hogeboom, G. H. (1950). *J. biol. Chem.* **183**, 123.
- Stahl, T. J. & Tapley, D. F. (1963). *Endocrinology*, **73**, 271.
- Sweat, M. L. & Bryson, M. J. (1960). *Biochim. biophys. Acta*, **44**, 217.
- Talalay, P. (1957). *Physiol. Rev.* **37**, 362.
- Taub, D., Hoffsommer, R. D., Slates, H. L., Kuo, C. H. & Wendler, N. L. (1960). *J. Amer. chem. Soc.* **82**, 4012.
- Theorell, H. & McKinley-McKee, J. S. (1961). *Acta chem. scand.* **15**, 1797.
- Tomkins, G. M. (1959). *Ann. N.Y. Acad. Sci.* **82**, 836.
- Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1957). *Manometric Techniques*. Minneapolis: Burgess Publishing Co.
- Warburg, D. & Christian, W. (1936). *Biochem. Z.* **287**, 291.
- Wellman, K. M. & Djerassi, C. (1965). *J. Amer. chem. Soc.* **87**, 60.
- Westheimer, F. H., Fisher, H. F., Conn, E. E. & Vennesland, B. (1951). *J. Amer. chem. Soc.* **73**, 2403.