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THE EFFECT OF BUDESONIDE AND TRIAMCINOLONE ACETONIDE ON HEPATIC MICROSOMAL TESTOSTERONE METABOLISM IN THE RAT

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Abstract—The hepatic microsomal metabolism of testosterone was studied in male rats after treatment with either budesonide or triamcinolone acetonide for 13 weeks. The *in vitro* metabolism was determined using a testosterone concentration of 35 nM which is comparable to the levels found in plasma. It was shown that the total microsomal testosterone metabolism was decreased in budesonide-treated rats and increased in rats treated with triamcinolone acetonide. The testosterone metabolites produced were measured and thus it was revealed that budesonide treatment brought about its effect through a 50% decrease in the activity of steroid 5α-reductase, but did not affect other reductive enzymes, or the oxidation of testosterone. Triamcinolone acetonide treatment decreased steroid 5α-reductase activity by 95% and also decreased the activities of steroid 3α- and 3β-reductases by more than 90%. In addition, treatment with triamcinolone acetonide caused a 50% increase in the oxidative metabolism of testosterone, which resulted in the observed increase in total testosterone metabolism. The presence of 0.1 μM budesonide in the microsomal incubations was without effect on testosterone metabolism. However, 0.1 μM triamcinolone acetonide inhibited testosterone oxidation by 65%, without affecting the reductive pathway of testosterone metabolism.

Budesonide and triamcinolone acetonide (TAAC‡) are synthetic glucocorticoids that are commonly used in the treatment of a number of inflammatory diseases, including asthma and various skin disorders [1–3]. Locally applied budesonide has a higher therapeutic ratio than TAAC and other synthetic glucocorticoids, probably as a result of its rapid clearance from the systemic circulation due to rapid biotransformation in the liver [1, 4]. Budesonide contains an asymmetric 16α,17α-acetal group resulting in an equal mixture of two epimers with 22*R* and 22*S* configuration (Fig. 1). The 22*R* epimer has two to three times the anti-inflammatory potency of the 22*S* epimer [1]. TAAC differs from budesonide in that it contains a symmetrical 16α,17α-acetal group and a fluorine substituent in ring B (Fig. 1).

It has been reported that glucocorticoid treatment may alter the activity of some enzymes involved in steroid metabolism. For example, in the rat, several anti-inflammatory steroids have been shown to inhibit hepatic cytosolic 3α-hydroxysteroid dehydrogenase [5]. The most potent inhibitors also had the highest anti-inflammatory activity [5]. Betamethasone and dexamethasone have been shown to be potent non-competitive inhibitors of this enzyme,

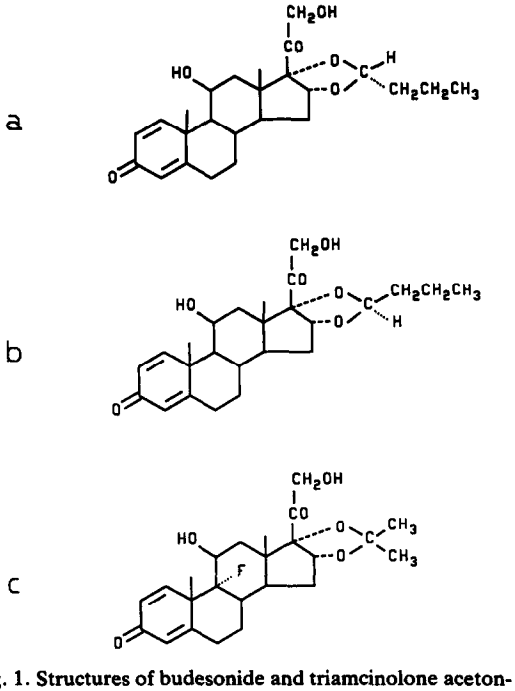

Fig. 1. Structures of budesonide and triamcinolone acetonide. The 22*R* (a) and 22*S* (b) configurations of budesonide compared with the structure of triamcinolone acetonide (c).

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‡ Abbreviations: TAAC, triamcinolone acetonide; 4MA, *N,N*-diethylcarbamoyl-4-methyl-4-aza-5α-androstan-3-one; 5-DHT, 5α-dihydrotestosterone; 2α-OHT, 2α-hydroxy-testosterone; 3α-DIOL, 5α-androstan-3α,17β-diol; 3β-DIOL, 5α-androstan-3β,17β-diol; 6α-OH-5-DHT, 6α-hydroxy-5α-dihydrotestosterone; MOX, methoxyamine-HCl in pyridine; BSTFA, bis(trimethylsilyl) trifluoroacetamide; TMS, trimethylsilyl, MO, *O*-methyloxime; GC-MS, gas chromatography–mass spectrometry.

and triamcinolone a weak competitive inhibitor [6]. Some isoenzymes of cytochrome P450, involved in hepatic steroid metabolism, have also been shown to be affected: Schuetz *et al.* [7] have shown that TAAC and other synthetic glucocorticoids can induce hepatic cytochrome P450p (CYP3A1 or 3A2: the cytochrome P450 nomenclature according to Nebert *et al.* [8] is given in parentheses) in the rat, an isoenzyme thought to be responsible for catalysing the 6β-hydroxylation of steroids [9]. Dexamethasone has also been shown to reduce the levels of hepatic cytochrome P450h (CYP2C11) and cytochrome P450i (CYP2C12) [10, 11]. These isoenzymes of cytochrome P450, and others, have been shown to catalyse the hydroxylation of testosterone and other steroids [12], however, *in vitro* studies using physiological levels of testosterone have yet to be performed.

The oral administration of budesonide or TAAC at a dose of 50 μg/kg/day for 8 weeks to male Sprague–Dawley rats has been shown to cause a significant increase in plasma testosterone, levels from 0.95 ± 0.19 ng/mL (mean ± SEM) to 1.78 ± 0.17 ng/mL and 1.93 ± 0.24 ng/mL, respectively (Ahren, personal communication). It is possible that such treatments may modulate the activity of enzymes involved in steroid metabolism in general and testosterone metabolism in particular. Hence, an *in vitro* assay for the metabolism of testosterone to its principle metabolites was devised. The assay employed a low concentration of testosterone so that the effects of budesonide and TAAC treatment of rats on the hepatic microsomal metabolism of testosterone could be investigated at physiologically relevant levels of this compound.

MATERIALS AND METHODS

Materials. Budesonide was a kind gift from Dr A. Ryrfeldt (AB Astra, Sodertalje, Sweden) and 17β-*N,N* - diethylcarbamoyl - 4 - methyl - 4 - aza - 5α - androstan-3-one (4MA) was kindly provided by Dr G.H. Rasmusson (Merck, Sharp and Dohme Research Laboratories, Rahway, U.S.A.). Ketoconazole, TAAC and unlabelled testosterone were obtained from the Sigma Chemical Co. (Poole, U.K.). [$^{14}C_4$]Testosterone with a specific activity of 52 mCi/mmol was purchased from New England Nuclear (Stevenage, U.K.) and was 96% pure as determined by HPLC; the impurities were evenly distributed over the HPLC elution profile. The steroids 5-DHT, 2α-OHT, 2β-OHT, 6α-OHT, 6β-OHT, 7α-OHT, 11β-OHT, 14α-OHT, 16α-OHT, 16β-OHT and 19-OHT were from Steraloids (Croydon, U.K.); 5β-dihydrotestosterone, 3α-DIOL, 3β-DIOL, 5β-androstan-3α, 17β-diol, androstenedione, 5α-androstanedione, 5β-androstanedione, 5β-androstan-3α-ol-17-one, 5β-androstan-3β-ol-17-one, androsterone and epiandrosterone were from Sigma; 6α-OH-5-DHT, 7α-OH-5-DHT, 9α-OH-5-DHT and 11α-OH-5-DHT, were from the MRC Steroid Reference Collection, and 17β-hydroxy-4,6-androstadiene-3-one, synthesized in this department, was a kind gift from Dr John Williams. Acetonitrile grade S was obtained from Rathburn (Walkerburn, U.K.) and all other solvents (analytical reagent grade) were obtained from BDH Ltd (Poole, U.K.). All HPLC equipment was purchased from Millipore (Harrow, U.K.). Scintillation fluid (Instagel) was purchased from Canberra Packard (Pangbourne, U.K.). Hexamethyldisilazane : trimethylchlorosilane : pyridine (9:3:1) (Trisil) and MOX were from Pierce and Warriner, U.K. Ltd. BSTFA was obtained from Sigma. All other chemicals were purchased from Sigma or BDH Ltd and were of analytical grade or the best equivalent.

Treatment of animals. Treatment of rats was carried out by Hazleton Laboratories America Inc. Groups of male Sprague–Dawley rats (Charles River Breeding Laboratories Inc., NY, U.S.A.), 35–40 days old, were treated with either budesonide (approximately 100 μg/kg/day) or TAAC (approximately 50 μg/kg/day) in their drinking water, the consumption of which was monitored and the addition of the drugs adjusted accordingly. Control rats were housed and fed under the same conditions, but did not receive budesonide or TAAC. Groups of rats were treated for 2 weeks, 13 weeks or 2 years. The effects of treatment for 13 weeks are described here. At the end of the treatment period, the animals were killed, their livers removed and rapidly frozen for stage at −60°.

Preparation of hepatic microsomal fractions. All the preparative steps were performed at 4°. Hepatic microsomes were prepared using the method of Boobis *et al.* [13] except that the microsomal pellets were washed once with four volumes of 0.25 M potassium phosphate, 0.15 M potassium chloride, 1 mM EDTA, pH 7.25. The final pellet was resuspended in one volume of 0.25 M potassium phosphate buffer, pH 7.25, containing 30% (v/v) glycerol and stored at −80° in portions of 400 μL. The samples were thawed on ice prior to addition to incubation mixtures. To ensure consistency in enzyme activities, samples were used directly from storage and were not re-frozen after use. There was no detectable decrease in enzyme activity after storage of the samples for up to 3 years.

Incubation conditions. The *in vitro* rate of testosterone metabolism was measured in 10 mL incubation mixtures containing 50 mM potassium phosphate buffer, pH 7.4, 1 mM NADPH, 3 mM magnesium chloride, 0.5 mg of microsomal protein and 35 nM [^{14}C]testosterone or [^{14}C]5-DHT. The reaction mixture was warmed to 37° in a shaking water bath and the reaction initiated by the addition of radiolabelled steroid. Aliquots of 1 mL were removed after 0.5 min, 1 min, and then at 1 min intervals up to 5 min. Each aliquot was immediately mixed with 9 mL of diethyl ether. Unlabelled testosterone (1.7 nmol) was added as a carrier, a marker for spectrophotometric detection following HPLC, and an internal standard for quantification. After vortex mixing for 30 sec and centrifugation at 1000 *g* for 10 min, the organic phase was transferred to a glass conical tube, evaporated under a stream of nitrogen and the residue dissolved in 100 μL of methanol for HPLC, as described below.

In the experiments where the effect of various inhibitors was investigated, the incubation mixture contained, in addition to those reagents stated above, either 0.1 μM budesonide, 0.1 μM TAAC, 10 μM ketoconazole, 0.1 μM 4MA or no additions. The

incubation time was 4 min and the reaction stopped by addition of 9 mL of diethyl ether to a 1 mL aliquot of the incubation mixture. Otherwise, the reaction conditions were the same as described above.

HPLC. The method of Cochran *et al.* [14] was used, except that the flow rate was 1.5 mL/min. The chromatography was achieved using a μBondapak C_{18} column (3.9 × 300 mm) and C_{18} μBondapak guard column under isocratic elution conditions with 40% (v/v) acetonitrile. The eluent was monitored at 205, 240, 254 or 284 nm, as appropriate to the absorption characteristics of individual steroids. Under these conditions, the retention times of testosterone (11.0 min) and the following oxidized and reduced metabolites of testosterone were determined using authentic standards: 7α-OHT (3.0 min), 6α-OHT (3.0 min), 19-OHT (3.2 min), 6α-OH-5-DHT (3.2 min), 6β-OHT (3.4 min), 14α-OHT (3.4 min), 16α-OHT (3.4 min), 7α-OH-5-DHT (3.6 min), 11α-OH-5-DHT (4.4 min), 11β-OHT (4.5 min), 9α-OH-5-DHT (4.9 min), 16β-OHT (5.2 min), 2α-OHT (5.4 min), 2β-OHT (5.4 min), 17β-hydroxy-4,6-androstadiene-3-one (9.4 min), 3β-DIOL (12.5 min), androstenedione (14.8 min), 5β-androstan-3α,17β-diol (15.2 min), 3α-DIOL (17.1 min), epiandrosterone (17.6 min), 5β-dihydrotestosterone (18.5 min), 5β-androstan-3β-ol-17-one (18.5 min), 5-DHT (19.6 min), 5β-androstan-3α-ol-17-one (21.0 min), androsterone (25.3 min), 5α-androstanedione (26.4 min) and 5β-androstanedione (26.8 min).

Extracts resulting from the incubations carried out with radiolabelled testosterone were separated by HPLC as described above. The injection volume was 50 μL and the eluent was collected in fractions of 1.5 mL. To each fraction 10 mL of Instagel was added and analysed by liquid scintillation spectroscopy to quantify testosterone and its metabolites. Under these conditions, 98% of radiolabel was recovered. A typical HPLC profile is shown in Fig. 2. There was a clear separation of testosterone, 5-DHT, 3α-DIOL and 3β-DIOL. However, under the conditions employed, the oxidized metabolites of testosterone were not resolved, although they were clearly separated from the reduced metabolites. Resolution of the oxidized metabolites was not required as it was convenient to consider the oxidized metabolites collectively.

Testosterone and its metabolites were quantified by internal standard correction, using the ratio: (radioactivity in testosterone or metabolite peak)/(UV peak height measured at 240 nm of unlabelled testosterone). Standard solutions of radiolabelled testosterone were extracted from incubation mixtures as described above to produce a standard curve (Fig. 3) with testosterone concentrations ranging from 3.5 to 35 nM ($r = 0.987$). The coefficient of variation for both the production of testosterone metabolites and the rate of testosterone metabolism was less than 10%. In each experiment, appropriate controls were included where NADPH was omitted, radiolabelled testosterone was omitted or there were no omissions but the reaction was stopped at zero time. Under all of these conditions, no testosterone metabolites were detected.

Synthesis and purification of [^{14}C]*testosterone metabolites.* To produce sufficient quantities of the metabolites for further analysis, 20 mL incubation mixtures, each containing 350 nM radiolabelled testosterone, were used. The incubation time was 4 min for metabolites 1 and 2, and 1 min for metabolite 3 (see Table 1 for the identification of these metabolites) and the reactions were each stopped by the addition of 40 mL of diethyl ether. Unlabelled tes-

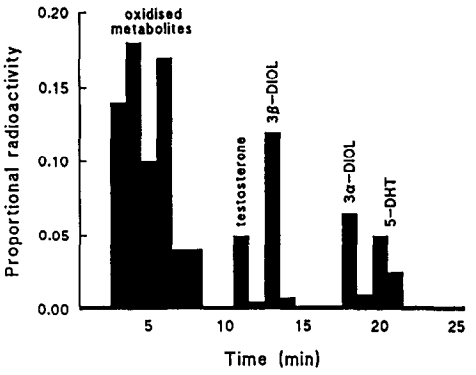

Fig. 2. Example of the elution profile of testosterone and its metabolites following separation by HPLC. Radiolabelled testosterone was incubated with a hepatic microsomal preparation obtained from untreated rats as described in Materials and Methods. The testosterone metabolites generated were then separated by HPLC. Eluent fractions of 1.5 mL were collected every 1 min and were analysed by scintillation spectroscopy. The radioactivity in each fraction is expressed as a proportion of the total amount of radioactivity recovered in the 25 fractions. The identity of each of the metabolites was determined by comparison with the retention time of authentic standards.

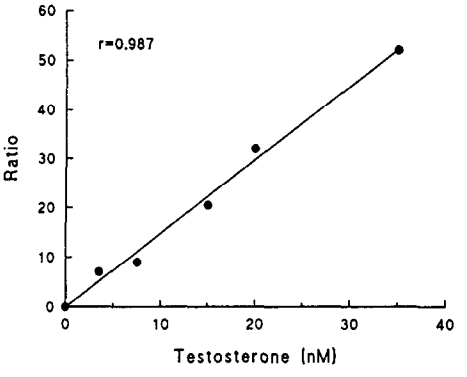

Fig. 3. Typical standard curve for the determination of testosterone and its metabolites. Standard solutions of radiolabelled testosterone were dissolved in the incubation mixture and then extracted, in the presence of 1.7 nmol of unlabelled testosterone, as described in Materials and Methods. During HPLC, the absorbance of unlabelled testosterone was measured by on-line UV spectroscopy at 240 nm and the amount of radiolabelled testosterone was determined by scintillation spectroscopy of the eluent fractions. The results are expressed as a ratio of the radioactivity in the fraction containing testosterone to the peak UV absorbance, and are the mean of duplicate determinations. Testosterone metabolites were quantified in an analogous manner.

Table 1. Analysis of testosterone and its metabolites by GC-MS

Compound	Ether derivative	Retention time (sec)	Molecular ion (m/z)	Fragment ions (m/z)
Testosterone	MO–TMS	616, 622	389	383, 374, 358, 349, 268
5-DHT	MO–TMS	591, 599	391	376, 360, 301, 286, 270
3α-DIOL	TMS	410	436	421, 346, 331, 256, 241
3β-DIOL	TMS	463	436	421, 346, 331, 256, 241
Metabolite 1 ([^{14}C]3β-DIOL)	TMS	463	438	423, 348, 333, 258, 243
Metabolite 2 ([^{14}C]3α-DIOL)	TMS	410	438	423, 348, 333, 258, 243
Metabolite 3 ([^{14}C]5-DHT)	MO–TMS	591, 599	393	378, 362, 303, 288, 272

Each of the steroids was derivatized to either its TMS or MO–TMS ether prior to separation and analysis by GC-MS. The gas chromatography retention times are shown. The structure specific region of the resulting mass spectra has been summarized by stating the size of the molecular ion and those fragments ions where the ratio m/z was larger than 200. Where two figures have been given for the retention time the derivative was composed of two geometric isomers that were separated by gas chromatography. In these cases the sizes of the molecular and fragmentation ions were the same for each isomer.

tosterone was not added. After evaporation of the ether extracts, each of the residues was dissolved in 1 mL of diethyl ether, pooled as appropriate, and again evaporated to dryness. The residues were then dissolved in 100 μL of methanol. The metabolites were purified from the appropriate incubation mixture extracts by HPLC as described above. In each case, two separate injections of approximately 50 μL, were applied to the HPLC column. The eluent fractions corresponding to the metabolite of interest were pooled and dried under vacuum before reconstitution in 1 mL of methanol. Each of the purified metabolites gave a single peak on analytical HPLC. [^{14}C]5-DHT (metabolite 3) was used in incubation experiments, as described above. The concentration of [^{14}C]5-DHT was determined by scintillation spectroscopy. The yield of 5-DHT was 14% of the amount of radiolabelled testosterone added to the reaction mixture.

Gas chromatography–mass spectrometry (GC-MS). Standard solutions of 100 ng of testosterone, 5-DHT 3α-DIOL and 3β-DIOL were chemically derivatized using Trisil or MOX and their spectra were compared with the major metabolites purified by HPLC. Preliminary work showed that Trisil was suitable for the derivatization of 3α-DIOL and 3β-DIOL, but not testosterone or 5-DHT, which were successfully derivatized by MOX/BSTFA.

Derivatization by Trisil was performed by adding 20 μL of Trisil to the dried steroid samples. The mixtures were heated for 10 min at 70° and then evaporated under a stream of nitrogen before reconstitution in 5 μL of dodecane immediately prior to GC-MS.

Derivatization by MOX/BSTFA was performed by adding 50 μL of MOX to the dried steroid samples. The mixtures were incubated overnight at room temperature and then extracted with 200 μL water and 500 μL ethyl acetate. The organic phase was transferred to a glass vial and evaporated under a stream of nitrogen. The residues were dissolved in 100 μL of BSTFA and incubated overnight at room temperature. BSTFA was then removed by evaporation under a stream of nitrogen and the residues were dissolved in 10 μL of dodecane immediately prior to GC-MS.

GC-MS analysis was performed using a Finnigan 4500 quadropole mass spectrometer operating in electron impact mode. The gas chromatograph was equipped with an SE54 fused silica capillary column (0.25 × 30 mm; Jones chromatography, Llanbradach, U.K.) which was routed through the separator oven and directly into the mass spectrometer ion source. Helium (at a head pressure of 140 kPa) was used as the carrier gas. The gas chromatograph was fitted with a Grob type capillary injector operated in splitless mode and maintained at a temperature of 270°. The gas chromatograph oven temperature was held at 200° for 1 min, raised to 240° at 20°/min and then raised to 325° at 5°/min. The mass range was scanned between m/z 50–650 every 1 sec and 2.5 μL of sample was injected each time. Selected ion monitoring was also performed under these conditions. Data acquisition and manipulation were performed by an INCOS data system using IDOS 2 software.

Other methods. The concentration of microsomal protein was determined by the method of Lowry *et al.* [15] using bovine albumin as standard.

The cytochrome P450 content of hepatic microsomes was determined using the method of Omura and Sato [16]. The concentration of cytochrome P450 was calculated assuming an extinction coefficient of 91 $mM^{-1}\,cm^{-1}$, between 450 and 490, for reduced versus reduced and carbon monoxide-complexed cytochrome P450.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis [17] was performed using slab gels containing 8.5% (w/v) acrylamide. Microsomal samples

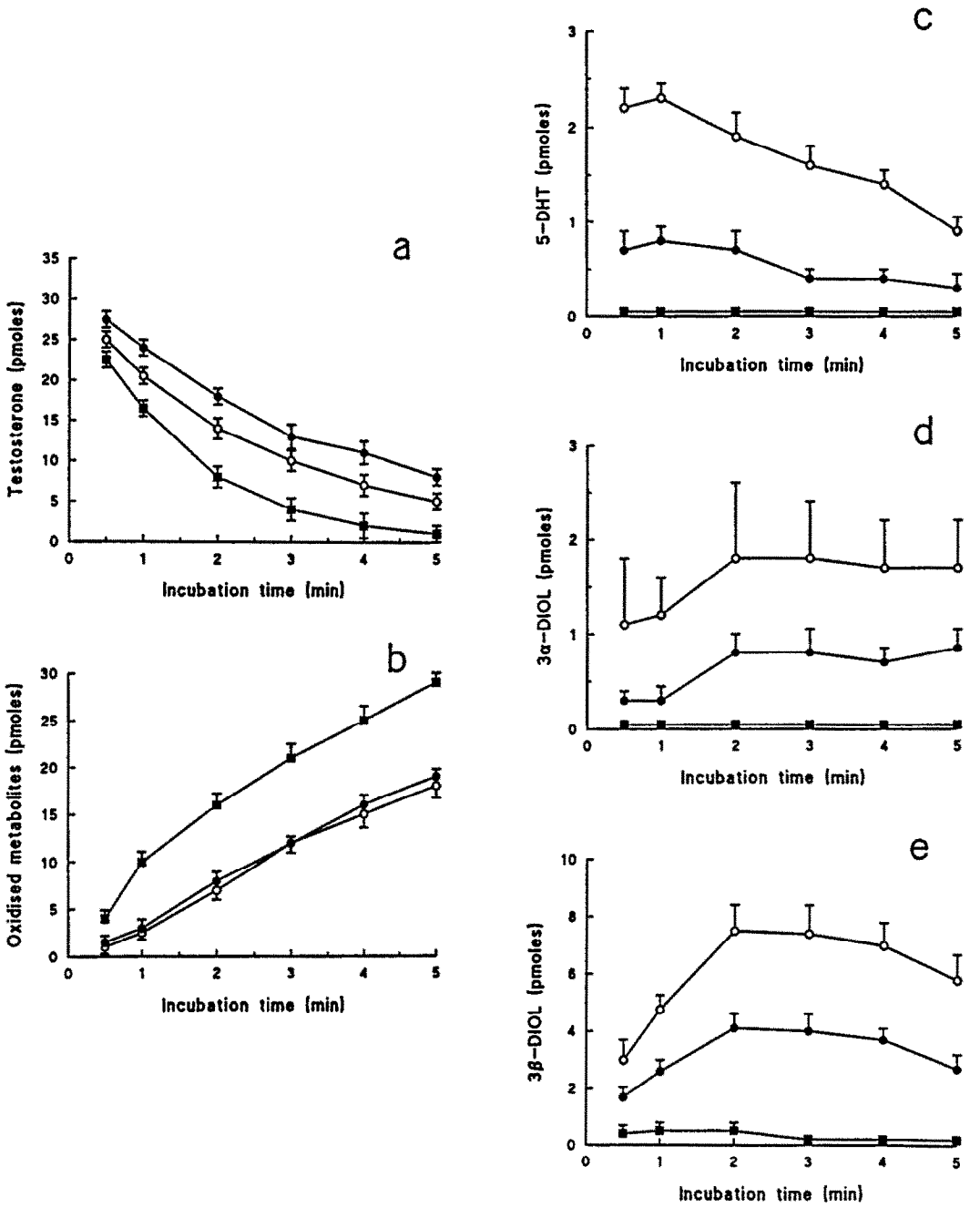

Fig. 4. The effect of treatment of rats with either budesonide or TAAC on the metabolism of testosterone by hepatic microsomal fractions. Incubations were performed with hepatic microsomal fractions prepared from rats treated with budesonide (closed circles, N = 6), TAAC (closed squares, N = 3), or were untreated (open circles, N = 6). Samples were removed from the incubation mixtures at intervals and the quantities of (a) testosterone, (b) oxidized testosterone metabolites, (c) 5-DHT, (d) 3α-DIOL and (e) 3β-DIOL, were determined as described in Materials and Methods. The results are shown as mean values ± SEM.

were reduced by the addition of 1% (v/v) 2-mercaptoethanol and 30 μg was loaded on to the gel. The gels were stained for 1 hr with 0.2% (w/v) Coomassie Brilliant Blue R dissolved in 50% (v/v) methanol, 10% (v/v) acetic acid and destained overnight in 25% (v/v) methanol, 7.5% (v/v) acetic acid.

Statistical analysis. Results were analysed using a computer package, Statgraphics 2.1 (STSC Inc., Rockville, MD, U.S.A.) employing Student's

unpaired t-test, except for the results shown in Fig. 4 which were subjected to 2-way analysis of variance. All results are presented as mean values with standard errors.

RESULTS

Incubation of radiolabelled testosterone with microsomal fractions prepared from rat liver resulted in the production of a number of different metabolites of testosterone which were separated by HPLC (Fig. 2). The group of metabolites that were eluted at 2–8 min correspond to the retention times determined for many oxidized metabolites of testosterone. These compounds were not fully separated by the chromatography conditions used here and, henceforth, are referred to, collectively, as oxidized metabolites. The second peak of radioactivity eluted was testosterone and had a retention time of 11–12 min. Three other peaks were found at 13–14, 17–18 and 19–21 min (metabolites 1, 2 and 3, respectively), which correspond to the retention times of 3β-DIOL, 3α-DIOL and 5-DHT, respectively.

Each of these late-running metabolites was purified by HPLC and their identity confirmed by comparison with authentic standards using GC-MS (Table 1). The TMS ethers of 3α-DIOL and 3β-DIOL had the same molecular ion size and both steroids also fragmented into ions of the same sizes. 3α-DIOL and 3β-DIOL could, however, be distinguished from one another by the relative intensity of the fragment ions (results not shown), and they also had different retention times measured using gas chromatography (Table 1). The TMS ether of metabolite 1 had the same retention time (463 sec) as 3β-DIOL. The molecular ion and the major ion fragments of metabolite 1 were all two mass units higher than the authentic 3β-DIOL standard as a result of the ^{14}C present in the radiolabelled substrate. The TMS ether of metabolite 2 had the same molecular ion and fragmentation pattern as metabolite 1, but its retention time (410 sec) on gas chromatography corresponded to that of 3α-DIOL. The TMS ether of testosterone could not be detected by GC-MS. However, the O-methyloxime–trimethylsilyl (MO–TMS) ether of testosterone was easily identified. This derivative of testosterone produced two geometric isomers with syn- or anti-configuration, which were resolved under the gas chromatography conditions used (Table 1). The MO–TMS ether of 5-DHT also produced two geometric isomers which were separated by gas chromatography (Table 1). Both of the isomers had the same molecular and fragmentation ions following mass spectroscopy. The MO–TMS ether of metabolite 3 was also resolved into two components on gas chromatography. The retention times measured were the same as those of authentic 5-DHT. The molecular and fragmentation ions were also the same as authentic 5-DHT, except that the ions of the radiolabelled metabolite were, again, two mass units heavier for the reasons explained above.

The metabolism of testosterone to 5-DHT, 3α-DIOL, 3β-DIOL and oxidized steroid metabolites was measured in incubations containing microsomal fractions prepared from rats treated for 13 weeks with either budesonide or TAAC and compared with those from untreated rats. In untreated rats, approximately two-thirds of the metabolites produced in the incubations were oxidized and one-third reduced (Fig. 4). Rats treated with budesonide had a significantly decreased microsomal testosterone metabolism ($P < 0.0005$) (Fig. 4a). This decrease appeared to be due to a slowing of metabolism through the reductive pathways, as there was a significant decrease in the amounts of 5-DHT ($P < 0.0001$) (Fig. 4c), 3α-DIOL ($P < 0.0005$) (Fig. 4d) and 3β-DIOL ($P < 0.0001$) (Fig. 4e) produced, whilst the production of oxidized metabolites was unchanged compared with untreated animals (Fig. 4b). In contrast, treatment with TAAC resulted in a significant increase in the rate of microsomal testosterone metabolism compared with age matched controls ($P < 0.0005$) (Fig. 4a). There was a significant increase in the production of oxidized metabolites ($P < 0.0001$) (Fig. 4b) and this was accompanied by a decrease in testosterone metabolism via reductive pathways, with significant decreases in the production of 5-DHT ($P < 0.0001$) (Fig. 4c), 3α-DIOL ($P < 0.0005$) (Fig. 4d) and 3β-DIOL ($P < 0.0001$) (Fig. 4e).

The addition of 10 μM ketoconazole to the reaction mixture caused a substantial inhibition of oxidative metabolism in untreated, budesonide-treated and TAAC-treated rats ($P < 0.001$, in call cases) (Fig. 5b). This resulted in a general increase in testosterone reduction with statistically significant ($P < 0.05$) increases in the production of 5-DHT (Fig. 5c) in untreated and budesonide treated rats, and 3α-DIOL (Fig. 5d) in untreated rats. Ketoconazole decreased the total metabolism of testosterone in all three groups of rats, with the most marked decline seen in TAAC-treated rats ($P < 0.001$) (Fig. 5a). This is consistent with the higher contribution of oxidative metabolism in this group (Figs 4b and 5b). In the absence of the oxidative route of metabolism, reductive metabolism was compared between the treatments. There was a decrease in the production of 5-DHT (Fig. 5c), 3α-DIOL (Fig. 5d) and 3β-DIOL (Fig. 5e) in both budesonide and TAAC-treated rats compared with untreated rats ($P < 0.02$, in all cases), indicating that treatment with either of these drugs caused a decrease in the activities of the reductive enzymes.

The addition of 0.1 μM 4MA to the reaction mixture caused almost complete inhibition of testosterone reduction with virtually no 5-DHT (Fig. 5c), 3α-DIOL (Fig. 5d) or 3β-DIOL (Fig. 5e) produced ($P < 0.05$, in all cases). There was no effect on total testosterone metabolism (Fig. 5a) or testosterone oxidation (Fig. 5b) at this concentration of 4MA.

5-DHT was used as a substrate in order to investigate if reductive enzymes other than steroid 5α-reductase were affected in rats following treatment with budesonide or TAAC. Budesonide treatment had no effect on the rate of disappearance of 5-DHT (Fig. 6a), nor on its metabolism to oxidized metabolites (Fig. 6b). The production of 3α-DIOL (Fig. 6d) and 3β-DIOL (Fig. 6e) was the same as in untreated rats, indicating that the activities of ster-

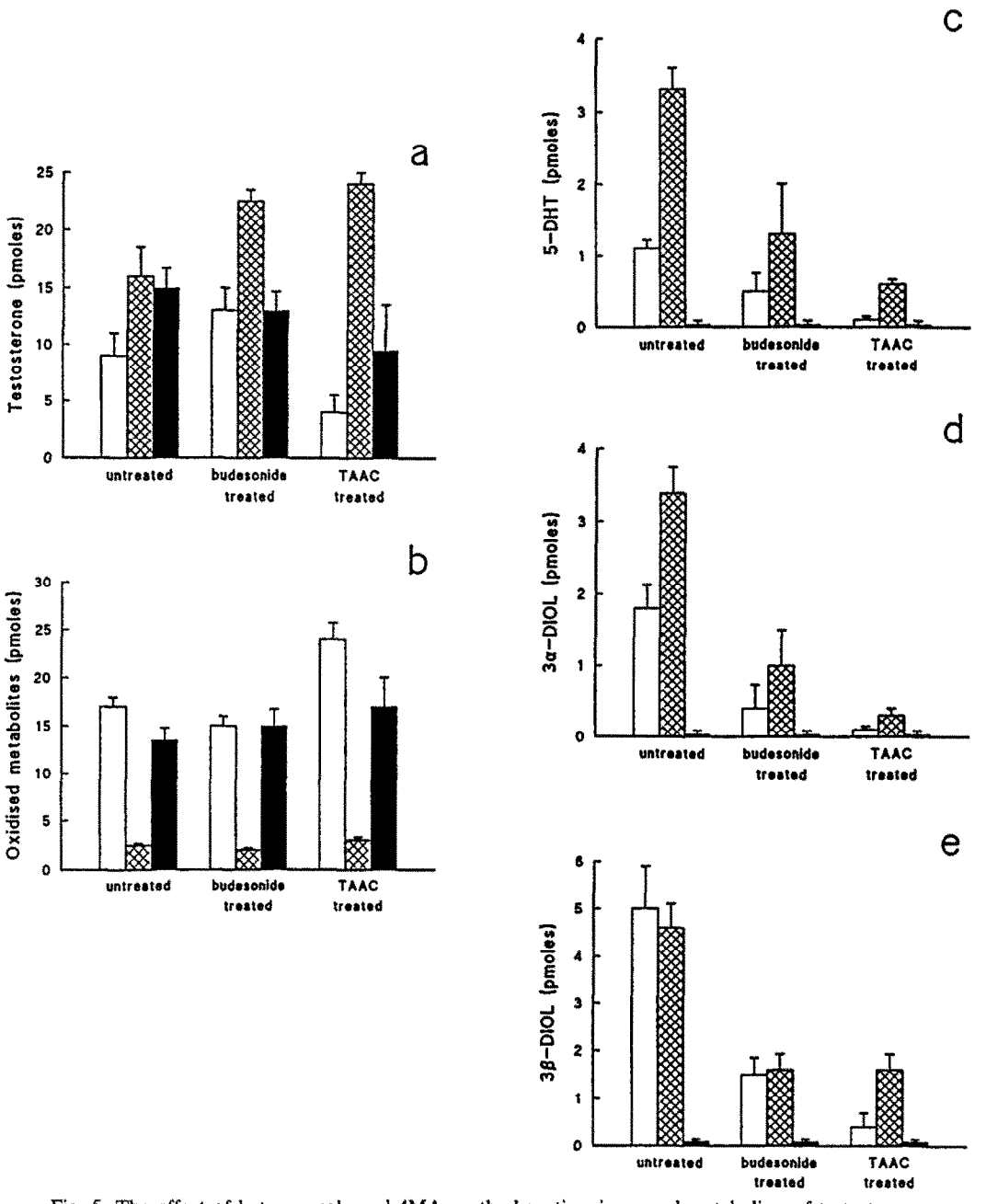

Fig. 5. The effect of ketoconazole and 4MA on the hepatic microsomal metabolism of testosterone. Incubations were performed with hepatic microsomal fractions prepared from untreated rats (N = 6), and rats treated with either budesonide (N = 6) or TAAC (N = 3). The incubations were performed in the absence of inhibitor (open bars), or in the presence of 10 μM ketoconazole (cross-hatched bars) or 0.1 μM 4MA (closed bars). The mixtures were incubated for 4 min, after which time the quantity of (a) testosterone, (b) oxidized testosterone metabolites, (c) 5-DHT, (d) 3α-DIOL and (e) 3β-DIOL, was determined as described in Materials and Methods. The results are shown as mean values ± SEM.

oid 3α-and 3β-reductases were unaffected. There appeared to be a decrease in the amount of testosterone produced from 5-DHT (Fig. 6c), however, this did not reach statistical significance. In the rats treated with TAAC, there was no testosterone detected in any of the incubation mixtures (Fig. 6c). There was also a decrease in the levels of 3α-DIOL $P < 0.05$) (Fig. 6d) and 3β-DIOL ($P < 0.02$)

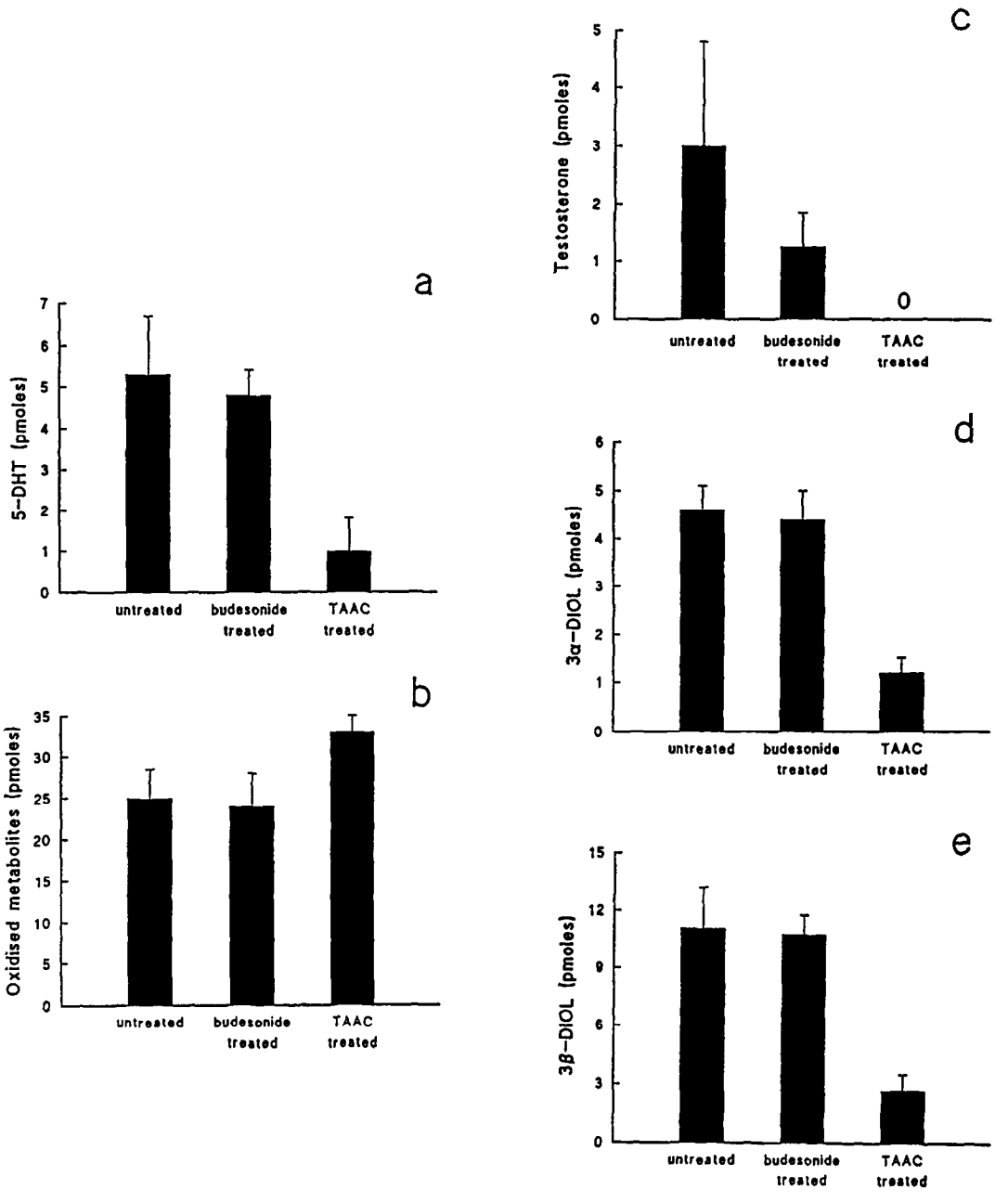

Fig. 6. The effect of treatment of rats with either budesonide or TAAC on the metabolism of 5-DHT by hepatic microsomal fractions. Incubations were performed with hepatic microsomal fractions prepared from untreated rats (N = 6), and rats treated with either budesonide (N = 6) or TAAC (n = 3), in the presence of 35 nM [^{14}C]5-DHT. The mixtures were incubated for 3 min, after which time the quantity of (a) 5-DHT, (b) oxidized 5-DHT/testosterone metabolites, (c) testosterone, (d) 3α-DIOL and (e) 3β-DIOL, was determined as described in Materials and Methods. The results are shown as mean values ± SEM.

measured (Fig. 6e), indicating a decrease in the activity of not only steroid 5α-reductase, but also steroid 3α- and 3β-reductases. Total 5-DHT metabolism was also increased compared with untreated controls, but this did not reach statistical significance (Fig. 6a). The products of oxidative metabolism also appeared to be increased (Fig. 6b), but again this was not statistically significant.

The possibility that budesonide or TAAC had a direct inhibitory effect on microsomal testosterone

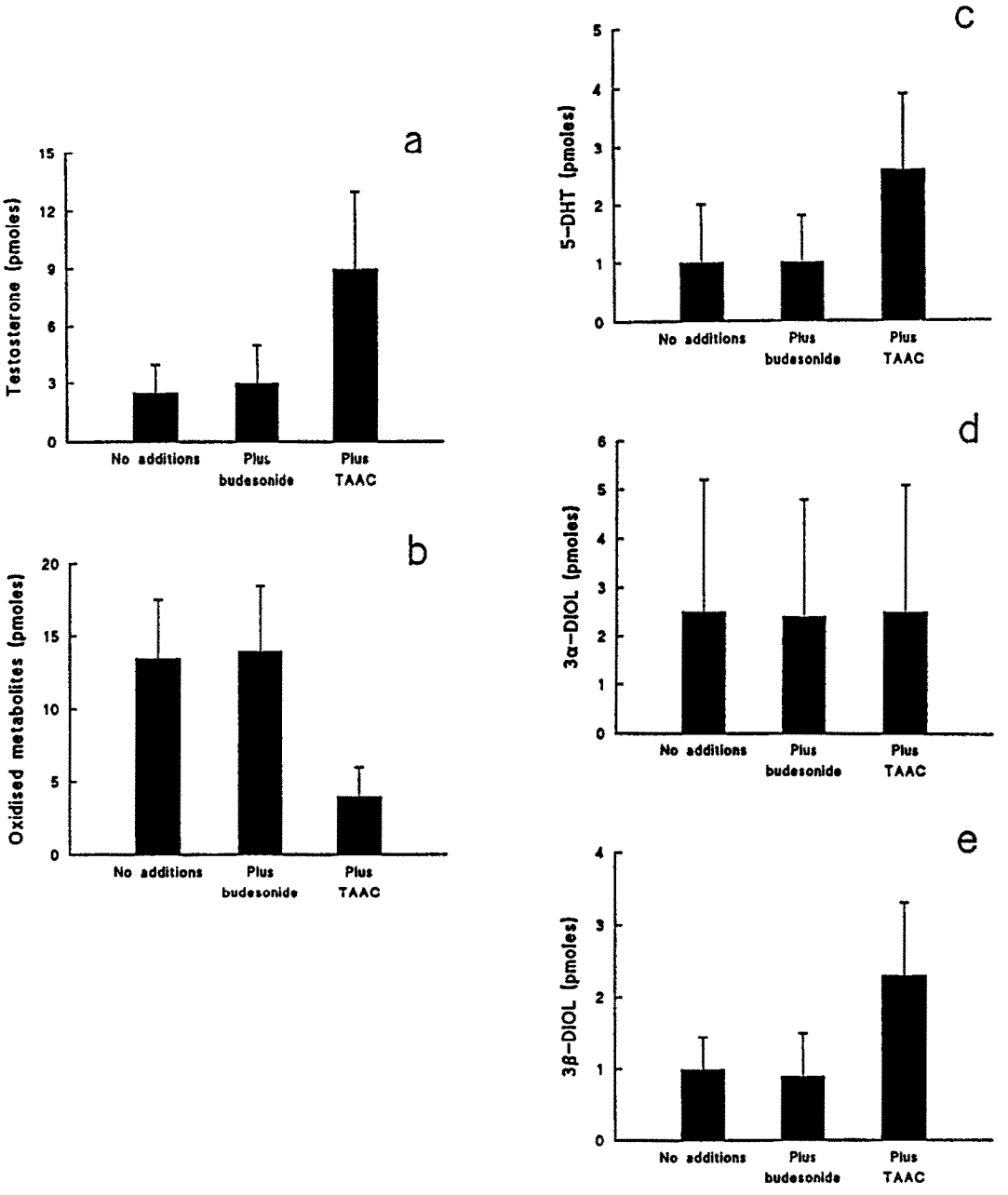

Fig. 7. The effect of the presence of budesonide and TAAC on hepatic microsomal testosterone metabolism. Incubations were performed with hepatic microsomal fractions prepared from untreated rats (N = 3). The effect of 0.1 μM budesonide and 0.1 μM TAAC were compared with no additions. The mixtures were incubated for 4 min, after which time the quantity of (a) testosterone, (b) oxidized testosterone metabolites, (c) 5-DHT, (d) 3α-DIOL and (e) 3β-DIOL, was determined as described in Materials and Methods. The results are shown as mean values ± SEM.

metabolism was investigated by addition of these drugs, at concentrations measured in the plasma, to incubation mixtures containing microsomal fractions prepared from untreated rats. Budesonide (0.1 μM) had no effect on the formation of any of the metabolites (Fig. 7). However, TAAC (0.1 μM) was found to inhibit testosterone oxidation ($P < 0.05$) (Fig. 7b), but had no statistically significant effect on the production of 5-DHT (Fig. 7c), 3α-DIOL or 3β-DIOL (Fig. 7e). Although there was an indication that the overall metabolism of testosterone was inhibited (Fig. 7a), this did not reach statistical significance.

Neither budesonide nor TAAC appeared to be inducers of cytochrome P450, as the specific hepatic contents of cytochrome P450 were unchanged after treatment with these glucocorticoids. The levels

measured were 0.52 ± 0.06 (mean ± SEM) (N = 6) and 0.56 ± 0.06 (N = 3) nmol cytochrome P450/mg microsomal protein, respectively, compared with 0.48 ± 0.05 nmol/mg measured in untreated rats (N = 6). In addition, analysis of the microsomal proteins by sodium dodecyl sulphate–polyacrylamide gel electrophoresis did not reveal any differences between the untreated and treated rats (results not shown). However, it is still possible that small changes in the levels of individual cytochrome P450 isoenzymes may have occurred which were not detected by the methods used here.

DISCUSSION

In this study *in vitro* incubations employing 35 nM testosterone were used in an attempt to model testosterone metabolism at physiologically relevant levels. The concentration used is comparable to the levels of testosterone found in plasma samples [18–20]. Physiological levels of testosterone were used in order to avoid the possibility, often seen in work with cytochrome P450, of bringing into play low affinity enzymes which do not have a significant role in metabolism at the low concentrations found *in vivo*. As there was almost complete utilization of the substrate under these conditions, analysis of the results using Michaelis–Menten kinetics was clearly not possible. However, the metabolic fate of testosterone through the different enzyme pathways could be determined under these conditions. Hence the effect of treatment of rats with budesonide or TAAC on stosterone metabolism could be determined at near physiological levels of testosterone. In contrast, a number of workers [12, 21–24] have used higher testosterone concentrations (25–693 μM) and have measured the rates of metabolism to specific hydroxylated and reduced metabolites under conditions suitable for analysis by Michaelis–Menten kinetics.

The treatment of rats with budesonide caused a decrease in the total hepatic microsomal metabolism of testosterone compared with untreated rats. The metabolism of testosterone via the reductive route was decreased by 50%, whereas, testosterone oxidation was unaffected. In comparison, treatment of rats with TAAC led to a striking change in both routes of testosterone metabolism. There was an overall increase in the rate of testosterone metabolism, as a result of a 50% increase in the rate of oxidative metabolism, whereas the reductive route was strongly inhibited. There was a 95% decrease in the activity of steroid 5α-reductase activity and steroid 3α- and 3β-reductase activities were also decreased by more than 90%.

These observations were confirmed by selective inhibition of the oxidative and reductive pathways. To block testosterone oxidation, ketoconazole, an inhibitor of cytochrome P450 [25] was employed and to eliminate the reduction of testosterone, 4MA, a steroid 5α-reductase inhibitor was used [26].

Ketoconazole has been shown to inhibit a number of reactions catalysed by cytochrome P450 [27–29]. Sheets *et al.* [25] reported that the production of 6β-, 7α-, 16α- and 16β-hydroxyandrostenedione by hepatic microsomal fractions prepared from male rats was potently inhibited by ketoconazole, whereas the activity of steroid 5α-reductase was not affected. The concentration of ketoconazole which caused maximum inhibition of testosterone oxidation with minimum effect on testosterone reduction under the conditions used in this study was determined. At a concentration of 10 μM, ketoconazole inhibited testosterone oxidation by >80% (Fig. 5b). This resulted in an increase in the production of 5-DHT (Fig. 5c) and 3α-DIOL (Fig. 5d). The production of 3β-DIOL was relatively unaffected (Fig. 5e), which indicates that there may be a slight inhibitory effect of ketoconazole on steroid 3β-reductase, as inhibition of the oxidative pathway was expected to result in a proportional increase in the metabolism to all reduced metabolites, due to substrate diversion. Although it is possible that the incubation time may have been too short to see the effect of substrate diversion on the production of 3β-DIOL. Ketoconazole caused a small decrease in the overall rate of testosterone metabolism (Fig. 5a). Higher ketoconazole concentrations caused further decreases in the rate of testosterone metabolism, as a result of inhibition of 5-DHT, 3α-DIOL and 3β-DIOL production in microsomal incubations in all groups of rats (results not shown). Thus, 10 μM ketoconazole caused near complete inhibition of testosterone oxidation with only a marginal effect on testosterone reduction, and was thus used in these studies.

In the presence of ketoconazole, the effect of the treatment of the rats on their capacity to perform reductive metabolism of testosterone could be more clearly demonstrated. The results show an obvious decrease in the activity of reductive enzymes following treatment with either budesonide or TAAC. In budesonide-treated rats the production of 5-DHT was decreased, indicating a lower activity of steroid 5α-reductase. The production of 3α-DIOL and 3β-DIOL was also decreased, but this could have been due to the decrease in steroid 5α-reductase activity, and the consequently lower concentration of 5-DHT available. This was shown to be the case as, when radiolabelled 5-DHT was used as a substrate, budesonide-treated rats produced the same amount of 3α-DIOL and 3β-DIOL as untreated rats. Hence, the activities of steroid 3α- and 3β-reductases were not affected by budesonide treatment.

In TAAC-treated rats, the production of all 3 reductive metabolites was decreased, and to a greater extent than budesonide treatment. There was a 95% decrease in the amount of 5-DHT produced, indicating a substantial lowering of steroid 5α-reductase activity. In contrast to budesonide-treated rats, when radiolabelled 5-DHT was used as a substrate, TAAC-treated rats produced only 30% of the amount of 3α-DIOL and 3β-DIOL, compared with untreated rats, indicating that the activities of steroid 3α- and 3β-reductases were also both inhibited.

The profile of metabolites produced in incubations containing microsomal fractions from untreated rats in the presence of ketoconazole did not resemble that seen in the incubations with microsomes from budesonide or TAAC-treated rats. The effect of ketoconazole was to decrease oxidation and increase the reduction of testosterone. In contrast, TAAC treatment caused an increase in testosterone oxi-

dation and a decrease in its reduction. Treatment with budesonide also caused a decrease in testosterone reduction, but had no effect on its oxidation. This indicated that the effect of either of the two glucocorticoids on testosterone metabolism was unlikely to be the same as that of the oxidative inhibitor, ketoconazole and that their main effect was on testosterone reduction.

4MA is a potent inhibitor of hepatic steroid 5α-reductase activity [26, 30]. Sonderfan and Parkinson [31] reported that 0.1 μM 4MA inhibited hepatic microsomal testosterone 5α-reductase activity by 95%, without inhibiting testosterone oxidation. In the present study, 0.1 μM 4MA was shown to inhibit testosterone reduction by >90%. This concentration of 4MA potently inhibited the production of 5-DHT (Fig. 5c), 3α-DIOL (Fig. 5d) and 3β-DIOL (Fig. 5e). Testosterone oxidation was not altered significantly by the presence of 4MA (Fig. 5c), however, as the reductive pathway was almost completely blocked by 4MA, the proportion of testosterone oxidized would be expected to increase. This may be explained by a slight inhibition of testosterone oxidation or by changes in the oxidative products of testosterone caused by 4MA at the concentration used here. Thus, the addition of 0.1 μM 4MA caused near total inhibition of testosterone reduction with a possible slight effect on testosterone oxidation and was thus used in further studies.

In the presence of 4MA, the reductive pathway of testosterone metabolism was blocked, allowing the effect of treatment with rats with budesonide and TAAC on oxidative metabolism to be determined. In neither treatment group was there any difference in oxidative metabolism, indicating that this was not the main effect of the glucocorticoid treatment on testosterone metabolism. However, the profile of the metabolites produced in incubations of microsomal fractions from untreated rats in the presence of 4MA was similar to that found in glucocorticoid-treated rats, indicating that it was the reductive metabolism of treated rats that was affected.

The effect of budesonide and TAAC in microsomal incubations was investigated to determine if either were able to inhibit testosterone metabolism directly. Budesonide at 0.1 μM was shown to have no effect on the rate of disappearance of testosterone, or the rate of appearance of any of the metabolites measured. Therefore, it is concluded that budesonide should not be an inhibitor of the hepatic microsomal enzymes involved in testosterone metabolism *in vivo*. This is in contrast to the effect of TAAC on testosterone metabolism. TAAC was shown to inhibit testosterone oxidation directly, without affecting the reductive routes of metabolism. This was similar to the effects caused by ketoconazole. Thus, TAAC, like ketoconazole, appears to be an inhibitor of isoenzymes of cytochrome P450 involved in testosterone oxidation. The specific hydroxylation reactions involved and the isoenzymes which catalyse them were not determined here and could form the basis of future studies. In contrast to dexamethasone [32], TAAC was not found to be a strong inducer of cytochrome P450, as after the treatment period the specific content of this enzyme was unchanged and there were no obvious changes in the composition of the microsomal proteins. However, the possibility of induction of individual cytochrome P450 isoenzymes cannot be excluded.

In conclusion, treatment of rats with either budesonide or TAAC changed the microsomal metabolism of testosterone. The two glucocorticoids had opposite effects on the overall rate of testosterone metabolism; budesonide decreased its metabolism, whereas TAAC treatment caused an increase. However, both treatments caused an inhibition of steroid 5α-reductase activity, although TAAC was shown to be more potent than budesonide. TAAC treatment was also shown to decrease the activities of both steroid 3α- and 3β-reductases, and increase the oxidative metabolism of testosterone, whereas budesonide treatment had no effect on these activities. Finally, from *in vitro* experiments it was shown that TAAC, but not budesonide was an inhibitor of testosterone oxidation. Hence, the suggested effect on steroid metabolism in rats following treatment with glucocorticoids has been confirmed at the microsomal level. The role of the cytosolic enzymes involved in steroid metabolism has not been studied here and has yet to be determined.

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