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Trenbolone growth promotant: covalent DNA binding in rat liver and in *Salmonella typhimurium*, and mutagenicity in the Ames test*

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Abstract. *DNA binding in vivo:* [6,7-^{3}H]β-trenbolone (β-TBOH) was administered p.o. and i.p. to rats. After 8 or 16 h, DNA was isolated from the livers and purified to constant specific radioactivity. Enzymatic digestion to deoxyribonucleotides and separation by HPLC revealed about 90% of the DNA radioactivity eluting in the form of possible TBOH-nucleotide adducts. The extent of this genotoxicity, expressed in units of the Covalent Binding Index, CBI = (μmol TBOH bound per mol nucleotide)/(mmol TBOH administered per kg body weight) spanned from 8 to 17, i.e. was in the range found with weak genotoxic carcinogens. *Ames test:* low doses of β-TBOH increased the number of revertants in *Salmonella* strain TA100 reproducibly and in a dose-dependent manner. The mutagenic potency was 0.2 revertants per nmol after preincubation of the bacteria (20 min at 37° C) with doses between 30 and 60 μg per plate (47 and 94 μg/ml preincubation mixture). Above this dose, the number of revertants decreased to control values, accompanied by a reduction in survival. The addition of rat liver S9 inhibited the mutagenicity. *DNA binding in vitro:* calf thymus DNA was incubated with tritiated β-TBOH with and without rat liver S9. Highest DNA radioactivities were determined in the *absence* of the "activation" system. Addition of inactive S9 (without cofactors) reduced the DNA binding by a factor of up to 20. Intermediate results were found with active S9. *DNA binding in Salmonella:* β-TBOH was irreversibly bound to DNA isolated from *S. typhimurium* TA100 after incubation of bacteria with [^{3}H]β-TBOH. *Conclusions:* Covalent DNA binding appears to be the mechanism of an activation-independent ("direct") mutagenicity of TBOH which is not easily detected because of the bactericidal activity. The genotoxicity risk arising from exposure of humans to trenbolone residues in meat was estimated using the in vivo data and compared to that from the exposure to unavoidable genotoxins aflatoxin B_1 and dimethylnitrosamine. It is concluded that trenbolone residues represent only a low genotoxic risk.

Key words: Trenbolone – Anabolic agent – DNA binding – Genotoxicity – Ames test – Salmonella typhimurium

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Introduction

Trenbolone (β-TBOH, 17β-hydroxy-4,9,11-androstatrien-3-one) is an androgenic steroid hormone analogue used as a growth promotant in beef cattle. It is administered as trenbolone acetate (TBA) which is readily deacetylated to β-TBOH, hormonally the most active form. Further metabolism includes the formation of the α-isomer and oxidation to the 17-keto derivative.

Short-term in vitro studies on genotoxicity were, in general, considered negative (Ingerowski et al. 1981; Schiffmann et al. 1985; Scheutwinkel et al. 1986). Although the administration of tritiated β-TBOH in a DNA binding experiment in the rat resulted in radiolabelled liver DNA, the level of DNA radioactivity was similar to the one obtained with natural steroid hormones such as estradiol, and hence it was concluded that the xenobiotic TBOH does not represent a specific genotoxic hazard (Barraud et al. 1984). On the other hand, β-TBOH was reported to transform Syrian hamster embryo fibroblasts in culture (Schiffmann et al. 1985) and to induce micronuclei (Schiffmann et al. 1988). In the view of these conflicting results it was considered worth investigating the potential genotoxicity of TBOH in more detail.

Materials and Methods

Materials. [6,7-^{3}H]β-trenbolone with a specific activity of 55 Ci/mmol was from Roussel-Uclaf, France. The original radiochemical purity was >99%, as determined by radiothin layer chromatography with cyclohexane: ethyl acetate 1:1 (v/v; R_f = 0.2). This sample was used for the DNA-binding experiments with rats. For the DNA-binding experiments in vitro and in Salmonella performed 1 year later, the tritiated β-TBOH was repurified on a silica gel 60 column, eluting with cyclohexane:ethyl acetate 1:9. The radiochemical purity then was >97%.

DNA binding in rat liver

Animal treatment. [^{3}H]β-Trenbolone was dissolved in ethanol and administered by oral gavage to female Sprague-Dawley rats (Iva:SIV-50.SD from Ivanovas, Kissleg, FRG), or by i.p. injection to male Wistar rats (Crl:(WI)BR from Charles River Wiga, Sulzfeld, FRG). The chemical and radioactivity dose is shown in Tables 1 and 2. After 8 h (SD rats), or 16 h (Wistar rats), the animals were killed by an ether overdose.

Table 1. Binding of [^{3}H]β-trenbolone to liver DNA and chromatin protein, 8 h after oral administration of [^{3}H]-labelled drug to female Sprague-Dawley rats

	Trenbolone		Controls	
			No. 1: Incub.	No. 2: Backgr.
Animal no./weight (g)	1/203	2/209	3/191	4/196
Chemical dose (μg/kg body weight)	255	204	–	–
Radioactivity dose (dpm/kg)	$1.19 \cdot 10^{11}$	$0.95 \cdot 10^{11}$	–	–
Specific activity of chromatin protein (dpm/mg protein)	33 060	31 980	1520	–
DNA				
Amount in vial (mg)	0.10[a]	2.16	1.76	1.72
Total activity (cpm)	136	4200	25	15
Net activity (dpm)	294	11426	28	–
Specific activity (dpm/mg DNA)	3000	5290	16	–
Covalent Binding Index	8	17[b]	(0.04)	–
Mean ± SD	12 ± 6			

[a] Less sample available because of three rounds of DNA purification
[b] DNA nucleotides analysed by HPLC

Table 2. Binding of [^{3}H]β-trenbolone to liver DNA and chromatin protein, 16 h after i.p. administration of [^{3}H]-labelled drug to male Wistar rats

	Trenbolone		Controls	
			No. 1: Incub.	No. 2: Backgr.
Animal no./weight (g)	5/203	6/196	7/199	8/155
Chemical dose (μg/kg body weight)	191	267	–	–
Radioactivity dose (dpm/kg)	$0.89 \cdot 10^{11}$	$1.25 \cdot 10^{11}$	–	–
Specific activity of chromatin protein (dpm/mg protein)	18 600	54 100	1460	–
DNA				
Amount in vial (mg)	2.01	1.97	1.93	1.56
Total activity (cpm)	2120	2240	20	16
Net activity (dpm)	5860	6220	10	–
Specific activity (dpm/mg)	2910	3160	5	–
Covalent Binding Index	10[a]	8	(0.012)	–
Mean ± SD	9 ± 2			

[a] DNA nucleotides analysed by HPLC

Isolation of liver chromatin. The livers were excised, rinsed with ice-cold saline (0.85% NaCl), and homogenized in a teflon Potter-Elvehjem-type homogenizer at 4° C. Chromatin was prepared according to Sagelsdorff et al. (1983), essentially by precipitation with the non-ionic detergent Nonidet P 40 (BDH Chemicals Ltd, Poole BH12 4NN, UK). This pellet, containing about 2–3 mg DNA and 20–30 mg protein per g liver, was washed until the suspension contained < 1 μCi total radioactivity in order to remove the majority of non-covalently bound radioactivity.

Isolation of DNA. These steps were performed according to Sagelsdorff et al. (1983). Essentially, the chromatin pellet was homogenized in a Waring blender in 25 ml lysing medium [1% (w/v) SDS, 10 mM EDTA, 8 M urea in 0.24 M sodium phosphate, pH 6.8]. Protein was extracted from the homogenate with chloroform/isoamyl alcohol/phenol (CIP), phenol removed with diethylether, and the DNA purified by adsorption on a hydroxylapatite column, dialysis, and precipitation with ethanol. The highly purified DNA (less than 0.2% protein, as shown with radiolabelled amino acid incorporation (Caviezel et al. 1984)) was dissolved in a 20 mM sodium succinate buffer, containing 8 mM $CaCl_2$, pH 6.0. The amount of DNA was determined by assuming an absorbance of 20 at 260 nm for a solution of 1 mg DNA/ml. Scintillation counting (Packard scintillation counter Tricarb 460 CD) was performed on an aliquot of the DNA solution after addition of 10 ml Insta-Gel.

Repetitive purification of DNA. The remaining DNA solution was mixed with 25 ml lysing medium, extracted twice with CIP and ether, purified by hydroxylapatite, dialysed and precipitated with ethanol. The DNA was again dissolved in a 20 mM sodium succinate buffer, containing 8 mM $CaCl_2$, pH 6.0, and the specific activity of DNA was determined as described above.

Isolation of chromatin protein. Chromatin protein was precipitated with acetone from the first CIP extract (see

above) and redissolved in 1% (w/v) aqueous SDS. The precipitation and redissolving steps were repeated 5 times. The last solution was diluted to 0.1% SDS and 1 ml was used for scintillation counting. The amount of protein was determined with the Folin reagent.

Control experiment No. 1: DNA binding during work-up. The chromatin pellet isolated from the liver of an untreated rat was incubated for 15 min at 4° C with the radiolabelled supernatant from the first chromatin precipitation step of the DNA preparation from a treated animal. This allowed a check of whether radiolabel could contaminate DNA during the process of DNA isolation.

Control experiment No. 2: Background radioactivity. DNA was isolated from an untreated animal. The radioactivity count – upon comparison with historical controls – was used to show that the work-up of the DNA samples was performed without external contamination with radiolabels.

Analysis of the nucleotides by reverse phase chromatography. DNA 0.5–1 mg in 2 ml 20 mM sodium succinate buffer, pH 6.0, containing 8 mM $CaCl_2$, was digested enzymatically with 2.5 units/ml micrococcal endonuclease (Sigma no. N3755; E. C. 3.1.31.1.) and 0.05 units/ml spleen exonuclease (Boehringer Mannheim no. 108251; E. C. 3.1.16.1.) for 16 h at 37° C. The resulting nucleotide mixture was separated by HPLC on a Lichrosorb RP18 (10 µm) column (diameter 8 mm for rat liver DNA sample, 4 mm for *Salmonella* DNA × 250 mm length), eluting with a flow of 3.5 ml/min of 5% methanol in 50 mM sodium phosphate buffer (pH 6.8 for rat liver DNA, pH 5.5 for *Salmonella* DNA) for 5 min, followed by a linear gradient to 100% methanol in 40 min. The absorbance was recorded at 254 nm. Fractions of 2 min were collected. Scintillation counting was performed after the addition of 10 ml Insta-Gel (above conditions for 8-mm diameter column).

Calculation of the Covalent Binding Index (CBI). The specific activity of the DNA (dpm/mg) was divided by the radioactivity dose administered (dpm/kg) and multiplied by 309×10^6 to change to the molar units of the CBI = (µmol test compound bound per mol DNA-nucleotide)/(mmol test compound administered per kg body weight). The CBI indicates the number of adducts per 10^6 nucleotides expected (under the assumption of a linear dose-response relationship) after a dose of 1 mmol per kg body weight.

Ames test with TA100, TA98, and TA102

Salmonella typhimurium strains TA98, TA100, and TA102 were kindly provided by B. N. Ames, University of California, Berkeley CA, USA. Liver homogenate fractions [9000 g supernatant (S9) prepared according to Ames et al. (1975)] from aroclor 1254-induced male Sprague-Dawley rats were used. Ampoules containing 5 ml S9 were stored in liquid nitrogen. The protein concentration was 40 mg/ml. The influence of the S9 concentration in the S9 mix [0, 5, and 30% (v/v)] on the mutagenicity of trenbolone was tested with the preincubation method (20 min at 37°) of Yahagi et al. (1977). The incubation mixture consisted of 0.5 ml sodium phosphate buffer pH 7.4, 0.1 ml overnight culture of bacteria and 0.05 ml trenbolone in ethanol per plate. The number of revertants was determined in three replicate plates after addition of 2.1 ml top agar per plate.

DNA binding in vitro. DNA was incubated with tritiated β-TBOH in duplicate for 30 min at 37° C in 50 ml centrifuge tubes in a total volume of 5 ml. The concentration of calf thymus DNA was 1 mg/ml in 0.1 M sodium phosphate buffer pH 7.4 containing 33 mM KCl and 8 mM $MgCl_2$. S9 was added to give a 10% (v/v) concentration. For the experiments with active S9, 4 mM NADP and 5 mM glucose-6-phosphate was present. For the two experiments with inactive S9, no cofactors were added. In one of these experiments, the mixture was preincubated with 25 µM unlabelled β-TBOH for 30 min at 37° C to exhaust all residual cytochrome-P450 activity in the S9 fraction before the addition of the radiolabelled TBOH. The reaction was started by the addition of 50 µCi [^{3}H]β-TBOH in 50 µl ethanol, containing sufficient unlabelled β-TBOH to give a final chemical concentration of 25 µM. The reaction was stopped by putting the tubes in ice and adding 2 volumes of ethanol to precipitate the DNA. The tubes were kept at −20° C for 90 min and then centrifuged at 2000 g for 20 min. The DNA pellet was purified as described above (Isolation of DNA). The radioactivity background was determined on the basis of a replicate which contained unlabelled β-TBOH only.

DNA binding and mutagenicity in Salmonella. For the simultaneous determination of mutagenic effects and binding to bacterial DNA, the liquid preincubation method of Yahagi et al. (1977) was modified as described by Bösch et al. (1987). Bacteria were grown overnight in three roundbottom flasks, each containing 500 ml nutrient broth. The suspension was concentrated before use 10-fold by centrifugation at 3000 g for 20 min and resuspension in 150 ml fresh medium. Based on the composition used for one plate in the Ames agar plate assay, a 500-fold volume (325 ml) was used. Each flask contained 270 ml buffer, 50 ml concentrated bacterial suspension and 5 ml ethanol as solvent for the tritiated trenbolone. The incubation lasted 30 min in an round-shaking water bath (100 cycles/min; 37° C). Aliquots of 5 ml were centrifuged at 2000 g for 20 min, the bacteria were washed once with cold saline (0.85% NaCl) and resuspended in 5 ml 0.85% NaCl for the determination of mutagenicity and cell survival. Washed suspension (2 ml) was mixed with 8 ml top agar and 3.3 ml was pipetted onto each of three replicate plates. For the determination of viable cell counts, 200 µl of the suspension was diluted 10^6-fold with 0.85% NaCl and plated onto minimal agar plates together with top agar supplemented with 0.3 mM histidine and 0.05 mM biotin (100 µl diluted suspension in 2 ml agar per plate). Two independent dilutions per incubation mixture were made.

After centrifugation of the remaining incubation mixture at 8000 g for 10 min, DNA was isolated from the bacterial pellet, as described above for the isolation of DNA from chromatin. The hydroxylapatite step was omitted during the repetitive purification of the bacterial DNA.

Results

DNA binding in rat liver

[6,7-^{3}H]β-Trenbolone was investigated for DNA-binding in vivo, both under the conditions used in our earlier investigations with steroid hormones (Caviezel et al. 1984) and under the conditions used by Barraud et al. (1984). Eight hours after oral gavage of [^{3}H]β-trenbolone to female

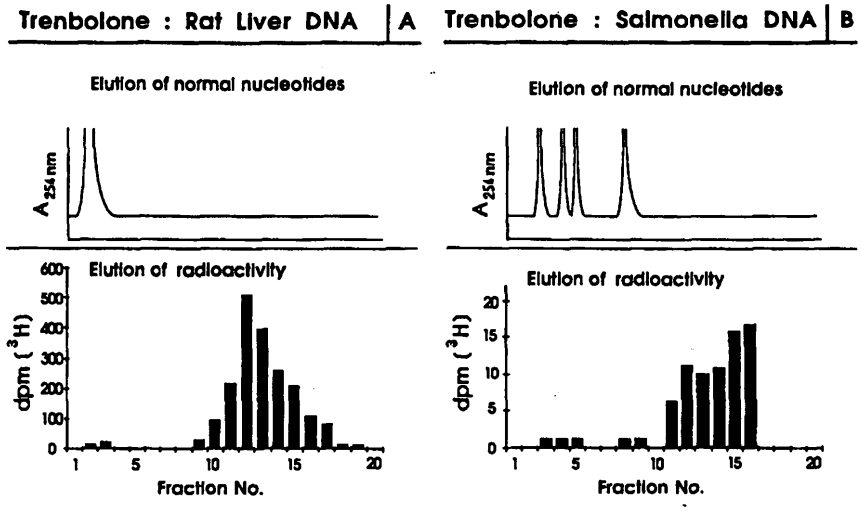

Fig. 1. HPLC elution profiles of absorbance at 254 nm (schematic) and [^{3}H]radioactivity of 3'-deoxyribonucleotides obtained from enzymatically degraded DNA. A Liver DNA isolated from a male Wistar rat (no. 5), 16 h after intraperitoneal administration of [6,7-^{3}H]β-trenbolone. The natural nucleotides eluted in fractions 2 and 3 (pH 6.8). Fractions 9–18 did not show detectable absorbance at 254 nm.
B DNA isolated from *Salmonella typhimurium* after incubation with [6,7-^{3}H]β-trenbolone. The natural nucleotides eluted in fractions 3–5 and 8–9 in the sequence dCp, dGp, dTp, dA pat pH 5.5. Fractions 11–16 did not show detectable absorbance at 254 nm

Sprague-Dawley rats (Table 1) and 16 h after i.p. administration to male Wistar rats (Table 2), all DNA samples isolated from the livers were radiolabelled. The specific activity of three of the four DNA samples remained constant upon repetitive purification. The DNA isolated from animal no. 1 also showed constant specific activity after a third round of DNA purification. This is a strong indication that non-covalently bound radiolabelled compounds have been completely removed. The control incubation of an unlabelled chromatin pellet with a radiolabelled supernatant (column 3 in Tables 1 and 2) led to negligible DNA radioactivity. It is therefore highly unlikely that the DNA radioactivity in the treated animal was due to a work-up artefact. The specific activity of chromatin protein was 6–17 times higher than that of DNA. A contamination of the DNA with protein could not, therefore, be responsible for the DNA radioactivity (contamination <0.2%; Caviezel et al. 1984). Enzymatic degradation of the DNA to the deoxyribonucleotides and HPLC analysis revealed very little radioactivity eluting with the natural nucleotides (Fig. 1). Most radiolabel eluted in regions without detectable optical density, a characteristic of adducts derived from covalent binding of the radiolabelled test compound to DNA nucleotides.

For a comparison of the DNA-binding ability of trenbolone with other compounds, the specific DNA activity was divided by the radioactivity administered and the result converted to the units of the Covalent Binding Index (CBI). Values ranging from 8 to 17 were found (Tables 1 and 2). This range is found with weak genotoxic carcinogens (Lutz 1979).

In the view of this DNA damage observed in vivo, the generally negative results with the Ames test were rather intriguing. Four explanations could be put forward: 1) Rat liver S9 is not an appropriate activation system. 2) TBOH or its reactive metabolites are not taken up by the bacteria. 3) TBOH or its reactive metabolites bind to DNA also in the bacteria but the adducts do not lead to observable mutations. 4) TBOH is mutagenic in *Salmonella* but cannot be tested adequately because of its bactericidal activity.

Ames test with TA100, TA98, and TA102

Three different strains of *Salmonella typhimurium* were incubated with different concentrations of trenbolone in the presence or absence of various concentrations of rat liver 9000 g supernatant (S9). Bactericidal effects were already noted at 111 μg TBOH per plate with TA100 without S9. At this dose level, the living cell count was reduced to less than 50%. At 333 μg per plate, growth was inhibited in all strains. An evaluation of a potential mutagenicity of TBOH was therefore possible only in a very small dose range. With TA100 and TA102, the number of revertants was slightly increased in the absence of S9 (Table 3). The addition of rat liver S9 at 5% inhibited this mutagenicity. The observable activation-independent effect was so small, however, that additional data were required to investigate the mutagenic potential of TBOH.

Dose response with TA100: mutagenic potency

In order to investigate whether the increased number of revertants was a significant and reproducible result, the dose range of 0–50 μg TBOH per plate was investigated more carefully. The total number of revertants in *Salmonella* TA100 using the preincubation method (20 min, 37° C) with 0, 5, 10, 20, 30, 40, and 50 μg TBOH per plate was 153, 175, 161, 174, 187, 199, and 191 revertants, respectively (means of triplicate plates; relative standard deviation within 10%). The linear regression for this dose-response relationship followed the equation

$$\text{Number of revertants} = 160 + 0.78 \times \mu\text{g TBOH per plate}$$

The slope of 0.78 revertants per μg was highly significantly different from zero ($p = 0.008$; t-test).

In a second set of experiments, repetitive determinations of the mutagenicity at 30 and 60 μg per plate (47 and 94 μg/ml preincubation mixture) were performed. Using 13–15 replicate incubations at each dose level, the number of revertants for 0, 30 and 60 μg TBOH per plate was 191 ± 4 (± standard error; $n = 15$), 202 ± 6 ($n = 13$), and 234 ± 6 ($n = 15$), respectively. The slope of the linear regression was 0.71 revertants per μg TBOH which was,

Table 3. Investigation of the mutagenic activity of trenbolone in the Ames *Salmonella*/rat liver microsome assay. Test with preincubation (20 min, 37°C)

	Salmonella strain/concentration of S9								
	TA100 S9 (%; v/v)			TA98 S9 (%; v/v)			TA102 S9 (%; v/v)		
	0	5	30	0	5	30	0	5	30
Dose [μg per plate]	Total number of revertants (means of three replicate plates[a])								
0 (control)	187	206	186	38	36	42	261	468	532
12	191	193	185	35	41	34	294	469	547
37	211	201	205	30	40	31	278	445	467
111	213[b]	207	215	30	40	27	249	505	545
333	118[b]	30[b]	188[b]	17[b]	17[b]	16[b]	121	176[b]	560
1000	109[b]	131[b]	155[b]	15[b]	13[b]	15[b]	116[b]	131[b]	349[b]

[a] The relative standard deviation was approximately 10% of the total number of revertants
[b] Bactericidal effects (inhibition of background growth)

Table 4. Covalent binding of [^{3}H]trenbolone to DNA of *Salmonella* TA100 and mutagenicity after incubation with 80 μg/ml for 30 min at 37°C

Experiment no.	1	2	Controls	
			Unlab. TBOH	No TBOH
DNA binding				
Radioactivity incubated (mCi)	1.50	1.54	0	
DNA specific activity (dpm/mg)	110	108	= background	
TBOH-DNA binding (pmol/mg DNA)	3.1	3.0		
Mutagenicity				
Total number of revertants	216	223	209	183
Induced number of revertants	33	40	26	–
Living cell count per ml ($\times 10^9$)	2.86	3.06	3.25	3.32

Positive control for mutagenicity: 1.25 μg NaN_3/plate: 1032 induced revertants

again, highly significantly different from zero ($p<0.001$) and in excellent agreement with the slope of the dose-response curve discussed above. In molar units, the mutagenic potency under the given conditions therefore was 0.2 TA100 revertants per nmol TBOH, and there can be little doubt that the increased number of revertants is due to the presence of TBOH in the bacterial suspension.

The indication of a weak activation-independent mutagenicity prompted us to investigate the DNA-binding activity in vitro.

DNA binding in vitro

When calf thymus DNA was incubated with tritiated TBOH in the presence or absence of active or inactive rat liver S9, DNA was always radiolabelled. Repetitive purification of the DNA did not reduce the specific activity in any situation, indicating that the radiolabel was covalently bound. Highest DNA radioactivities were determined in the *absence* of S9 (471 ± 23 dpm/mg DNA). It is concluded that TBOH can covalently bind to DNA without enzymatic activation. In the view of the fact that 25 μCi was used per incubation, the yield of DNA adducts must have been extremely low, indicating that the reaction proceeds very slowly. This is supported by additional results showing that the DNA binding is proportional to the period of incubation (data not shown).

Addition of S9 *without cofactors* led to an almost 20-fold reduction of the specific DNA radioactivity (25 ± 11 dpm/mg). Preincubation of the mixture with *unlabelled* TBOH before the addition of the radiolabelled TBOH did not further reduce the radioactivity bound to DNA (23 ± 2 dpm/mg). The presence of *active* S9 only partially inhibited DNA binding (280 ± 38 dpm/mg).

DNA binding in Salmonella

It remained to be shown whether DNA binding would also occur in bacteria. Large batches of TA100 bacteria were incubated for 30 min at 37° C with tritiated TBOH at 80 μg/ml, i.e., in a concentration low enough to avoid bactericidal effects. The living cell count was not reduced in the treated groups. After the removal of aliquots for the determination of the living cell count and the number of revertants, DNA was isolated. DNA was clearly radiolabelled (Table 4). Enzymatic degradation of the DNA to the deoxyribonucleotides revealed that more than 90% of the radioactivity eluted without optical density in the region of the more lipophilic nucleotide-[^{3}H]TBOH-adducts (Fig. 1).

The number of induced revertants per dose was slightly lower than in the standard preincubation test. This has been observed before with emodin (Bösch et al. 1987) and is probably due to the centrifugation and washing steps after the incubation period.

All data reported in this communication indicate that TBOH has the potential to bind covalently to DNA without metabolic activation. This activity is in agreement with the increase in the number of TA100 revertants observed in the first set of experiments shown in Table 3.

Discussion

DNA binding in vitro

The DNA-binding experiments in vitro indicate that trenbolone reacts very slowly with DNA without metabolic activation. The nature of this activation-independent reaction of trenbolone with DNA is unknown. The most plausible explanation might be an addition reaction of nucleophilic centers in DNA to the polyunsaturated ketone moiety.

The presence of protein (inactive S9) reduced DNA binding about 20-fold. This is probably due to non-specific associations of TBOH to protein, thereby reducing the concentration of free TBOH available in solution for reaction with DNA. Active S9 did not reduce DNA binding as effectively as inactive S9. It cannot, therefore, be excluded that activation-dependent DNA binding, possibly via epoxidation, might also take place as a minor pathway in vitro.

DNA binding in Salmonella and mutagenicity

The data indicate that TBOH can penetrate the bacterial membrane and react with DNA. The resulting adduct is a promutagenic lesion, as the number of revertants is also increased. The toxicity to the bacteria prevents the use of doses higher than about 60 μg TBOH per plate. It is therefore not possible with TBOH to induce a doubling of the spontaneous number of revertants, a criterion which is often used to define a mutagenic response. This definition is, however, not generally accepted (McCann et al. 1984), and it is possible that TBOH is only one example of a number of compounds where severe bactericidal activity limits the testable dose range.

For a comparison of the mutagenic potency of TBOH with activation-independent standard mutagens, only experiments using a preincubation can be evaluated. A 1-h preincubation of TA100 with N-methyl-N'-nitro-N-nitrosoguanidine at 37° C resulted in 2000 revertants/nmol. Methyl methanesulfonate (MMS) induced about 0.1 rev./nmol (Kerklaan et al. 1985), i.e., was not more potent than TBOH (0.2 rev./nmol after a 20-min preincubation period). Since MMS can be tested at higher dose levels than TBOH without interfering toxicity, its mutagenicity is generally accepted.

The negative literature data on the Ames test with TBOH are not in disagreement with the present findings if the published results are closely examined. Ingerowski et al. (1981) used a lowest dose of 1 mg per plate, i.e. a highly toxic concentration for the bacteria. Schiffmann et al. (1985) did find an increase of the number of revertants but they considered the data as negative, probably because of the "twice background" rule. Therefore, we rather interpret existing data in a different manner.

DNA binding in rat liver

The CBI values around 10, as determined for TBOH in this study, are close to the values published by Barraud et al. (1984). When using the natural hormones 17β-estradiol and testosterone, these authors found similar DNA radioactivities, while we were unable to detect estradiol-DNA adducts at a limit of detection of CBI = 0.1 (Caviezel et al. 1984). With estradiol (but not with trenbolone) we noticed an extremely high level of protein radioactivity so that minute contamination of the DNA with protein could simulate DNA adducts. As Barraud and coworkers did not analyse the DNA for adducts, it cannot be excluded that in their samples the DNA radioactivity of the estradiol-treated rats was due to protein contamination while with trenbolone the radioactivity was indeed due to DNA adducts.

Tentative approach for a genotoxicity risk estimate

The following estimation is based exclusively on the DNA binding aspect. Chromosomal damage and hormonal effects will require a separate assessment.

While Ames test data can only be used for screening purposes, the DNA-binding data in rat liver might be useful for a genotoxicity risk evaluation in mammals (Lutz 1979). A relatively good correlation exists between carcinogenic potency and the CBI for liver DNA for the mutagenic carcinogens (Lutz 1986). Compounds exhibiting a CBI of over 1000 are potent carcinogens, those with a CBI on the order of 100 are moderately strong carcinogens, and compounds with a CBI below 10 are weakly carcinogenic. Tumor induction in a long-term bioassay by chemicals with a CBI lower than 1 is unlikely to be due to genotoxicity mediated solely by DNA binding. Under the assumptions that the data obtained for rats can be extrapolated to man, it is tempting to compare the DNA damage of a single trenbolone exposure to the risk run by the intake of aflatoxin B_1 (AFB_1; CBI for rat liver DNA = 10000) or dimethylnitrosamine (DMNA; CBI = 6000). An average daily burden is on the order of about 100 ng DMNA (about 1 nmol) per day (Preussmann and Eisenbrand 1984) and 1–10 ng (about 0.03 nmol) aflatoxin B_1 per day. The level of DNA damage from a single dose can be estimated by multiplying the Covalent Binding Index, CBI, with the appropriate single dose. The corresponding DNA adduct risk will therefore be at $10000 \times 0.03 = 300$ and $6000 \times 1 = 6000$ units for AFB_1 and DMNA, respectively. For the use of trenbolone as an anabolic drug in humans (Parabolan®: 50 mg ampoules for intramuscular injection), the corresponding value of $10 \times 200000 = 2000000$ (CBI × nmol) represents an unacceptably high genotoxic risk.

In contrast, for trenbolone residues in veal meat (50 ng/kg taken for this estimation), a daily intake of 100 g meat would result in a daily burden of 5 ng (about 0.02 nmol) TBOH. Multiplied by the DNA-binding potency of CBI = 10, the risk would amount to 0.2 DNA adduct risk units. Since this is many orders of magnitude below the risk run from essentially unavoidable exposure to one mycotoxin and one nitroso compound, we consider the risk from trenbolone residues in meat to be a low priority genotoxicity problem.

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