extracts [3]. One possible mechanism for such binding would be the non-enzymatic decarboxylation of DFMO by pyridoxal phosphate which would generate a reactive species able to react with cellular nucleophiles or with water. This could also provide the means by which the two metabolites of DFMO observed in Fig. 2 were produced. It should also be noted that only about 50% of the total radioactivity present in the SV-3T3 cells after 12 hr of exposure to labeled DFMO was present as the unchanged compound. It is most unlikely that all of the remainder was attached to ornithine decarboxylase since calculation of the number of ornithine decarboxylase molecules per cell indicates that this enzyme is an extremely small fraction of the cellular protein [3]. Therefore, some other degradation of DFMO must take place, possibly non-enzymatically as discussed above. This may represent a very minor proportion of the drug *in vivo* because it is excreted very rapidly [15], but it could have significance in experiments with cultured cells exposed to high levels of DFMO for prolonged periods of time.

In summary, DFMO entered the cell by a passive diffusion mechanism, and its uptake was not prevented by polyamines or basic amino acids. Therefore, the reversal of the effect of DFMO by polyamines was not due to interference with uptake.

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Metabolism of tamoxifen by isolated rat hepatocytes: anti-estrogenic activity of tamoxifen *N*-oxide

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Tamoxifen [*trans*-1-(*p*-*β*-dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene, compound **1**] is used in the palliative treatment of advanced breast cancer [1] and is thought to act by competing with estradiol (E_2) for the cytoplasmic receptor (ER).

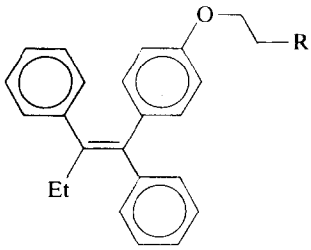
There has been considerable interest in the role(s) of the metabolites of tamoxifen in the expression of biological activity [2, 3]. Fromson *et al.* concluded that 4-hydroxytamoxifen (**2**) was the major serum metabolite in laboratory animals [4] and female patients [5], and that elimination was largely through biliary excretion of conjugates of hydroxylated derivatives. A more recent study [6] also concluded that the major extractable metabolites present in plasma and liver after injection of [^{3}H]tamoxifen into rats were 4-hydroxytamoxifen and other hydroxylated derivatives. In marked contrast, Adam *et al.* [7, 8] reported that the major metabolite of tamoxifen in human serum was the *N*-desmethyl derivative **3** and in the plasma of

female patients undergoing tamoxifen therapy of advanced breast cancer only small amounts of the 4-hydroxy metabolite **2** could be detected [9].

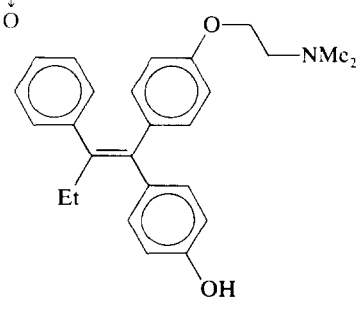
In studying the balance between hydroxylation (→**2**) and *N*-demethylation (→**3**) we found that when tamoxifen (**1**) was incubated with the rat liver microsomes [10] *N*-demethylation greatly preponderated over aromatic hydroxylation and a substantial amount of a hitherto undetected metabolite, tamoxifen *N*-oxide (**4**) was formed. We now report on the metabolism of tamoxifen by freshly isolated rat hepatocytes. This *in vitro* experimental system should give results which resemble more closely liver metabolism *in vivo* [11, 12].

In addition to unchanged tamoxifen (peak 4, Fig. 1B) ether extracts after hepatocyte metabolism contained metabolites (peaks 1, 5 and 3) with retention times on high performance liquid chromatography (HPLC) corresponding to those of authentic 4-hydroxytamoxifen (**2**), *N*-desmethyltamoxifen (**3**) and tamoxifen *N*-oxide (**4**) (Fig. 1C) and their identities were confirmed by mass spectrometry. Application of reverse-phase thin-layer chromatography (RPTLC) in complementary experiments (see Mass Spectrometry) indicated that there could be an additional hydroxytamoxifen metabolite at the position of peak 2.

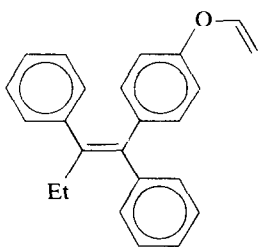
The major hepatocyte metabolite (Table 1) was *N*-desmethyltamoxifen (**3**) as was the case with microsomal metabolism [10], but the amount of 4-hydroxytamoxifen (**2**) formed by hepatocytes was proportionately higher than by microsomes and at the expense of **3** (Table 1). Similar amounts of tamoxifen *N*-oxide (**4**) were formed by microsomes and hepatocytes. Treatment of the hepatocyte incubate with β-glucuronidase or sulfatase (Table 1) did not increase the proportion of any of the ether-extractable metabolites. Since the same hepatocyte preparations formed glucuronide and sulfate conjugates when incubated with harmine [12] the results indicated that 4-hydroxytamoxifen was not conjugated with glucuronic acid or sulfate in isolated hepatocytes.



1 R = NMe_2 (tamoxifen)
3 R = NHMe (*N*-desmethyltamoxifen)
4 R = NMe_2 (tamoxifen *N*-oxide)
↓
O



2 (4-hydroxytamoxifen)



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The formation of substantial amounts of 4-hydroxytamoxifen (**2**) by hepatocytes supports the contention [2] that it may contribute to the anti-tumour activity of tamoxifen in the rat since it is a stronger competitor of E_2 binding to ER and a more potent anti-estrogen than tamoxifen [2, 13], and has anti-tumour activity [2, 14, 15].

In seeking to assess the contribution of tamoxifen *N*-oxide (**4**) to the anti-tumour activity of tamoxifen (**1**) we have investigated its anti-estrogenic activity. The relative binding affinity (*RBA*) of tamoxifen *N*-oxide for rat mammary tumour ER was low (0.2–0.5% of that for E_2) and comparable to that (0.2–0.5%) for tamoxifen. Both estrogens and anti-estrogens bind to ER but anti-estrogenic activity can be assessed on the basis of inhibition of the growth *in vitro* of ER-positive and ER-negative cell lines derived from human breast cancers [16, 17]. Neither tamoxifen nor tamoxifen *N*-oxide inhibited the growth of the ER-negative cell line Evsa-T indicating that they had no general cytostatic properties. The results in Table 2 show that the growth of an ER-positive cell line MCF-7 was inhibited to the same extent by tamoxifen and tamoxifen *N*-oxide and that this effect was reversed by the addition of E_2 to the culture medium. In a separate experiment (Table 3), and as would be expected from the *RBA* values, *N*-desmethyltamoxifen (**3**, *RBA* ~ 0.5%) showed growth inhibitory activity similar to that of tamoxifen and 4-hydroxytamoxifen (**2**, *RBA* ~ 100%, cf. [18]) was a much more potent inhibitor.

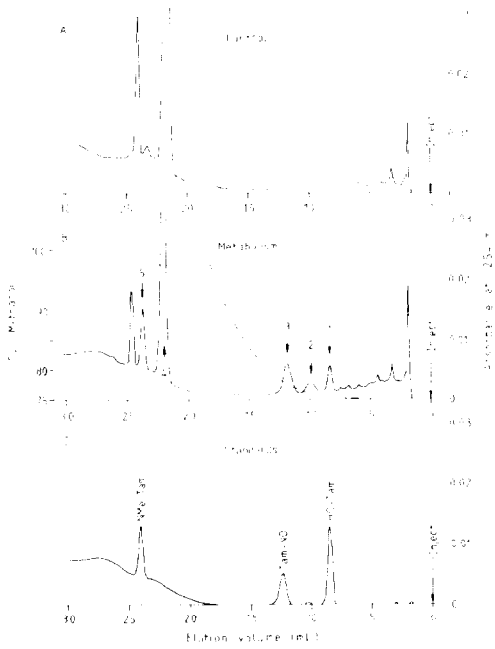

Fig. 1. Metabolism of tamoxifen by isolated hepatocytes prepared from phenobarbital-treated rats. Hepatocytes (1–3 × 10^6 cells/ml, buffer B) were incubated for 60 min with 100 μg/ml tamoxifen citrate. HPLC traces of ether extracts of incubates adjusted to pH 9 after removal of protein: A control, B after metabolism, C synthetic standards. HPLC conditions as in Ref. 10, except that a solvent gradient methanol–water–diethylamine (75:25:0.1 → 97.5:2.5:0.1, at 1 ml/min) was used.

Table 1. Proportions* of tamoxifen metabolites present in ether extracts of hepatocyte incubations

	Percentage of total ether-extracted tamoxifen and metabolites†			
	60 min incubate	Treated incubate‡		
		buffer	β-glucuronidase	sulfatase
Tamoxifen (**1**)	76.3 ± 4.3	77.3 ± 4.7	76.0 ± 5.5	73.7 ± 5.6
4-Hydroxytamoxifen (**2**)	5.2 ± 0.9	5.0 ± 1.0	4.7 ± 1.2	5.0 ± 1.5
N-Desmethyltamoxifen (**3**)	11.8 ± 2.1	11.7 ± 2.3	13.0 ± 2.5	13.3 ± 2.2
Tamoxifen *N*-oxide (**4**)	6.7 ± 1.5	6.0 ± 1.5	6.3 ± 1.9	7.7 ± 2.2

* The proportions for the microsomal metabolism [10] were, **1** 72%, **2** 1.5%, **3** 20.5%, **4** 6%.

† Mean values ± S.E. determined from peak areas in HPLC analyses of three separate hepatocyte preparations.

‡ Separate samples of incubate from each hepatocyte preparation were treated with β-glucuronidase, or sulfatase, or buffer alone.

Table 2. Reversibility* of the inhibitory effect of tamoxifen (**1**) and tamoxifen *N*-oxide (**4**) on the growth of MCF-7 cells by estradiol (E_2)

		DNA (μg)	
	Molarity	Compound alone	Compound + 10^{-8} M E_2
None		12.9 ± 2.2 (100)†	
E_2	10^{-8}	14.9 ± 0.8 (115)	
Tamoxifen (**1**)	10^{-6}	4.2 ± 0.7 (33)	14.8 ± 1.5 (115)
	10^{-7}	9.2 ± 1.5 (71)	14.4 ± 1.1 (112)
	10^{-8}	12.3 ± 1.6 (95)	13.4 ± 1.7 (104)
Tamoxifen	10^{-6}	6.3 ± 0.3 (49)	14.0 ± 1.3 (108)
N-oxide (**4**)	10^{-7}	11.2 ± 1.0 (87)	15.2 ± 1.0 (118)
	10^{-8}	13.0 ± 1.3 (101)	13.8 ± 0.4 (107)

* Variance analysis showed that tamoxifen and its *N*-oxide significantly reduced MCF-7 cell growth ($P < 0.01$). The Newman–Keuls test [22] showed that tamoxifen was active at 10^{-7} and 10^{-6} M and the *N*-oxide at 10^{-6} M only ($P < 0.01$). These effects were suppressed by E_2 (interaction $P < 0.01$).

† Figures in brackets are the percentages of the control value.

Table 3. Reversibility* of the inhibitory effect of tamoxifen (**1**), 4-hydroxytamoxifen (**2**) and *N*-desmethyltamoxifen (**3**) on the growth of MCF-7 cells by estradiol (E_2)

		DNA (μg)	
	Molarity	Compound alone	Compound + 10^{-8} M E_2
None		9.4 ± 0.5 (100)†	
Tamoxifen (**1**)	10^{-6}	5.6 ± 1.3 (60)	
	10^{-7}	8.0 ± 0.8 (85)	
4-Hydroxytamoxifen (**2**)	10^{-6}	0.7 ± 0.5 (7)	0.8 ± 0.7 (8)
	10^{-7}	2.6 ± 1.6 (28)	4.8 ± 0.4 (51)‡
	10^{-8}	4.7 ± 0.6 (50)	8.5 ± 1.4 (90)**
N-Desmethyltamoxifen (**3**)	10^{-6}	6.6 ± 1.1 (70)	9.1 ± 1.6 (97)‡
	10^{-7}	9.2 ± 0.9 (98)	7.9 ± 0.7 (84)
	10^{-8}	7.6 ± 0.4 (81)	8.4 ± 0.9 (89)

* Variance analysis showed that tamoxifen and the metabolites **2** and **3** significantly reduced MCF-7 cell growth ($P < 0.01$). E_2 reduced significantly the effects of the metabolites (interaction ‡$P < 0.05$, **$P < 0.01$). The Newman–Keuls test [22] showed that **1** and **3** were active at 10^{-6} M and **2** was active at all the concentrations tested.

† Figures in brackets are the percentages of the control value.

Gas chromatography–mass spectrometry (GC–MS; 3% OV-1 on Gas Chrom Q at 240° with helium as carrier gas; EI mode at 70 eV) of ether extracts of plasma from two patients undergoing chronic treatment with tamoxifen afforded evidence that tamoxifen *N*-oxide (**4**) was present since the unique vinylether **5**, formed [10] from **4** *via* Cope elimination, was detected by selective ion monitoring at *m/z* 326 (M^+ for **5**). Quantification of tamoxifen *N*-oxide in plasma should allow an assessment of its contribution to the anti-tumour activity of tamoxifen. Studies along these lines are in progress.

Metabolism

Hepatocytes (70–80% viable, 4–9 × 10^6 cells/g tissue) were obtained [12] from phenobarbital pretreated male Wistar albino rats. Incubations (1–3 × 10^6 cells/ml) were carried out under O_2:CO_2 (95:5) in buffer B (5 ml; 118 mM NaCl, 4.8 mM KCl, 0.96 mM KH_2PO_4, 1.2 mM $MgSO_4$, 23.8 mM $NaHCO_3$ and 2.5 mM $CaCl_2$, pH 7.4) for 60 min at 37°. After a preincubation period of 30 min tamoxifen citrate (500 μg) was added as a solution in *N,N*-dimethylformamide (10 μl). Hepatocyte viability was retained during the subsequent incubation for 60 min. Controls involved adding tamoxifen citrate after incubation.

The incubations were terminated by the addition of AR ethanol (4 vol.). Denatured protein was removed by centrifugation at 1200 g_{av} for 5 min at room temperature and most of the ethanol from the supernatant at 30° under vacuum. The concentrate was adjusted to pH 9 by the addition of 1 M NaOH and then extracted with diethyl ether [19] (2 × 4 vol.). The combined extracts were dried (Na_2SO_4), concentrated, and a solution of the residue in AR methanol was subjected to reverse-phase HPLC as described previously [10] except that the column was eluted at 1 ml/min using an exponential solvent gradient methanol–water–diethylamine 75:25:0.1 → 97.5:2.5:0.1 (Waters Model 660 solvent programmer system, gradient programme 10 in 18 min) (Fig. 1).

Some concentrates were incubated [12] with β-glucuronidase or aryl sulfatase and the HPLC profiles of the subsequent ether extracts were compared with those from incubations performed in the absence of enzyme.

Mass spectrometry

Samples were concentrated from methanol solutions onto the direct insertion probe of a VG 7070H mass spectrometer. Mass spectra were determined in the CI mode (ionizing voltage 100 eV, trap current 500 μA, ion source temperature 150°) using isobutane or ammonia as reagent gas, or in the EI mode (ionizing voltage 70 eV, trap current 100 μA).

The major metabolite (peak 5, Fig. 1B) was isolated by HPLC and characterized as **3** by its EI mass spectrum [10]. The less abundant metabolites (peaks 1–3, Fig. 1B) were more conveniently isolated in the quantities required for mass spectrometry by RPTLC (Whatman $KC_{18}F$, 20 × 10 cm plates) using methanol–water–diethylamine (80:20:1). The R_f values for **2** (0.20), **4** (0.13) and **3** (0.06, unresolved from **1**) accorded with their relative retention times in HPLC. The component with R_f 0.17 probably corresponded to peak 2 on the HPLC trace (Fig. 1B) and its EI mass spectrum contained a molecular ion at *m/z* 387 (M^+, 10%) and major fragments at *m/z* 72 ($[CH_2CH_2NMe_2]^+$, 25%) and 58 ($[CH_2NMe_2]^+$, 100%), suggesting that it was a monohydrotamoxifen isomeric with **2**. The component corresponding in R_f to the *N*-oxide **4** was readily characterized by its CI mass spectrum using ammonia as the reagent gas [*m/z* 388 (MH^+ for **4**, 28%), 372 (MH^+ for **1**, 86%), 358 (MH^+ for **3**, 22%), 72 (36%) and 58 (100%)]. Authentic **4** gave *m/z* 388 (12%), 372 (91%), 358 (29%), 327 (MH^+ for the Cope elimination product **5**, 33%), 72 (39%) and 58 (100%). The variations between the spectra of **4** and the metabolite are due to the variable extents of thermal decomposition variously to **1**, **3** and **5**.

Relative binding affinities [20]

Rat mammary tumour ER, partially purified by ammonium sulfate precipitation, was incubated with 6 × 10^{-10} M [^{3}H]-(E_2) (saturating amount) in the presence of increasing amounts of tamoxifen or metabolite or E_2 (control). Unbound compounds were then removed using dextran-coated charcoal and the ER-bound [^{3}H]-E_2 was measured. The relative concentrations of tamoxifen (or metabolite) and E_2 required to achieve 50% inhibition of [^{3}H]-E_2 binding is the relative binding affinity.

Cell culture assays

The human breast cancer cells MCF-7 (ER-positive) and Evsa-T (ER-negative) were grown in Falcon plastic flasks (75 cm^2) containing Earle's minimal essential medium supplemented with L-glutamine (0.6 mg/ml), gentamicin (40 μg/ml), penicillin (100 μg/ml), streptomycin (100 μg/ml) and 10% fetal calf serum. At confluency the cells were removed by trypsinisation (0.05% trypsin, 0.025% EDTA), suspended (50–200 × 10^3 cells/ml) in the growth medium supplemented with charcoal-stripped fetal calf serum (0.5% charcoal, 0.005% dextran in 1.5 ml of medium/ml serum; incubation overnight at 4°). Cells were then plated in 35 mm Petri dishes containing the same culture medium and cultured at 37° in humidified air–CO_2 (95:5). After 24 hr, a solution of tamoxifen or metabolite in medium was added (0.1% final concentration of ethanol) and, after 48 hr, the medium was replaced by fresh tamoxifen (or metabolite)-containing medium. After a further 72 hr the cells in each dish were harvested, washed twice with Earle's base (2 ml) and suspended in trypsin–EDTA (1.5 ml). The DNA was precipitated with 0.5 M perchloric acid and quantified by the diphenylamine method [21]. Quadruplicate cultures were used throughout.

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Diuretic action of BTS 39542 (dihydrophthalazin-1-ylacetic acid) is consistent with inhibition of Na + K + Cl cotransport

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BTS 39542 (dihydrophthalazin-1-ylacetic acid) is a potent diuretic agent in rats, rabbits and dogs [1] which is structurally unrelated to other high-efficacy diuretics [1]. The exact renal tubular locus and mechanism of action of BTS 39542 are, however, unknown. Simple pharmacological model systems of diuretic action such as anuran skin [2] or human red cells [3] have been identified whose major ion transport systems reflect the functionally important ion transport of microdissected, microperfused thick ascending limb and distal renal tubules [3, 4]. In the present paper we have attempted to characterise the molecular action of BTS 39542 by comparing its effect with loop diuretics such as furosemide upon the passive Na + K + Cl contransport system in cultured human carcinoma cells (HeLa), cultured canine renal epithelial cells (MDCK) and human red blood cells.

Materials and methods

Human red cells were obtained by venepuncture from healthy volunteers. After washing four times in a medium containing 150 mM NaCl, 10 mM Tris–HCl, 6.0 mM KCl, 10 mM glucose, pH 7.4 and removal of the buffy coat, red cells were used for ^{86}Rb influx measurements at approximately 70% haematocrit.

HeLa cells were grown in Eagle's Basal Medium supplemented with 10% new-born calf serum [5]. For experimental purposes, monolayers of cells were grown to confluency in 60 mm Sterilin plastic petri dishes (approximately 2×10^6 cells/plate).

MDCK cells form monolayers possessing epithelial structure and capable of transmonolayer ion transport [6]. Cultures of MDCK cells were obtained at 60 serial passages from Flow Laboratories (Irvine, U.K.) and maintained in serial culture in Roux flasks in Minimum Essential Eagles (M.E.M.E.) supplemented with non-essential amino acids, (2 mM glutamine, 1 unit/cm^3 gentamycin antibiotic and 10% v/v foetal bovine serum (Flow)) at 37° in an air–5% CO_2 atmosphere. For experiments, cells were grown in 60 mM Sterilin plastic petri dishes to form subconfluent cell monolayers.

K influx measurements were made using ^{86}Rb as a tracer in all experiments [5, 7, 8]. Influx determinations (30 min) were made in human red cells at 37° by a slight modification of the method of Lauf and Joiner [9] using centrifugation through dibutylphthalate to separate cells from the radioactive influx solution. For cultured cells, the ^{86}Rb influx into cell monolayers was measured at 37° during a 10 min (HeLa) or 5 min (MDCK) incubation in Krebs' solution containing 137 mM NaCl; 5.4 mM KCl; 2.8 mM $CaCl_2$; 1.2 mM $MgSO_4 \cdot 7H_2O$; 0.3 mM NaH_2PO_4; 0.3 mM KH_2PO_4; 12 mM HCl; 14 mM Tris base and 11 mM glucose supplemented with 1.0% v/v new-born calf serum, pH 7.4 and 0.2 μCi/ml ^{86}Rb. At the end of the influx period, the monolayer was rapidly rinsed four times with ice-cold Krebs' solution to remove extracellular isotope and then trypsinised to form a single cell suspension. After trypsinisation, the cell number and mean cell volume of each experimental plate was determined on a Coulter Counter (Model ZF) fitted with a Channelyser attachment (Model C1000). The ^{86}Rb content of all samples was measured in a liquid scintillation spectrophotometer (Packard, model 3255) by the Cerenkov method.

The effect of chronic BTS 39542 treatment upon Na–K pump function was determined in HeLa cells by measuring specific [^{3}H]ouabain binding to determine Na^+ pump number [5] from a media containing 2×10^{-7} M ouabain in K-free Krebs solution or one containing 15 mM KCl for 20 min [5]. At the end of the incubation period the cells were rinsed four times with ice-cold Krebs solution and the cell number, mean cell volume determined, as well as the ^3H content. Intracellular Na + K contents were also measured by flame photometry after rinsing cell layers four times with ice-cold choline chloride followed by a 3-hr incubation in deionised water. Cell number and cell volume were determined on parallel samples as above.