is not a white, chalk-like powder, but rather a clear, viscous, sugar-water gel. This characteristic explains the formation of the third layer in the high-concentration sugar samples.

The increase in volume of the coacervate phase in the high-concentration theophylline samples (Table I) indicates a general increase in the structuring of this phase. In this case, the additives also act as general structure makers causing an increased nonpolarity of the equilibrium phase. This hypothesis is consistent with the decrease in electrical conductivity of these samples (Fig. 4), indicating a more structured system.

If the coacervate state is a model for human cytoplasm, then clinical parallels should be found in man. The decrease in the volume of the coacervate layer in the high-concentration sugar samples may be related to the known delayed healing of wounds in diabetics with low insulin and therefore high blood sugar levels. Ecanow *et al.* (7) have hypothesized that this delay in healing is partially caused by a rupturing of the cells in the wounded area from the high sugar concentrations acting as a structure breaker of these cells. This hypothesis is consistent with the decrease in the volume of the coacervate in the high-concentration sugar samples (acting as a structure breaker in the highly structured coacervate phase) without noticeable effect on the loosely bound equilibrium phase.

For each additive, the conductivity of the coacervate phase is reduced compared with the equilibrium phase (Figs. 2–5). This decreased conductivity of coacervates is consistent with both the hypothesis of Ecanow *et al.* (7) that conditions like malignancy occur in a nonpolar aqueous matrix (coacervate) and the findings of Lowenstein and Kanno (8) that malignant cells have a reduced action potential. Also, a low conductivity of the sugars is shown which may be related to the known depressant effect of sugars on the central nervous system, culminating in diabetic coma with high glucose concentrations (4). The effect of high concentrations of sucrose on membrane interface disorientation (structure breaking) can account for the known low absorption rate of sucrose in the intestine, compared to glucose (9).

The conductivity of caffeine was highest, followed by theobromine and theophylline (Fig. 2). These data may have a relationship to the decurarizing effects of these three drugs with caffeine showing the greatest effect, followed by theobromine and theophylline (10). With higher concentrations (Fig. 4) the conductivity was generally highest in theobromine, then caffeine and theophylline. These data may then be related to the effects of these drugs on urine production with theobromine showing the greatest effect followed by caffeine and theophylline, as reported by Scott *et al.* (11).

The increase over control values of the caffeine (Figs. 2, 4) followed by a reversal of a decrease with high concentrations (Fig. 4) finds a clinical parallel in the known increased cardiac contraction effect of caffeine at concentrations of 0.25–1.50 mM and a decreased contraction effect with concentrations >2.0 mM as reported by Guboreff and Sleator (12).

This discussion has shown a number of clinical parallels in the conductivity changes from the drug and sugar series and provides evidence in favor of the usefulness of a coacervate system as a model for human cytoplasm.

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Urinary Metabolites of the Antiprotozoal Agent *cis*-3a,4,5,6,7,7a-Hexahydro-3-(1-methyl-5-nitro-1*H*-imidazol-2-yl)-1,2-benzisoxazole in the Rat

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Abstract □ ^{1}H-NMR and MS were employed to identify 13 rat urinary metabolites of ^{14}C-labeled *cis*-3a,4,5,6,7,7a-hexahydro-3-(1-methyl-5-nitro-1*H*-imidazol-2-yl)-1,2-benzisoxazole (MK-0436). The major free (unconjugated) metabolite was *cis*-3a,4,5,6,7,7a-hexahydro-3-carboxamido-1,2-benzisoxazole; it was also the second most abundant metabolite released during hydrolysis of the conjugated fraction. All other identified metabolites were hydroxylated analogues substituted at C(4)—C(7a) of the cyclohexane ring. The 4-equatorial,5-axial,7a-triol was the second most abundant metabolite excreted in an unconjugated form. Four monohydroxy (5-axial, 6-axial, 6-equatorial, 7-equatorial) metabolites of the drug were identified; they were found in the conjugated fraction only and were released by hydrolysis. The 5-axial hydroxy compound is the major conjugated metabolite and is overall the most abundant of all the metabolites. Six dihydroxy metabolites were identified: one was found exclusively in the free state, three as conjugates only (including the 7-axial,7a-diol, which is the major dihydroxy species), and two both free and conjugated. A second triol was found both free and conjugated.

Keyphrases □ *cis*-3a,4,5,6,7,7a-Hexahydro-3-(1-methyl-5-nitro-1*H*-imidazol-2-yl)-1,2-benzisoxazole—urinary metabolites, rat, NMR, MS □ Hydroxylation—conjugation, *cis*-3a,4,5,6,7,7a-hexahydro-3-(1-methyl-5-nitro-1*H*-imidazol-2-yl)-1,2-benzisoxazole, NMR, MS

Elliott and co-workers (1) reported that in the rabbit, the urinary metabolites of cyclohexane were cyclohexanol and cyclohexane-*trans*-1,2-diol (as conjugates); Renwick and Williams (2) showed that cyclohexylamine was converted to mono- and dihydroxy metabolites (free and conjugates). Testa and Jenner (3) also discussed the metabolic hydroxylation of cyclohexyl ring systems. Substituted nitroimidazoles are known to undergo hydroxylation on their hydrocarbon moieties: metronidazole is hydroxylated on the 2-methyl group (4), ipronidazole on the 2-isopropyl group (5). The antipro-

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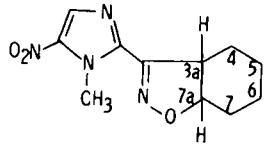
tozoal agent *cis*-3a,4,5,6,7,7a-hexahydro-3-(1-methyl-5-nitro-1*H*-imidazol-2-yl)-1,2-benzisoxazole (MK-0436, I) is metabolized by the dog to a series of alicyclic ring hydroxylation products (6, 7). The *in vitro* [rat liver post mitochondrial fraction (S9)] metabolism of I was reported recently (8). As in the dog study, metabolite formation in all cases involved biotransformations on C(4)—C(7a) of the cyclohexane ring. Two dihydroxy and seven monohydroxy metabolites were identified; the major *in vitro* metabolite is the 5-axial hydroxy analogue. The rat urinary metabolite study reported herein was executed to allow a direct comparison of the *in vitro* and *in vivo* metabolism of I, which possesses trypanocidal activity (1, 9-12) and is reported (6)[1,2] to be converted to bioactive metabolites.



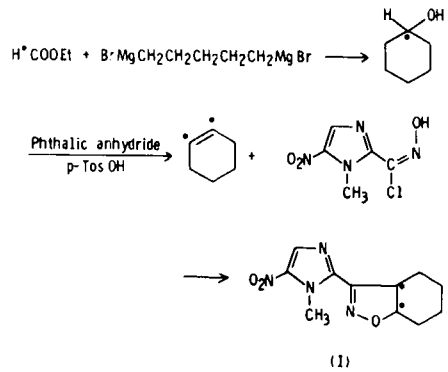
I

EXPERIMENTAL SECTION

Two Sprague-Dawley CD rats were pretreated with phenobarbital (0.1% in their drinking water) for 8 consecutive days. The animals were then dosed twice (16 h apart) at 500 mg/kg ip with I labeled with carbon-14 in the 3a- and 7a-positions (*vide infra*). Urine, collected until 24 h following the second dose of labeled I, was extracted with dichloromethane. The spent urine was incubated for 5.5 h at 37°C with β-glucuronidase and sulfatase[3] and then extracted with dichloromethane. The solvent was removed under reduced pressure to give two fractions containing the "free" and "liberated" metabolites of I (fractions 1 and 2, respectively). These fractions were subjected to TLC[4] with solvent[5] system A (toluene–ethyl acetate, 3:1) to yield partially purified metabolite fractions. Further purification was achieved by repeated use of this system and by TLC with two other solvent systems (system B: dichloromethane–methanol, 10:1; system C: trimethylpentane–2-propanol, 4:1). Metabolite zones were scraped from the plates, and metabolite fractions were isolated as described previously (8). The latter were assayed for radioactivity by use of the liquid scintillation counting technique[6].

Electron-impact[7] mass spectra (low resolution) of metabolite fractions were obtained using a 3.5-kV accelerating potential, 60-μA trap current, 70-eV ionizing energy, and 250°C source temperature. High-resolution (exact-mass)[8] mass spectrometric data were obtained using an 8-kV accelerating potential, 100-eV ionizing potential, and 200°C source temperature. Chemical-ionization[9] mass spectrometric data were obtained using methane as the reagent gas (1 Torr) and a 150-eV ionizing potential, 0.8-mA emission current, 1.8-kV electron multiplier, and 130°C source temperature. ^{1}H-NMR spectra[10] were obtained in deuterochloroform using tetramethylsilane as the internal standard. The chemical shifts and coupling patterns of H_{3a} and H_{7a} together with any new signals in the δ 3.5–5.5 ppm region (*i.e.*, diagnostic of *H*COH) were used to assign the location and stereochemistry of the hydroxyl groups of the transformation products.

Labeled I was prepared as shown in Scheme I. Ethyl [^{14}C]formate was treated with Grignard reagent prepared from 1,5-dibromopentane and magnesium to give [1-^{14}C]cyclohexanol, following the procedure of Fields *et al.* (13). Treatment of the [1-^{14}C]cyclohexanol with phthalic anhydride and *p*-toluenesulfonic acid (catalyst) then gave [1,2-^{14}C]cyclohexene (14). Reaction of the labeled cyclohexene with 1-methyl-5-nitro-1*H*-imidazol-2-hydroxyamoyl chloride (15) gave [3a,7a-^{14}C]I.



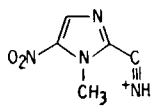
(I)

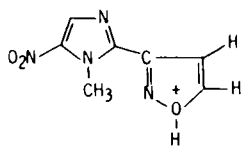
Scheme I—Synthesis outline; asterisks indicate position(s) of the ^{14}C-label.

RESULTS AND DISCUSSION

The pre- and posthydrolysis extractions resulted in the isolation of most (70%) of the total urinary radioactivity, with 70% of this associated with the posthydrolysis extraction fraction. Analytical TLC (solvent system A) of the pre- and posthydrolysis extraction fractions (1 and 2, respectively) demonstrated their multicomponent nature. The fractions were subjected to preparative-scale TLC with the same solvent system. Fraction 1 gave three major radioactive zones (R_f ~0.05, ~0.10, and ~0.27) with no evidence of more than a trace of the parent drug (R_f 0.70). The liberated metabolites (fraction 2) were subjected to preparative TLC as above; a broad, smeared zone (R_f ~0.05–0.10) and three more defined zones (R_f ~0.15, ~0.20, and ~0.28) were obtained, each of which contained drug-related species. These seven metabolite zones were further purified by TLC using solvent systems A–C to yield a total of 16 metabolites, 13 of which (accounting for >98% of the total of isolated metabolites) were identified (Table I). Five of the isolated, identified metabolites (or hydrolysis products thereof) were shown to be compounds previously identified as biotransformation products of I, and their designations (III and VI–IX, respectively) are as previously described (7, 8). The monohydroxy compounds (VI, VII, and IX) were found as rat *in vitro* metabolites (8), and III was found in dog urine (7). Compounds IX and VI were found to be the major and second most abundant rat *in vitro* metabolites, respectively. The dihydroxy metabolite VIII was found both as a rat *in vitro* metabolite (8) and a dog urinary metabolite (unconjugated) (7).

The electron-impact mass spectrometric behavior of I and several of its metabolites (those possessing hydroxyl groups on the cyclohexane ring) was discussed previously (6, 7). Not only do increases of 16 mass units in the molecular ions signify the number of oxygen atoms added to form hydroxyl groups, but the continuing presence of the fragment ions of *m/z* 153 and 195 appears to demonstrate that metabolism has occurred on the cyclohexane ring (6, 7). Thus for I and its mono-, di-, and trihydroxy metabolites, the molecular ions are *m/z* 250, 266, 282, and 298, respectively, and with these compounds the fragment ions *m/z* 153 and 195 are observed (see Table II for representative spectral data).





With respect to the identified rat urinary metabolites, two [the 5-equatorial,7a dihydroxy (XVI) and 4-equatorial,5-axial,7-axial trihydroxy (XVII) metabolites] were found only in the free form. Seven were found as conjugates only [the 5-axial hydroxy (IX), 6-axial hydroxy (III), 6-equatorial hydroxy (VII), 7-equatorial hydroxy (VI), 5-axial,6-equatorial dihydroxy (XVIII), 7-axial,7a dihydroxy (XIX), and 7-equatorial,7a dihydroxy (XX) metabolites]. Four of the metabolites [5-axial,7a dihydroxy (VIII), 5-axial,7-axial dihydroxy (XII), 4-equatorial,5-axial,7a trihydroxy (XXI), and the carboxamide (XXII) metabolites] were found in both free and conjugated forms. Identification of the eight newly recognized metabolites (XII and XVI–XXII; each isolated by TLC and homogeneous in three solvent systems) is discussed below (*cf.* Table I).

Metabolites XVI and XVII, under electron-impact mass spectrometry conditions, possess molecular ions of *m/z* 282 and 298, respectively, and thus these compounds are di- and trihydroxy analogues of I (for comparison, I yields

[1] B. M. Miller, R. Meurer, and W. J. A. VandenHeuvel, unpublished results.
[2] H. Skeggs, R. M. Berglund, W. J. A. VandenHeuvel, P. G. Wislocki, H. Mrozik, and F. J. Wolf, unpublished results.
[3] Each milliliter contained 1200 U of β-glucuronidase and 36,000 U of arylsulfatase; Calbiochem.
[4] Silica gel F; Brinkmann.
[5] Distilled in glass; Burdick and Jackson.
[6] Tricarb Liquid Scintillation Spectrometer and INSTA-GEL cocktail; Packard.
[7] LKB 9000.
[8] MAT 731; Varian.
[9] Model 3200; Finnigan.
[10] Model SC 300-MHz spectrometer equipped with a Fourier transform accessory; Varian.

Table I—Identified Rat Urinary Metabolites of I

Form in Urine	Compound	Fraction of Total Identified Metabolites, %[a]	R_f Values[b] A[c]	B[d]	C[e]
Free only	XVI 5 eq,7a-$(OH)_2$	1	0.02	0.57	0.35
	XVII 4 eq,5 ax,7 ax-$(OH)_3$	1	0.03	0.66	0.21
Conjugate only	IX 5 ax-OH	41	0.10	0.77	0.52
	III 6 ax-OH	1	0.09	0.78	0.36
	VII 6 eq-OH	4	0.07	0.70	0.41
	VI 7 eq-OH	9	0.20	0.82	0.53
	XVIII 5 ax,6 eq-$(OH)_2$	1	0.02	0.63	0.31
	XIX 7 ax,7a-$(OH)_2$	3	0.11	0.73	0.54
	XX 7 eq,7a-$(OH)_2$	1	0.07	0.68	0.48
Free/conjugate	XXII Amide[f]	30	0.27	0.86	0.59
	VIII 5 ax,7a-$(OH)_2$	2	0.03	0.67	0.45
	XII 5 ax,7 ax-$(OH)_2$	2	0.03	0.72	0.35
	XXI 4 eq,5 ax,7a-$(OH)_3$	3	0.08	0.70	0.44

[a] The identified metabolites account for >98% of the isolated metabolites. [b] Silica gel TLC. [c] Toluene–ethyl acetate, 3:1. [d] Dichloromethane–methanol, 10:1. [e] Trimethylpentane–2-propanol, 4:1. [f] 3a,4,5,6,7,7a-Hexahydro-3-carboxamido-1,2-benzisoxazole.

Table II—Pertinent Ions Found in the Mass Spectra[a] of I and Three of Its Urinary Metabolites

Compound	Substitution	Relative Intensity of Ion *m/z* 298	*m/z* 282	*m/z* 266	*m/z* 265	*m/z* 264	*m/z* 263	*m/z* 250	*m/z* 249	*m/z* 247	*m/z* 233	*m/z* 232	*m/z* 231	*m/z* 205	*m/z* 195	*m/z* 153	*m/z* 107
I	—	—	—	—	—	—	—	100	—	—	44	23	—	19	37	73	24
IX	5-ax-OH	—	—	35	—	—	—	—	19	17	—	—	7	—	100	60	15
XVIII	5-ax,6-eq-$(OH)_2$	—	27	—	15	12	—	—	—	18	—	—	—	—	100	41	14
XVII	4-eq,5-ax,7-ax-(OH_3)	6	—	—	—	—	3	—	—	—	—	—	—	—	100	10	5

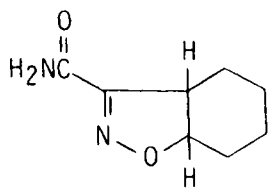
[a] Electron-impact low-resolution[7].

a molecular ion of *m/z* 250). The presence of fragment ions *m/z* 153 and 195 in the mass spectra of the metabolites strongly suggests (6) that hydroxylation occurred on the cyclohexane ring. Pertinent NMR data for the two metabolites are as follows: XVI: δ 3.86 (m, H_{6ax}) and 3.50 ppm (dd, 11.5, 7.2, H_{3a}); XVII: δ 4.99 (dd, 11.4, H_{7a}), 4.36 (m, H_{7eq}), 4.22 (br s, H_{4ax}), and 3.80 ppm (dd, 11, 9, H_{3a}).

The hydrolysis products of XVIII–XX each exhibit molecular ions of *m/z* 282 and fragmentation patterns indicative of hydroxylation of the alicyclic ring; these metabolites are thus dihydroxy analogues of I. NMR data critical for locating the hydroxy groups and defining their stereochemistry in these compounds include: XVIII: δ 4.69 (m, H_{7a}), 4.04 (br s, H_{5eq}), 3.57 (m, H_{6ax}), and 3.74 ppm (dt, H_{3a}); XIX: δ 4.22 (dd, 6.5, 4.5, H_{7eq}), and 3.70 ppm (dd, 11, 7, H_{3a}); XX: δ 4.02 (dd, 10, 6, H_{7ax}) and 3.66 ppm (t 8.5, H_{3a}). The NMR, MS, and TLC data of XVIII match those for the synthetic 5,6-*cis* (5-axial,6-equatorial) diol of I[11].

Two of the newly identified metabolites found both free and as conjugates were also shown to be hydroxy analogues of I. Metabolites XII and XXI exhibit molecular ions of *m/z* 282 and 298, demonstrating that they are di- and trihydroxy species, respectively. The mass spectrometric fragmentation patterns for the two compounds demonstrated the general sites of substitution [C(4)—C(7a)], but NMR spectrometry once again pinpointed the exact position and stereochemistry of the hydroxyl groups. Thus, for the 5-axial,7-axial-diol (XII), the NMR data include: δ 4.60 (br d, 7, H_{7a}), 4.32 (m, H_{7eq} + H_{5eq}), and 4.02 ppm (dt, 10, 7, 7, H_{3a}). For the 4-equatorial,5-axial,7a-triol, the NMR data include: δ 4.09 (br s, H_{5eq}), 3.44 (dd, 9.0, 3.0, H_{4ax}), and 3.40 ppm (d, 9.0, H_{3a}). Metabolite XII was actually noted previously as an *in vitro* biotransformation product of I but was not identified in the earlier work (8).

Metabolite XXII is the major drug-related species in the unconjugated fraction and the second most abundant of the metabolites released from the conjugated fraction during hydrolysis. Its relatively large R_f in solvent system A suggested that it was not a hydroxy analogue of I. The mass spectrometric (electron-impact) behavior of XXII (apparent molecular ion of *m/z* 168, with none of the normally encountered metabolite fragment ions) strengthened this assumption. Chemical-ionization MS of XXII yielded a pseudomolecular ion of *m/z* 169, confirming 168 as the molecular weight of the metabolite. An exact mass determination on the molecular ion (*m/z* 168) gave an empirical formula of $C_8H_{12}N_2O_2$ (calc., 168.0898; found, 168.0905). The metabolite did not possess the UV spectral features (λ_{max} at 240 and 320 nm) characteristic of I and its hydroxy metabolites. A structure which fits all these data is presented below. NMR data [δ 6.54 and 5.39 (2, $CONH_2$), 4.58 (qn, 3.7, H_{7a}), and 3.26 ppm (q, 8.0, H_{3a})] confirmed that the metabolite is indeed *cis*-3a,4,5,6,7,7a-hexahydro-3-carboxamido-1,2-benzisoxazole.



XXII

Three other rat urinary metabolites of I were partially characterized. Compound XXIII, found only in the nonconjugated form, is a dihydroxy analogue of I. Compounds XXIV and XXV, released from the conjugate fraction by hydrolysis, are both monohydroxy metabolites.

All the rat urinary metabolites of I identified (and the three isolated but only partially characterized), except one, are hydroxy analogues of the drug with substitution at C(4)—C(7a) of the cyclohexane ring. The lone exception is the carboxamide XXII which arises *via* degradation of the nitroimidazole ring. Another carboxamide, acetamide, has been found as a metabolite resulting from the *in vitro* reduction of metronidazole by xanthine oxidase (16) and the *in vivo* (turkey) metabolism of another nitroimidazole, ronidazole (17). As XXII was not found as an *in vitro* (rat liver S9) metabolite, it may arise *in vivo via* a nonhepatic metabolic route, such as reduction by gut flora.

Urinary monohydroxy metabolites of I were found only as conjugates. Compounds IX and VI (released by hydrolysis) are the two most abundant (in that order; see Table I) hydroxy metabolites of I, paralleling the rat *in vitro* situation (8). Of the two other monohydroxy metabolites, III and VII (both found as conjugates), the former was previously identified as a canine urinary metabolite (7) and the latter as a rat *in vitro* biotransformation product (8).

Although dihydroxy metabolites of I are produced by the rat both *in vitro* (free only) and *in vivo* (free and conjugated), trihydroxy metabolites XVII and XXI are found only as urinary metabolites. Compound XVII was found exclusively in the unconjugated form, whereas XXI was found both free and conjugated. The latter is the second most abundant of the metabolites excreted free.

It is clear that the alicyclic hydrocarbon moiety of I is very susceptible to hydroxylation by the rat, both *in vivo* and *in vitro*. Nine hydroxy analogues

[11] H. Mrozik and P. Eskola, unpublished data.

of I were identified as rat liver S9 *in vitro* metabolites, and this current paper reports the identification of a dozen metabolites of I in which hydroxylation occurred at C(4) - C(7a) of the cyclohexane ring. The intact animal produces not only a greater total number of and several more-extensively hydroxylated metabolites, but also effects an entirely different and quantitatively significant metabolic conversion, *i.e.*, degradation of the nitroimidazole ring. The most common metabolic transformation both *in vivo* and *in vitro*, however, is hydroxylation to form the 5-axial hydroxy metabolite. Axial hydroxyl groups at C-5 are also found in three of the dihydroxy metabolites and both of the urinary trihydroxy metabolites.

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Acyloxyamines as Prodrugs of Anti-inflammatory Carboxylic Acids for Improved Delivery Through Skin

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Abstract □ An *N,N*-dialkylhydroxylamine derivative of indomethacin has been synthesized. It has been shown to improve the delivery of indomethacin through mouse skin (compared to indomethacin itself) by a factor of two, to be more effective than indomethacin in inhibiting thermal inflammation (two to three times) in animal models, but to be only as effective as indomethacin in inhibiting UV-B radiation erythema in human volunteers.

Keyphrases ■ Indomethacin—derivatives, erythema inhibition in humans, inflammation inhibition in rats, delivery vehicle comparison □ Erythema—inhibition by indomethacin derivatives in humans, inflammation inhibition in rats, delivery vehicle

It is well known that UV radiation on skin produces intense erythema, pain, and blistering (1). However, the proximal cause or causes of the response of skin to UV radiation is less than well understood. UV radiation is usually divided into three arbitrary regions: UV-A, 320–400 nm; UV-B, 320–290 nm, which is also known as sunburn UV radiation; UV-C, 290–200 nm. Regardless of the wavelength, there is a delay of onset of redness that is inversely proportional to the intensity of the radiation. Furthermore, the erythema will persist for hours or days depending directly on the intensity of the radiation. The intensity of the effect of the UV-B radiation has been shown to be particularly sensitive to treatment with nonsteroidal anti-inflammatory agents while UV-C radiation is less sensitive (2) and UV-A radiation is insensitive to nonsteroidal anti-inflammatory agents (3). For instance, topically administered indomethacin has been shown to decrease the redness, as determined visually, and the temperature, as determined by telethermometer readings, of sunburned or UV-B treated skin compared with controls (4).

Since the nonsteroidal anti-inflammatory agents are known to prevent inflammation by inhibiting prostaglandin synthesis, it was logical to suspect that prostaglandins (5) were the proximal cause of at least some of the effects of UV-B radiation because UV-B radiation was susceptible to treatment with nonsteroidal anti-inflammatory agents. Indeed, increased levels of arachidonic acid and prostaglandins E_2 and $F_{2\alpha}$ were found in human skin after treatment with UV-B (6) and UV-C radiation (7); the levels of prostaglandin E increased in a parallel manner with increased erythema over the first 4 h after exposure of guinea pig skin to UV-B radiation (8). However, after 4 h erythema scores stayed high but prostaglandin levels fell back to normal (8). Thus, oral or topical administration of indomethacin completely suppressed the elevation of the levels of the prostaglandins (9) but the erythema associated with the radiation damage was only partially suppressed compared with controls and then for only ~24 h, and the acute

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