compartments of a vesicle and even after degradation of the microparticle some drug may remain covalently linked to the degradation products. Thus after lysosomal degradation of the microparticle, that proportion of the stibogluconate remaining covalently linked would be less liable than free drug to diffuse from the lysosomal compartment, so helping to maintain effective intracellular antimony levels for longer periods.

The evidence suggests that the microparticulate modified starch is a good carrier system for the therapy of this intracellular infection. The efficacy described here is, as far as can be judged within methodological constraints, as high as that obtained with liposomal delivery in similar animal models of visceral leishmaniasis (Alving 1983).

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Cytochrome P450 metabolic intermediate complex of nefopam

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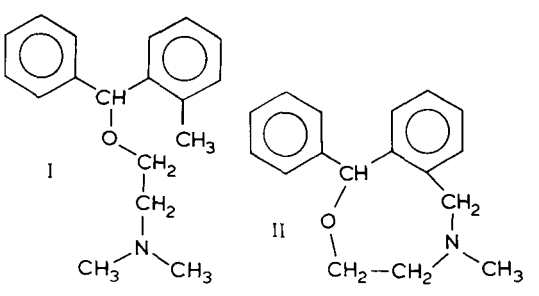
NADPH-catalysed biotransformation of nefopam in liver microsomes obtained from phenobarbitone-pretreated rats leads to the formation of an inactive cytochrome P450 metabolic intermediate (MI) complex. This complex can be detected spectrophotometrically by an absorbance maximum at 459 nm. The extent of the in-vitro MI complexation of 33 μM nefopam, a cyclic analogue of orphenadrine, was almost equal to the extent of the in-vitro MI complexation of 33 μM tofenacine, the mono-*N*-demethylated metabolite of orphenadrine. The time course of the MI complexation of nefopam and studies with two of its major metabolites suggest an initial biotransformation, which has to occur before MI complexation can take place. Maximal MI complexation of nefopam occurred at approximately 25 μM, whereas the MI complexation could not be detected at 100 μM nefopam.

Unexpected accumulation of the antiparkinsonian agent, orphenadrine, in man has been shown under chronic dosing conditions (Labout et al 1982). Product inhibition due to the formation of a cytochrome P450 metabolic intermediate (MI) complex during orphenadrine biotransformation has been suggested to cause this accumulation (Bast et al 1983a). The MI complex is probably a nitroxide radical or a nitroso metabolite, which binds irreversibly to the reduced form of cytochrome P450. The complex can be detected spectrophotometrically by an absorbance maximum near 455 nm (Bast et al 1983a).



A previous study with several orphenadrine analogues revealed some structural features necessary for MI complexation. In particular it was shown that secondary amines gave rise to extensive MI complexation (Bast et al 1984). In this study the in-vitro MI complex formation during biotransformation in phenobarbitone-induced rat liver microsomes of the analgesic, nefopam, is reported. Nefopam (I) is a cyclic analogue of orphenadrine (II) and contains a ring tertiary amine function.

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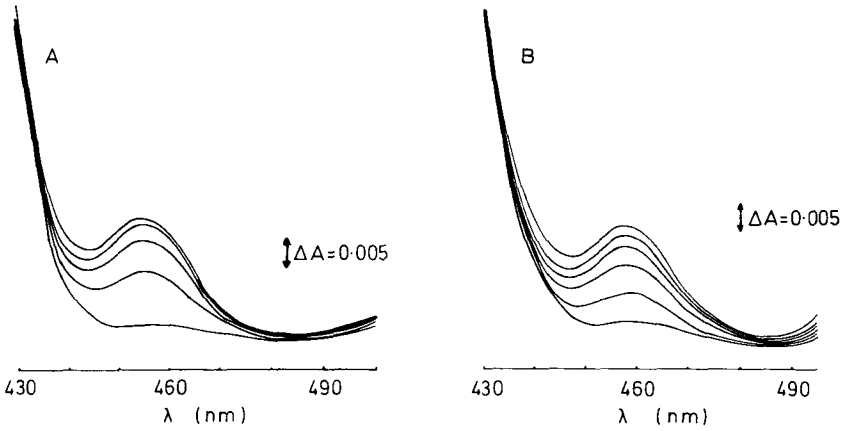

FIG. 1. Repetitive scanning of difference spectra (with a time difference of approximately 60 s between each scan) produced during the NADPH-dependent metabolism of 33 μM tofenacine (A) and nefopam (B) in phenobarbitone-induced rat liver microsomes at 37 °C. The reaction was started with the addition of NADPH at a final concentration of 400 μM in the sample cuvette. A representative experiment out of four is shown.

Materials and methods

Chemicals. Tofenacine hydrochloride was a gift from Gist-brocades N.V. (Delft, The Netherlands). Nefopam hydrochloride, nefopam-*N*-oxide and *N*-desmethylnefopam fumarate were gifts from Riker Laboratories, 3M Health Care (Loughborough, UK). β-NADPH was purchased from Sigma Chemical Co. (St Louis, USA). All other chemicals were of analytical grade purity.

Preparation of rat liver microsomes and pretreatment of animals. Liver microsomes were prepared from male Wistar rats (250–300 g, TNO, Zeist, The Netherlands) as described previously (Bast & Noordhoek 1980). The rats were pretreated with phenobarbitone, dissolved in saline, as metabolic intermediate complex formation is enhanced after induction with phenobarbitone (Bast & Noordhoek 1982). The pretreatment consisted of three daily intraperitoneal injections of 80 mg kg^{-1}. The rats were killed 24 h after the last injection.

Spectral measurements. The spectrophotometric observations were performed with the microsomal fraction (2 mg protein mL^{-1}, [cytochrome P450] = 4·8 μM) suspended in 50mM phosphate buffer (pH 7·4) containing 0·1 mM EDTA and 4·2 mM $MgCl_2$. The metabolic intermediate complex formation was started with the addition of NADPH at a final concentration of 400 μM in the sample cuvette and was followed by either dual wavelength spectroscopy (459 nm or 455 nm (tofenacine) relative to 490 nm) or split-beam spectroscopy. In the latter case both sample and reference cuvettes contained phenobarbitone-induced microsomes in buffer (see above) whereas the sample cuvette contained the substrate for MI complex formation.

The concentration of cytochrome P450 was estimated as described previously (Estabrook et al 1972). Both cuvettes were bubbled with carbon monoxide gas and sodium dithionite was added to the sample cuvette to prevent interference from haemoglobin.

Results and discussion

The optimum concentration for metabolic intermediate complexation in studies using orphenadrine analogues has often been found to be 33 μM (Bast et al 1984). Therefore the initial experiments were conducted with 33 μM of substrate. The known MI complex formation elicited during tofenacine metabolism (mono-*N*-demethylated orphenadrine, 33 μM) was used as a reference (Fig. 1A) (Bast & Noordhoek 1982). Repetitive scanning spectroscopy (430–500 nm) with the spectrophotometer in the split beam mode revealed that nefopam metabolism in phenobarbitone-induced hepatic microsomes at 37 °C led to the formation of an MI complex, which resulted in an absorbance maximum at

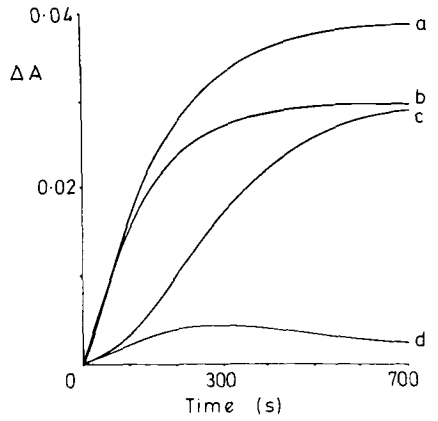

FIG. 2. Time course of the MI complexation elicited during microsomal metabolism of 33 μM of *N*-desmethylnefopam (a), tofenacine (b), nefopam (c) or nefopam-*N*-oxide (d). A representative experiment out of three is shown.

459 nm (Fig. 1B). The microsomal metabolism of 33 μM of two metabolites of nefopam, nefopam-*N*-oxide and *N*-desmethylnefopam, also gave rise to the formation of an MI complex, absorbing at 459 nm.

To study the time course and the extent of the MI complexation, the spectrophotometer was set in the dual wavelength mode (455 vs 490 nm for tofenacine and 459 vs 490 nm for nefopam and its two metabolites). Fig. 2 shows the time course of the MI complexation of 33 μM nefopam, nefopam-*N*-oxide, *N*-desmethylnefopam or tofenacine at 37 °C in phenobarbitone-induced microsomes. The extent of MI complexation of 33 μM nefopam was almost the same magnitude as that of 33 μM tofenacine, whereas the extent of the MI, complexation of 33 μM *N*-desmethylnefopam was greater. Using nefopam-*N*-oxide as substrate resulted in a small absorbance at 459 nm. Using a molar extinction coefficient of 65 $mM^{-1} cm^{-1}$ (Bast et al 1984) the percentage of cytochrome P450 complexed could be calculated and was 10·2% for tofenacine, 10·0% for nefopam and 16·0% for *N*-desmethylnefopam. This extensive MI complexation by nefopam was not expected since it contains a ring-membered tertiary nitrogen. It was already known that during the metabolism of orphenadrine, which also contains a tertiary nitrogen on an ethylamine side-chain, only a small absorbance change due to MI complexation could be detected, while the extent of MI complexation of several orphenadrine metabolites such as tofenacine, which has a secondary nitrogen, was much greater (Bast & Noordhoek 1982). Apparently, nefopam behaves differently from orphenadrine.

The lag phase observed during the time course of MI complexation of 33 μM nefopam (Fig. 2) is common for tertiary amines and can be explained by an initial *N*-demethylation, which has to occur before the complexing species can be produced. This explanation is supported by the absence of a lag phase in the time course and by the extent of the MI complexation of *N*-desmethylnefopam.

The extent of MI complex formation with orphenadrine or tofenacine has been shown to be dependent upon the substrate concentration (Bast & Noordhoek 1982). This is also demonstrated for nefopam and *N*-desmethylnefopam (Fig. 3). Maximal MI complexation of nefopam, detected as the absorbance change between 459 and 490 nm, occurred at approximately 25 μM. At 100 μM MI complexation of nefopam could not be detected. This phenomenon might be explained by a substrate inhibition, that is high concentrations of nefopam inhibit further biotransformation. For *N*-desmethylnefopam maximum MI complexation is not reached at a concentration of 100 μM, although the increase in absorbance at high concentrations is small (Fig. 3).

The results show that microsomal metabolism of the analgesic nefopam leads to the formation of an MI complex, absorbing at 459 nm. Study of the MI complexation of the two major metabolites, nefopam-*N*-oxide and *N*-desmethylnefopam, leads us to propose an interaction of a metabolic intermediate of nefopam with reduced cytochrome P450 similar to orphenadrine, where the MI complexation is caused by a nitroxide radical or nitroso metabolite of orphenadrine (Bast & Noordhoek 1982). It was shown that the MI complexation of orphenadrine also occurred after in-vivo administration (Bast & Noordhoek 1982) and that this complexation led to an inhibition of several cytochrome P450 catalysed reactions (Bast et al 1983b). The high degree of in-vitro MI complexation during biotransformation of nefopam and of *N*-desmethylnefopam suggests that similar effects may be expected with nefopam. However, some differences compared with orphenadrine-based MI complexation emerged as well with regard to maximal absorbance wavelength (nefopam at 459 nm, tofenacine at 455 nm) and the role of the tertiary amine function. In nefopam the tertiary amine was not restrictive whereas in orphenadrine a tertiary amine function precludes MI complex formation.

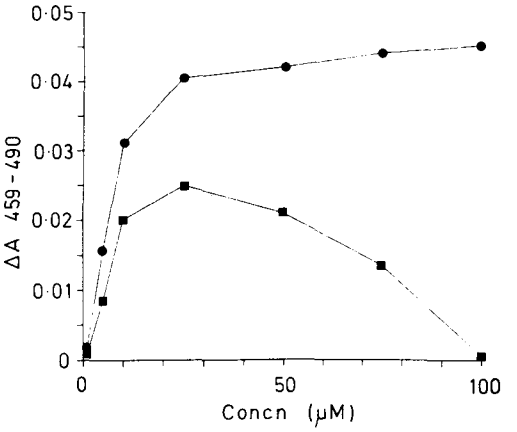

FIG. 3. Concentration dependence in the extent of MI complexation of nefopam (■) or *N*-desmethylnefopam (●). Each point is the mean of at least two experiments.

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