Biochemical Pharmacology, Vol. 21, pp. 2697–2703. Pergamon Press, 1972. Printed in Great Britain

USE OF ANTIFOAMS IN DRUG METABOLISM STUDIES *IN VITRO**

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(*Received* 3 *February* 1972; *accepted* 28 *April* 1972)

Abstract—The addition of very small amounts of antifoam or antifoam emulsions to rat hepatic microsomal suspensions was found to produce a type I substrate binding spectrum, and to increase the rate of NADPH–cytochrome P-450 reduction. The results also show that emulsifiers can themselves produce a type I substrate binding spectrum and can also alter the interaction of antifoam with microsomes.

SINCE THE identification of cytochrome P-450 by the absorption spectrum of its unique carbon monoxide complex,[1] the study of this cytochrome in microsomal suspensions has presented some practical problems. The relatively high protein content of the microsomal suspensions normally employed in optical determinations resulted in frothing when gassed with carbon monoxide. To overcome this problem, the practice of using antifoams on these occasions has arisen. This practice causes no alteration of the optical properties of the sodium dithionite reduced cytochrome P-450 CO complex. However, the extension of the use of antifoams during the study of other aspects of cytochrome P-450 function appears to have occurred without proper investigation. Our recent use of sonication to disperse insoluble substrates for drug metabolism in microsomal suspensions[2] led us to examine the effect of the presence of antifoams on other commonly determined properties of cytochrome P-450.

MATERIALS AND METHODS

All the antifoams were obtained from Sigma Chemical Co., Antifoam A concentrate is a 100% silicone polymer; Antifoam A and C emulsions are emulsions containing 30% silicone and undisclosed emulsifiers; Antifoam B emulsion contains 10% silicone and different emulsifiers from those in Antifoam A emulsion. Both Antifoam A and B emulsions contain the same silicone as Antifoam A concentrate. The mixture from Antifoam A aerosol was obtained by depressurizing the contents of the aerosol can.

Tween 80 was from Atlas Chemical Industries, Inc. and the WGS emulsifier was a sample of a non-ionic emulsifier (alkyl ether ester) supplied by Werner G. Smith Inc. NADPH was from P. L. Biochemicals, Inc.

Hepatic microsomes were prepared as described previously,[3] and all spectrophotometric determinations were obtained with an Aminco-Chance dual wavelength/split-beam spectrophotometer. The microsomes were suspended in 50 mM Tris–chloride buffer, pH 7·4, containing 150 mM KCl and 10 mM $MgCl_2$, since this is

* This work was supported by Grant GM 16488 from the National Institutes of Health.

the buffer normally employed to provide maximum rates of drug metabolism *in vitro*. The NADPH–cytochrome P-450 reductase activity was determined at 4° to overcome the limitations of the instrument response time encountered at 25°. Stopped flow experiments (J. Baron and J. A. Peterson, unpublished observations) have shown that at 25° the initial rate of the reduction of cytochrome P-450 by NADPH is much faster than can be satisfactorily determined using an Aminco–Chance spectrophotometer with the rapid mixing chamber assembly.

RESULTS AND DISCUSSION

The addition of antifoam emulsions to aerobic suspensions of rat liver microsomes from either control or phenobarbital-pretreated animals produces optical spectral changes characteristic of what have been termed by Remmer *et al.*[4] type I substrates. The similarity of the difference spectra obtained upon the addition of Antifoam A emulsion, Antifoam B emulsion, or a type I substrate, hexobarbital, is shown in Fig. 1 and Table 1. As can be seen in Fig. 1, Antifoam A emulsion produces a larger

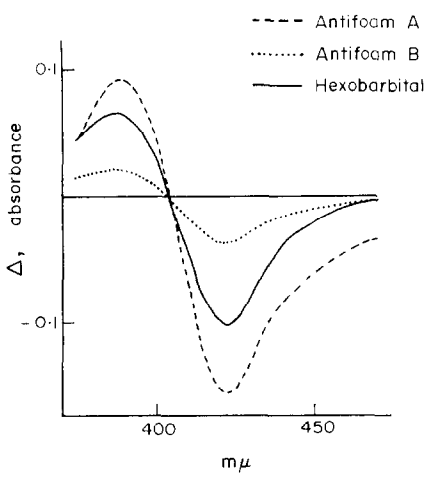

FIG. 1. Spectral changes occurring upon the addition of antifoam emulsions to aerobic suspensions of microsomes. Microsomes from phenobarbital-pretreated rats were suspended at 2 mg protein/ml (cytochrome P-450 = 2·4 nmoles/mg of protein) in 50 mM Tris–chloride buffer, pH 7·4, containing 150 mM KCl and 10 mM $MgCl_2$ and the suspension was divided between two cuvettes. After establishing a baseline of equal light absorbance, 10 μl of Antifoam A or Antifoam B emulsion or 5·4 mM hexobarbital (final concentration) was then added to the contents of the sample cuvette and the difference spectrum recorded 5 min later, after correcting for increased turbidity in the sample cuvette by offsetting the zero balance point at 500 nm.

type I difference spectrum than does saturating concentrations of hexobarbital. Both Antifoam A emulsion and Antifoam B emulsion are aqueous suspensions of silicones, Antifoam A emulsion containing three times more silicone than Antifoam B. However, they contain different emulsifying agents. Antifoam A from aerosol cans and Antifoam A concentrate, although more difficult to mix with microsomal suspensions, also give a type I substrate binding spectrum. The type I difference spectra of the antifoams, hexobarbital and two non-ionic detergents are indicated in Table 1. All these compounds give very similar spectra with regard to the wavelengths of the absorption

TABLE 1. CHARACTERISTICS OF THE TYPE I DIFFERENCE SPECTRA CAUSED BY NON-IONIC DETERGENTS, ANTIFOAM EMULSIONS AND HEXOBARBITAL WITH HEPATIC MICROSOMES*

Compound	Concentration (μl/ml)	Type I difference spectrum (after 5 min) Peak (nm)	Trough (nm)	Δ O.D. peak–trough
Hexobarbital	2·7 mM	387	422	0·17
	5·4 mM			0·17
Antifoam A	2·7	385	422	0·14
Antifoam A emulsion	1·4	388	422	0·23
	2·7			0·25
Antifoam A aerosol	2·7	386	422	0·14
	6·7			0·22
Antifoam B emulsion	2·7			0·06
	6·7	387	422	0·10
	13·4			0·10
Antifoam C emulsion	6·7	386	422	0·06
Tween 80	2·7	386	422	0·10
	6·7			0·08
WGS emulsifier	2·7	391	426	0·11

* Microsomes from phenobarbital-pretreated rats were suspended at 2 mg protein/ml (cytochrome P-450 = 2·3 nmoles/mg of protein) in 50 mM Tris–chloride buffer, pH 7·4, containing 150 mM KCl and 10 mM $MgCl_2$. The suspension was divided between two cuvettes and a baseline of equal light absorbance determined. The spectral changes were monitored up to 5 min after the addition of the various compounds to the contents of the sample cuvette. When Antifoam A concentrate and Antifoam A aerosol were used, the microsomal suspensions were briefly sonicated after the antifoam addition.

maxima and minima, although differing considerably in intensity. The concentration of Antifoam B emulsion required for 50 per cent formation of the maximum type I difference spectra is mugh higher than that for Antifoam A emulsion. The results for Tween 80 and the WGS non-ionic emulsifier confirm the reports of Bridges and Burke,[5] and of Denk *et al.*,[6] showing that non-ionic detergents can interact with microsomal pigments and produce specific spectral changes.

The results in Table 1 were obtained from the spectral changes observed 5 min after the addition of the compound to the microsomal suspension. It was of interest to determine whether the differences in intensity might be due to the time period used. As shown in Fig. 2, the type I difference spectrum produced by hexobarbital was maximal as soon as it was experimentally measurable. Those produced by Antifoam A emulsion and the WGS emulsifier (not shown) were 95 per cent complete within 1 min. However, the type I difference spectrum produced by Tween 80 and Antifoam B and C emulsions were not fully formed within 10 min. The similarity in the time of formation of the type I spectrum between Antifoam A emulsion and the WGS emulsifier, and between Antifoam B and C emulsions and Tween 80, and the dissimilarity between

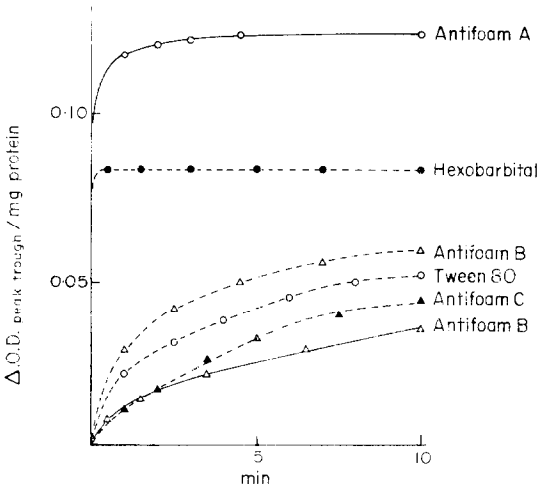

FIG. 2. Effect of time upon the type I difference spectrum formed by detergents, antifoams and hexobarbital with liver microsomes. Microsomes from phenobarbital-pretreated rats were suspended at 2 mg protein/ml (cytochrome P-450 = 2·3 nmoles/mg of protein) in 50 mM Tris–chloride buffer, pH 7·4, containing 150 mM KCl and 10 mM $MgCl_2$. The suspension was divided between two cuvettes. After establishing a baseline of equal light absorbance, hexobarbital (7·7 mM), Antifoam A emulsion (2·7 μl/ml), Antifoam C emulsion (5·4 μl/ml), Tween 80 (6·7 μl/ml), and Antifoam B emulsion (2·7 μl/ml, △——△; 6·7 μl/ml, △- - -△) were individually added to the contents of the sample cuvette and the difference spectra recorded up to 10 min after the addition.

the antifoam emulsions themselves, even though they presumably contain the same silicone, suggest that the type I substrate binding spectrum is partly a property of the emulsifier present in the antifoam emulsions. That there is a finite time required to form the type I difference spectrum with microsomal suspensions by the antifoam emulsions suggests a more complex interaction than is observed for substrates for mixed-function oxidation, such as hexobarbital. Attempts to produce a type I difference spectrum with Antifoam A concentrate, which contains no emulsifiers, were unsuccessful unless sonication (which in itself produced no spectral changes) was used to disperse the antifoam. Upon sonication, a type I difference spectrum attributable solely to Antifoam A (since no emulsifiers were present) was evident, and this did not change with time. However, the intensity of the type I difference spectrum was somewhat variable, depending upon the sonication techniques employed.

In an attempt to detect any differences in the interactions involved between Antifoam A and microsomal suspensions and Antifoam A emulsion (containing Antifoam A and emulsifiers) and microsomal suspensions, the effect of these compounds on the aniline type II difference spectrum of liver microsomes was investigated (Table 2). As has been shown for hexobarbital, a type I substrate,[7] the Antifoam A and Antifoam A emulsion both produce an increase in intensity and a shift in the wavelengths of the peak and trough in the difference spectrum obtained upon the addition of aniline. Thus, with regard to the spectral characteristics, the only difference between the presence and absence of an emulsifier with the antifoam is one of the intensity of the resultant spectrum. However, since it is difficult to disperse Antifoam A in the absence of emulsifiers, the differences in intensity may reflect a lack of sufficient dispersal to enable interaction between the antifoam and the microsomes to occur.

TABLE 2. CHARACTERISTICS OF THE MICROSOMAL ANILINE TYPE II DIFFERENCE SPECTRA IN THE PRESENCE AND ABSENCE OF TYPE I SUBSTRATES*

Compound (in addition to aniline)	Concentration	Type II difference spectrum		
		Peak (nm)	Trough (nm)	Δ O.D. peak–trough
None		431	397	0·22
Antifoam A	2·7 μl/ml	429	392	0·31
Hexobarbital	2·7 mM	427	391	0·34
Antifoam A emulsion	2·7 μl/ml	427	390	0·43

* Microsomes from phenobarbital-pretreated rats were suspended at 2 mg protein/ml (cytochrome P-450 = 2·7 nmoles/mg of protein) in 50 mM Tris–chloride buffer, pH 7·4, containing 150 mM KCl and 10 mM $MgCl_2$ and the additional compounds indicated. (With Antifoam A, the mixture was sonicated to disperse the antifoam.) The mixture was divided into two cuvettes and, after establishing a baseline of equal light absorbance saturating quantities (20 μl) of aniline were added to the contents of the sample cuvette, mixed and the spectral changes monitored.

The determination of an apparent K_s for Antifoam A emulsion (Fig. 3) shows that a concentration of 0·15 μl/ml or about one part in 7000 will produce 50 per cent of the maximal type I difference spectrum. With microsomal suspensions containing saturating concentrations of Antifoam A emulsion, no type I substrate binding spectrum was observable upon the addition of hexobarbital to the sample cuvette. The low apparent K_s (i.e. small volume) of Antifoam A emulsion is important, since in some laboratories Antifoam A is used to prevent foaming when gassing microsomal suspensions with carbon monoxide in order to determine NADPH–cytochrome P-450 reductase. Since compounds producing type I substrate binding spectra have been shown to enhance the rate of cytochrome P-450 reduction,[8] under anaerobic conditions,

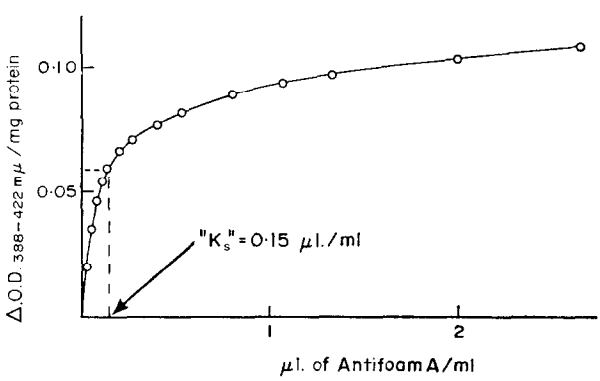

FIG. 3. Effect of various concentrations of antifoam A emulsion on the intensity of the type I difference spectrum of hepatic microsomes. Microsomes from phenobarbital-pretreated rats were suspended at 2 mg protein/ml in 50 mM Tris–chloride buffer, pH 7·4, containing 150 mM KCl and 10 mM $MgCl_2$, and the suspension was divided between two cuvettes. After establishing a baseline of equal light absorbance, Antifoam A emulsion was added to the contents of the sample cuvette and the difference spectrum recorded 1 min after each addition. Small spectral changes attributable to turbidity effects were corrected for by repeating the experiment with microsomal pigments reduced with sodium dithionite.

erroneously high rates of reduction may have been obtained for what have hitherto been termed "control microsomes"; in this case, the term "control" infers the absence of exogenous type I substrates. Thus, an increase in the rate of reduction by the presence of antifoam would cause a lessening in the stimulation of the NADPH–cytochrome P-450 reductase observed upon the addition of another type I substrate such as hexobarbital.

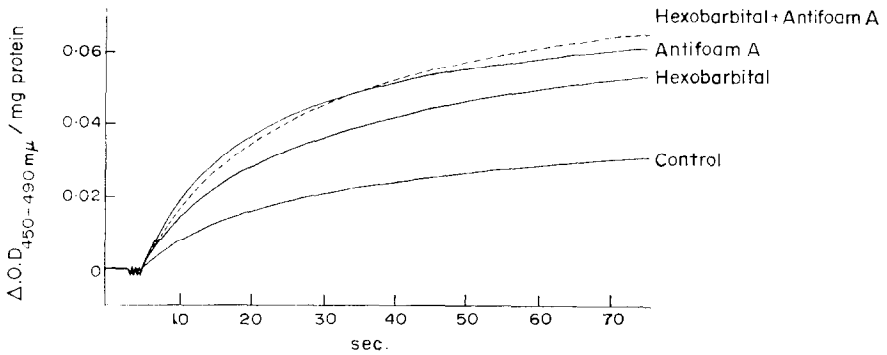

FIG. 4. Reduction of microsomal cytochrome P-450 by NADPH in the presence of antifoam emulsions and hexobarbital. Microsomes from untreated rats were suspended at 2 mg protein/ml (cytochrome P-450 = 1·2 nmoles/mg of protein) in ice-cold 50 mM Tris–chloride buffer, pH 7·4, containing 150 mM KCl and 10 mM $MgCl_2$ which had been saturated with CO. The microsomal suspension was then carefully gassed with CO for a further 3 min. The rates of formation of the cytochrome P-450-CO complex at 4° was then monitored by following the absorbance changes at 450 nm, relative to 490 nm. NADPH 400 μM was mixed with the microsomal suspension 4 sec after the start of the recorded trace. The experiment was repeated in the presence of hexobarbital (3·3 mM), Antifoam A emulsion (3·3 μl/ml), and hexobarbital and Antifoam A emulsion together.

As can be seen in Fig. 4, the presence of Antifoam A emulsion increases the rate of reduction of cytochrome P-450 above that observed in the presence of hexobarbital. The presence of both Antifoam A emulsion and hexobarbital produced no significant increase in the rate of reduction above that observed in the presence of Antifoam A emulsion alone. In additional experiments, the rate of reduction of cytochrome P-450 was also examined in the presence of Antifoam (aerosol), and while the effect of this form of antifoam may be somewhat limited by its dispersal, it did increase the rate of cytochrome P-450 reduction above that observed in its absence, but not as much as observed with the Antifoam A emulsion.

The results shown in this paper demonstrate that antifoams, either in the presence or absence of emulsifiers, act as type I substrates. This is evidenced by the production of type I spectrum upon interaction with microsomes, and upon saturation of the microsomes, preventing the observation of a hexobarbital type I difference spectrum. The antifoams also perturbate the aniline type II binding spectrum in a manner similar to hexobarbital and, like hexobarbital and many other type I substrates, increase the rate of cytochrome P-450 reduction by NADPH.

Acknowledgements—The author would like to thank Dr. Ronald W. Estabrook for his valuable advice and discussions throughout the preparation of this manuscript. Thanks are also due to Mrs. Nancy J. Mahanay for her skilled technical assistance.

REFERENCES

1. T. Omura and R. Sato, *J. biol. Chem.* **239,** 2370 (1964).
2. M. R. Franklin, *Xenobiotica,* **1,** 521 (1971).
3. M. R. Franklin and R. W. Estabrook, *Archs Biochem. Biophys.* **143,** 318 (1971).
4. H. Remmer, J. B. Schenkman, R. W. Estabrook, H. Sasame, J. R. Gillette, S. Narasimhulu, D. J. Cooper and O. Rosenthal, *Molec. Pharmac.* **2,** 187 (1966).
5. J. W. Bridges and M. D. Burke, *Chem. Biol. Interact.* **3,** 314 (1971).
6. H. Denk, J. B. Schenkman, P. G. Bacchin, F. Hutterer, F. Schaffner and H. Popper, *Expl. molec. Path.* **14,** 203 (1971).
7. J. B. Schenkman, *Biochemistry, N.Y.* **9,** 2081 (1970).
8. P. L. Gigon, T. E. Gram and J. R. Gillette, *Biochem. biophys. Res. Commun.* **31,** 558 (1968).