Toxicity of *n*-Alkanes, *n*-Alk-1-enes, *n*-Alkan-1-ols and *n*-Alkyl-1-bromides towards Yeasts

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SUMMARY

The toxicity of *n*-alkanes, *n*-alk-1-enes, *n*-alkan-1-ols and *n*-alkyl-1-bromides towards *Candida tropicalis*, *Candida* 107 and *Saccharomyces carlsbergensis* is related to their solubilities in an aqueous medium. *n*-Alkanes of chain-length longer than C₉, *n*-alkenes longer than C₁₂, *n*-alkyl bromides longer than C₁₀ and *n*-alkanols longer than C₁₄ generally do not inhibit growth and respiration. Toxicity may be reduced or relieved if oxidation by the yeasts can occur or if a non-toxic aliphatic compound is also added. Relief of inhibition is not dependent upon the oxidation of the non-toxic compound. It is also produced when the biologically inert hydrocarbon, pristane, is added. These effects are attributed to the reduction of the solubility of the toxic compound by the added compound in accordance with Raoult's and Henry's Laws. If the solubility of the toxic material is decreased below a critical level, growth on it as sole carbon source may occur with either of the two species of *Candida*. These results imply that small amounts of potentially deleterious compounds need not affect the growth of micro-organisms on petroleum hydrocarbons.

INTRODUCTION

n-Alkanes and their derivatives can be toxic to micro-organisms: fatty acids (Neiman, 1954; Barker, 1964), their sodium salts (Hurst, Stuttard & Woodruffe, 1960) and fatty alcohols (*n*-alkan-1-ols) (Quastel & Whetham, 1925) are bactericidal. The toxicity of *n*-alkan-1-ols increases with increasing chain-length up to C₈ but thereafter decreases (Hamilton, 1971). Unpublished work of Ishikura (see Foster, 1962) showed that *n*-alk-1-enes are more toxic than the corresponding *n*-alkane to Gram-positive bacteria, yeasts and fungi; those with a chain-length from C₅ to C₉ being the most potent. Gram-negative bacteria are, however, insensitive to the toxic effects of the hydrocarbons.

The reason for the toxicity of these compounds, particularly *n*-alkanes and *n*-alk-1-enes, is not certain although both solubility (Barker, 1964; Johnson, 1964; Klug & Markovetz, 1971) and surface tension (Cowles, 1938) have been discussed in this connexion.

In this report we present evidence of the importance of solubility in determining the toxicity of *n*-alkanes and their derivatives towards yeasts. The yeasts used are two species of *Candida* which can utilize *n*-alkanes above C₈ for growth and *Saccharomyces carlsbergensis* which cannot grow on any hydrocarbon.

METHODS

Organisms and growth. The yeasts used were *Candida tropicalis* NCYC4, *Saccharomyces carlsbergensis* NCYC530, and *Candida* 107, which is an untyped species used in previous work (Ratledge, 1968) and which is constitutive for alkane oxidation (Gill & Ratledge, 1972). They were grown in conical flasks at 30 °C with rotary shaking. Basal salts medium contained

NH_4Cl , 2.5 g; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 5.0 g; KH_2PO_4 , 7.0 g; and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g in 1 l distilled water, with the pH value adjusted to 5.5 with HCl. *n*-Alkanes or derivatives as carbon sources were at concentrations stated and were added to the medium with 0.2 g yeast extract/l before sterilization. Glucose as sole carbon source was at 3 g/l with 0.2 g yeast extract/l and was added after separate sterilization; all the glucose was consumed within 18 h of growth when the yeasts were harvested or used for further inoculation. Yeasts were harvested by centrifuging (5000g for 15 min) and washed twice in 0.2 M- KH_2PO_4 buffer at pH 5.5 before use.

When testing toxicity of aliphatic compounds, glucose was at 25 g/l with 2.5 g malt-extract/l of basal salts medium. This medium (20 ml) was in 100 ml flasks and inoculated with 0.1 ml of glucose-grown yeast.

Respiration measurements. A Beckman laboratory oxygen analyser (Model 777, Beckman Instruments Inc., Fullerton, California, U.S.A.) was used at 35 °C filled with up to 3.1 ml of a 5 % (v/v) emulsion of an aliphatic compound in water (prepared by ultrasonication). The final volume was made to 3.1 ml, if necessary, with water, and, when at equilibrium, 0.1 ml of a washed yeast suspension (about 20 mg dry wt/ml) was added.

Chemicals used. Most *n*-alkanes, *n*-alk-1-enes, *n*-alkan-1-ols and *n*-alkyl-1-bromides, each at 99 % purity (except for *n*-tetradec-1-ene which was described as 'practical grade'), and pristane (2,6,10,14-tetramethylpentadecane), at 97 % purity, were obtained from Koch-Light Laboratories Ltd, Colnbrook, Buckinghamshire. Other compounds were: *n*-dodec-1-ene and *n*-dodecan-1-ol (laboratory reagent grades) from Fisons Ltd, Loughborough, Leicester; *n*-undec-1-ene, *n*-undecyl-1-bromide and *n*-tridecyl-1-bromide (99 % purities) from K. & K. Laboratories Inc., Plainview, New York, U.S.A. and *n*-tridecan-1-ol from B. Newton-Maine Ltd, Melton Constable, Norfolk. All compounds except pristane were used without purification. With pristane, the impurities present permitted both alkane-utilizing yeasts to grow. It was purified by growing *Candida tropicalis* on basal salts medium containing 120 g pristane/l for 3 days. The alkane layer remained unemulsified and was separated from the remainder of the culture. After filtering through fibre glass paper, it was washed with 300 ml water, 300 ml 2 M-NaOH and a further three times with 300 ml water. The pristane was dried over MgSO_4 and finally filtered.

All other chemicals used were of the highest commercial grade available.

RESULTS AND DISCUSSION

The respiration rates of *Candida* 107 and *Saccharomyces carlsbergensis* were measured in the presence of *n*-alkanes, *n*-alk-1-enes, *n*-alkyl-1-bromides and *n*-alkan-1-ols (Fig. 1). In general, similar patterns of inhibition were observed with each series of compound and with each yeast suggesting that the toxicity was possibly due to a common effect on the yeast.

If solubility in water were a major factor in determining toxicity, then addition of a functional group to the alkane would enhance any common toxic effect by increasing the solubility of the derivative relative to that of the equivalent *n*-alkane, and in an homologous series of compounds, the maximum chain-length at which toxicity occurred would vary with the polarity of the functional group. The molar solubility of *n*-alkanes, *n*-alk-1-enes and *n*-alkan-1-ols (McAuliffe, 1964; Kinoshita, Ishikawa & Shinoda, 1958), when plotted as a logarithm against carbon chain-length, gives a series of parallel straight lines (Fig. 2). Values for the higher members of each series can be obtained by extrapolation (Fig. 2) although the effect of accommodation has been shown to affect the relationship between solubility and chain-length for alkanes longer than C_{10} (McAuliffe, 1969).

Fig. 1. Respiration rates of glucose-grown *Candida 107* (a) and *Saccharomyces carlsbergensis* (b) in the presence of 3.1 ml of 5% (v/v) emulsions of *n*-alkanes (○), *n*-alk-1-enes (□), *n*-alkyl-1-bromides (■) and *n*-alkan-1-ols (●).

Fig. 2. Solubility of *n*-alkanes (○), *n*-alk-1-enes (●) and *n*-alkan-1-ols (△) in water (data from McAuliffe, 1964; and Kinoshita *et al.* 1958).

The inhibition of yeasts growing on glucose by the four classes of compound (Table 1) showed that both the alkane-oxidizing yeasts (*Candida 107* and *C. tropicalis*) were affected similarly by *n*-alkanes, *n*-alk-1-enes and *n*-alkyl-1-bromides. With *n*-alkan-1-ols, *C. tropicalis* grew well on all substrates longer than C₁₁ and with *n*-undecan-1-ol growth also occurred if the culture was kept at room temperature (20 °C) and not shaken. Finnerty, Hawtrey &

Table 1. *Growth of yeasts on n-alkanes and derivatives in the presence and absence of glucose*

Medium as described in Methods; glucose at 25 g/l, alkanes and derivatives at 10% (v/v). All yeasts were grown for 1 day with the exception of *Candida* 107 which was grown for 3 days when alkanes and their derivatives were the only substrate. Growth was recorded on an arbitrary scale: — indicates no growth; + + + indicates good growth.

Yeast	No. of carbon atoms in substrate	Substrate							
		Alkane		Alkene		Alkyl bromide		Alkanol	
		With glucose	Alone	With glucose	Alone	With glucose	Alone	With glucose	Alone
<i>Candida</i> 107	8	—	—	.	.	.	.	.	.
	9	++	++	—	—	.	.	.	.
	10	+++	+++	—	—	—	—	.	.
	11	+++	+++	—	—	+++	—	.	.
	12	+++	+++	+++	+++	+++	—	—	—
	13	+++	+++	+++	+++	+++	—	—	—
	14	+++	+++	+++	+++	+++	—	+++	+++
<i>Saccharomyces carlsbergensis</i>	8	—	.	.	.	.	.	.	.
	9	—	.	—	—	.	.	.	.
	10	—	—	—	—	—	—	.	.
	11	+++	—	—	—	—	—	.	.
	12	+++	—	—	—	—	—	—	—
	13	+++	—	—	—	—	—	—	—
	14	+++	—	+++	—	+++	—	+++	—
<i>Candida tropicalis</i>	8	—	—	.	.	.	.	.	.
	9	++	+	—	—	.	.	.	.
	10	+++	+++	—	—	—	—	—	—
	11	+++	+++	—	—	+++	—	—	—
	12	+++	+++	+++	+++	+++	+	+++	+++
	13	+++	+++	+++	+++	+++	+	+++	+++
	14	+++	+++	+++	+++	+++	++	+++	+++

Table 2. *Relief of n-nonyl-1-bromide inhibition of respiration by alkanes and derivatives*

Respiration rates determined with an oxygen electrode. Both yeasts were grown on glucose prior to formation of washed suspensions.

Toxic substrate (0.5%, v/v, final concentration)	Non-toxic co-substrate (2.5%, v/v, final concentration)	Respiration rate (% endogenous rate*)	
		<i>Candida</i> 107	<i>Saccharomyces carlsbergensis</i>
<i>n</i> -Nonyl-1-bromide	None	0	21
	<i>n</i> -Tetradecane	87	70
	<i>n</i> -Hexadecan-1-ol	84	76
	<i>n</i> -Hexadecyl-1-bromide	90	69
	<i>n</i> -Dodec-1-ene	78	

* Endogenous rates of respiration: *Candida* 107, 17.4 nmol O₂/min/mg dry wt; *S. carlsbergensis*, 8.4 nmol O₂/min/mg dry wt.

Kallio (1962) have also shown that lowering the incubation temperature from 25 °C to 20 °C permitted two strains of *Micrococcus* to grow on *n*-decane whereas at the higher temperature they did not; they suggest that these results are obtained because of the lowered vapour pressure at 20 °C. With *Saccharomyces carlsbergensis* toxicity of the compounds

Fig. 3

Fig. 4

Fig. 3. Relief by *n*-tetradecane of *n*-heptane inhibition of respiration of *Candida* 107. The endogenous rate of respiration was 14.5 nmol O₂/min/mg dry wt. Each alkane was added as a 5% (v/v) emulsion in water. The final volume in the oxygen electrode chamber occupied by the alkanes was 3.1 ml.

Fig. 4. Effect of *n*-undecan-1-ol on the respiration rate of *Candida* 107, alone (○) and in the presence of an emulsion of either *n*-hexadecane (●) or *n*-hexadecan-1-ol (△), each at a final concentration of 1% (v/v).

persisted to longer chain-lengths than with the other two yeasts. Presumably, toxicity can be modulated if a yeast can oxidize these compounds as this will lower the effective concentration of the compound. *n*-Alk-1-enes, in the case of each yeast, appeared more toxic than predicted from the solubility results (Fig. 2) but the reason for this is obscure.

If the toxicity of an aliphatic compound is dependent upon its concentration in aqueous solution, then, in accordance with Henry's and Raoult's Laws, the addition of a non-toxic compound with which it is miscible would relieve the toxic effect by decreasing the vapour pressure of the toxic material and hence its concentration in the aqueous phase. Pasero, Duvnjak & Azoulay (1969) have shown relief of inhibition of growth of *Candida tropicalis* by intermediate chain-length fatty acids by adding sodium palmitate. Thus, relief of *n*-nonyl-1-bromide inhibition of respiration in *Candida* 107 and *Saccharomyces carlsbergensis* occurred with non-toxic members of all four groups of compounds (Table 2). Also relief of *n*-heptane toxicity on the respiration of *Candida* 107 by *n*-tetradecane (Fig. 3) and relief of *n*-undecan-1-ol toxicity by *n*-hexadecane and by *n*-hexadecan-1-ol (Fig. 4) reinforce the conclusion that solubility is a major influence on toxicity of alkyl compounds.

Besides being able to relieve the respiration of yeasts, the inhibition of growth of all three yeasts on glucose in the presence of toxic compounds is also reduced by adding a suitable amount of a non-toxic compound (Table 3). Non-metabolizable compounds were used to demonstrate this effect but obviously this relief would also occur if a metabolizable compound had been used. As a corollary to this experiment, if the solubility in water of a toxic aliphatic compound were decreased by mixing it with a biologically inert aliphatic material, the organism should now be able to grow on a previously toxic material. Thus, *Candida tropicalis* utilized *n*-undecan-1-ol, *n*-decan-1-ol and *n*-dec-1-ene as sole carbon sources for growth when these materials were mixed with the biologically inert hydrocarbon pristane (Miller & Johnson, 1966; Table 4). *n*-Octane appeared to support slow growth under similar conditions.

Table 3. *Relief of inhibition of growth of yeasts on glucose by non-metabolizable, non-toxic aliphatic compounds*

Conditions of growth as described in Table 1.

Yeast	Toxic compound	Concentration in medium (%, v/v)	Growth index	
			Glucose with toxic compound	Glucose with toxic compound and relieving compound at 10% (v/v)*
<i>Candida</i> 107	<i>n</i> -Decyl-1-bromide	1	—	—
		0.5	—	+++
		0.2	+	+++
		0.1	+++	+++
		0	+++	+++
<i>Saccharomyces</i> <i>carlsbergensis</i>	<i>n</i> -Decane	1	—	—
		0.5	—	++
		0.2	—	+++
		0.1	—	+++
		0	+++	+++
<i>Candida</i> <i>tropicalis</i>	<i>n</i> -Octane	0.5	—	+++
		0	+++	+++

* Relieving compounds: for *Candida* 107, *n*-hexadecyl-1-bromide; for *Saccharomyces carlsbergensis*, *n*-tetradecane; and for *C. tropicalis*, pristane.

Table 4. *Growth of yeasts on toxic aliphatic compounds in the presence of pristane*

Conditions of growth as described in Table 1.

Growth substrate	Concentration	Pristane (%, v/v)	Growth index	
			<i>Candida tropicalis</i>	<i>Candida</i> 107
None	—	10	—	—
Glucose	25 g/l	10	+++	+++
	25 g/l	—	+++	+++
<i>n</i> -Octane	2.5% (v/v)	10	+	—
	2.5% (v/v)	—	—	—
<i>n</i> -Dec-1-ene	5% (v/v)	10	++	—
	5% (v/v)	—	—	—
<i>n</i> -Undec-1-ene	5% (v/v)	10	—	—
	5% (v/v)	10	—	—
<i>n</i> -Decan-1-ol	0.25% (v/v)	10	++	.
	0.25% (v/v)	—	—	.
<i>n</i> -Undecan-1-ol	1.0% (v/v)	10	++	.
	1.0% (v/v)	—	—	.
<i>n</i> -Tridecan-1-ol	1.0% (v/v)	10	.	+++
	1.0% (v/v)	—	.	—

However, even with pristane present, *n*-undec-1-ene still could not be used for growth, which may account for the unexpected toxicity of this particular compound to the alkane-oxidizing yeasts. With *Candida* 107, the same relief of inhibition was only noted with *n*-tridecan-1-ol. Growth did not occur on any other materials mixed with pristane but this may be due to low growth rates of this yeast under shaking conditions (Thorpe & Ratledge, 1972).

These results imply that when micro-organisms are grown on commercial fractions of alkanes or petroleum hydrocarbons, small concentrations, probably up to 5 or 10% of the bulk of undesirable hydrocarbons or their derivatives are unlikely to be inhibitory to growth.

Either the potentially deleterious compound will be kept at too low a concentration in the medium to be toxic or alternatively it may be slowly metabolized. It is the concentration of long-chain aliphatic compounds in aqueous solution coupled with the ability of the organism to metabolize such materials that are the main factors determining their toxicity. The toxicity of intermediate chain-length aliphatic compounds is not explicable in terms of a common effect upon biological membranes as membrane-lipids would always preferentially absorb these hydrophobic compounds no matter what their solubility was in the aqueous phase. Indeed, yeasts growing on *n*-alkanes have been shown to adhere to droplets of alkane emulsion (Bakhuis & Bos, 1969). The explanation for the toxicity of alkanes, however, may lie in their ability to bind with proteins and presumably cause inhibition of metabolism (Mohammadzadeh-k, Feeney, Samuels & Smith, 1967). Bell (1971), examining the toxicity of fatty acids to *Candida tropicalis* growing on alkanes, also concluded that the solubility of the acids in the aqueous phase was the major factor in determining their toxicity. Again, this was attributed to a non-specific effect on enzymes rather than on the yeast membranes.

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