Anaerobic specimen transport system

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Summary. Degassed dimethyl silicone oil may be used as a transport medium for specimens containing anaerobic microorganisms.

Successful isolation of oxygen-sensitive anaerobic bacteria from clinical or environmental specimens requires anaerobic transportation of the specimen from collection site to laboratory. Methods used to obtain anaerobiosis include direct inoculation into pre-reduced media, immersion in oxygen-free gas, or catalytic oxygen reduction[1,2]. We have found that dimethyl silicone oil free of dissolved oxygen may be used as a medium for transporting oxygen-sensitive microbes.

Dimethyl silicone oil is a biologically inert, thermally stable fluid of density 0.97 g/ml. The solubility of oxygen in the oil is 26 ml O_2/dl, which is approximately 8 times that in water (3.16 ml O_2/dl). An oxygen electrode was used to determine the oxygen activity in water and dimethyl silicone oil at a variety of oxygen partial pressures. The equilibrium oxygen activities in a 2-phase water/oil system were identical in each phase. Since oxygen activity is the product of concentration and an activity coefficient, it follows that the activity coefficient of oxygen dissolved in dimethyl silicone oil is approximately $\frac{1}{8}$ that in water. Deoxygenated dimethyl silicone oil in contact with an aqueous phase will act as an 'oxygen sink' causing a decrease in the aqueous oxygen activity and concentration.

The anaerobe *Clostridium innocuum* ATCC 14501 was used to test this concept. Approximately 20 ml dimethyl silicone oil, with a viscosity of 1000 centistokes (Dow Corning 200 Fluid) was degassed and sterilized by evacuation at 130 °C for 4 h. Cultures were grown in thioglycollate broth medium and viable counts were made using pre-reduced thioglycollate agar in bottles gassed with anaerobic-grade CO_2. Bacterial cultures were diluted 1:10 with aerated nutrient broth and held for 5 min to simulate exposure to air during specimen collection. 2 ml samples were then incubated for 24 h at 23 °C in an empty tube or immersed in degassed dimethyl silicone oil. The table shows that immersion of samples in degassed silicone oil prevented a decline in viable counts.

Effect of degassed dimethyl silicone oil on survival of *Clostridium innocuum* ATCC 14501

	CFU/ml* (average of duplicates)
Original suspension in nutrient broth	5.8×10^4
Suspension under degassed silicone oil 24 h	6.8×10^4
Suspension exposed to air 24 h	9.5×10^2

* Colony forming units/ml.

The separation of dimethyl silicone oil from specimens was studied using guinea-pig tissues. 1 g samples of blood, kidney, muscle, liver or mesentery tissues, and swabs of intestinal contents were immersed in the oil. All tissues sank to the bottom of the tubes, consistent with the relative densities of oil and tissue. Tissues were removed with forceps and placed in nutrient broth. Gentle shaking caused the tissues to sink into the aqueous medium leaving a thin layer of oil on the surface. Oil also quickly separated from specimens when drops of blood and swabs were placed in the broth. Normal procedures may be used to Gram stain broth cultures after immersion in oil. Gram stains may be made on tissues or swabs after immersion in oil if care is taken to rub or roll the tissue or swab on the slide, in order to ensure the slide is wet.

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The mutagenic mode of action of benzimidazole

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Summary. By using a bacterial mutant strain with a known restricted reversion pattern, it has been shown that benzimidazole induces an unidirectional base change also in vivo. The in vitro demonstrated strict G → A transition has thus been confirmed.

Benzimidazole has been shown to induce mutations of the base substitution type in *Salmonella typhimurium*[2]. This behaviour could have been expected because of the purine-like structure of this compound. It could then be demonstrated, that indeed benzimidazole is incorporated into the nucleic acids of *Escherichia coli*[3,4]. An investigation into the possible mode of action yielded the somewhat unexpected result that benzimidazole is a very specific mutagenic agent; it had be concluded from in vitro results on the transcriptional use of benzimidazole nucleotide and on the translation of benzimidazole containing codons that this compound induced strictly G → A transitions in this direction only[5]. In view of the theoretical importance of such a mode of action it seemed indicated to test this conclusion by another way, if at all possible in an in vivo assay. One possibility would have been to investigate the reversion characteristics of benzimidazole induced mutants: such mutants should not be reverted by benzimidazole itself nor by hydroxylamine, which has a similar specificity. A much less tedious procedure, however, was found by using bacterial mutants whose reversion patterns in terms of possible base changes are already known. In its simplest application it should only be necessary to perform a mutagenicity test with such a mutant in order to obtain a clearcut 'yes' or 'no'

Table 1. Plate tests with benzimidazole and 2-aminopurine for mutagenic activity on strains *E. coli* trp A 46 and *S. typhimurium* his G 46

Compound	Concentration (M)	Colonies per plate Experiment 1 A 46	G 46	Experiment 2 A 46	G 46	Relative mutagenicity Experiment 1 A 46	G 46	Experiment 2 A 46	G 46	Mutagenicity A 46	G 46
None (spont. rev.)	–	6.0±1.5	4.5±1.5	7.0±2.0	1.5±1.0	1.0	1.0	1.0	1.0	–	–
2-AP	10^{-3}	–	–	9.5	2.0	–	–	1.4	1.3	–	–
	10^{-4}	24.0	12.0	21.0	3.5	4.0	2.5	3.0	2.3	+	+
	10^{-5}	–	–	6.5	2.0	–	–	0.9	1.3	–	–
	10^{-6}	6.0	5.5	–	–	1.0	1.2	–	–	–	–
	10^{-8}	6.5	3.5	–	–	1.1	0.8	–	–	–	–
Benzimidazole	10^{-1}	–	–	0.5	1.5	–	–	0.1	1.0	–	–
	10^{-2}	0.0	16.0	2.5	4.0	0.0	3.6	0.4	2.7	–	+
	10^{-3}	2.5	3.5	8.0	1.0	0.4	0.8	1.1	0.7	–	–
	10^{-4}	6.0	5.5	5.5	2.0	1.0	1.2	0.8	1.3	–	–
	10^{-5}	8.5	5.0	7.5	2.0	1.4	1.1	1.1	1.3	–	–
	10^{-6}	7.5	4.0	–	–	1.2	0.9	–	–	–	–

A significant ($p<0.05$) increase in colony number is reached at a relative mutagenicity of 1.5. The number of plates used was determined accordingly in advance[11] in order to allow the statistically significant detection of mutagenic activity at this level.

Table 2. Fluctuation tests with benzimidazole, hydroxylamine and methylmethanesulfonate for mutagenic activity on strains *E. coli* trp A 46 and *S. typhimurium* his G 46

Compound	Concentration (M)	% Tubes turbid[a] Experiment 1 A 46	G 46	Experiment 2 A 46	G 46	Experiment 3 A 46	G 46	Mutagenicity A 46	G 46
None (spont. rev.)	–	50	18	83	50	40	6	–	–
MMS	$8\cdot10^{-6}$			10	10			–	–
	$4\cdot10^{-6}$			99**	64*			+	+
	$2\cdot10^{-6}$			100**	64*			+	+
	$8\cdot10^{-7}$			96**	69**			+	+
Benzimidazole	10^{-3}	0	8	43	11	–	–	–	–
	10^{-4}	10	10	90	14	44	8	–	–
	$3\cdot10^{-5}$	–	–	–	–	40	6	–	–
	10^{-5}	36	26	90	20	35	12	–	–
	$3\cdot10^{-6}$	–	–	–	–	48	10	–	–
	10^{-6}	34	30*	88	91**	46	19**	–	+
	$3\cdot10^{-7}$	–	–	–	–	48	15*	–	+
	10^{-7}	44	16	86	96**	40	10	–	+
Hydroxylamine	(0.5)[b]	–	–	–	–	35[c]	24**[c]	–	+

[a] Statistical significance (χ^2-test): * $p<0.05$, ** $p<0.01$. [b] Pretreatment with hydroxylamine according to Yanofsky et al.[9]. [c] Calculated to 100% survival.

answer about the proposed base specificity of benzimidazole. A bacterial system, where a number of well characterized mutants exist, is the tryptophan synthetase A protein of *E. coli*[6]. We decided thus to test benzimidazole mutagenicity in 1 of these strains, and from the description of its attributes we chose strain A 46 for our experiments.

The tests were thus performed with the 2 strains *Salmonella typhimurium* his G 46 and *Escherichia coli* trp A 46. 2 types of experiments should allow the comparison between these 2 strains. In the 1st series we tested benzimidazole mutagenicity in the plate test, where known concentrations of the test agents are incorporated into the soft agar. This was done according to the procedures described by Ames[7]. As a positive control we used in this series the mutagenic base analogue 2-aminopurine, also in order to check the respective sensitivities of the 2 strains. In a 2nd series we used the more sensitive fluctuation test as described by Green et al.[8]. In this case we included methylmethanesulfonate as the positive control agent. The results of these experiments are summarized in the tables 1 and 2. It can be seen that strain *E. coli* A 46, although being more sensitive to 2-aminopurine and MMS than strain *S. typhimurium* G 46, shows in no case a significant positive response, indicating the mutagenicity of benzimidazole.

Since strain *E. coli* A 46 has a very restricted reversion pattern, this behaviour could have been predicted assuming the aforementioned unidirectional activity of benzimidazole. Reversions in this strain can occur at the original site of mutation from glutamic acid to valine, alanine and glycine, respectively, which changes correspond to base changes from A to T, C and G, respectively, in the 2nd codon position. Furthermore a second-site reversion from tyrosine to cysteine can also restore prototrophy; this corresponds to a change from A to G in the 2nd position of the respective codon[9]. As our previous studies indicate a G→A transition it is clear that this strain cannot be reverted by benzimidazole, if our in vitro results hold true also in vivo. The lack of mutagenic activity of benzimidazole in this strain thus indicates the validity of this assumption. In contrast to the results on the *E. coli* strain, *S. typhimurium* his G 46, which is said to respond probably to all possible base changes[7], could consistently be reverted by benzimidazole. The sensitivity of both strains towards the positive control agents was similar, so that the non-respon-

siveness of strain *E. coli* A 46 cannot be accounted for by its insensitivity against mutagenic agents. A control experiment to test the specificities of the respective strains was also set up. Hydroxylamine effects the single base change C → T, which is an analogous change to the G → A change effected by benzimidazole. Hydroxylamine should therefore be able to revert *S. typhimurium* G 46, but not *E. coli* A 46. For *E. coli* a very weak activity at the borderline of significance has been reported[9], whereas for *S. typhimurium* hydroxylamine has been described as a mutagen[7]. However, in a more recent publication, no mutagenic activity was found by using hydroxylamine hydrochloride in the Ames test[10].

We thus tested hydroxylamine according to the scheme given by Yanofsky et al.[9] in a fluctuation test, as the plate test has to be considered an unsuitable method for assaying hydroxylamine mutagenicity[12]. The treatment with 0.5 M hydroxylamine under addition of 10 mM EDTA, however, yielded a significant increase in the number of turbid tubes, but only so with strain *S. typhimurium* his G 46. The reversion of this strain is therefore possible by the transition C → T, whereas this transition does not revert the *E. coli* strain trp A 46. We can thus conclude that benzimidazole can effect only the GC → AT transition, as it reverts the *Salmonella* strain, which is also reverted by hydroxylamine, but cannot revert the *E. coli* strain, which cannot be reverted by hydroxylamine, too. The compound differs though from hydroxylamine in 1 important aspect; hydroxylamine acts directly on the DNA without the necessity of an active metabolism, whereas benzimidazole has to be metabolically converted to desoxyriboside and to be incorporated into DNA, before being able to exert its mutagenic activity. It shares however with hydroxylamine the singular property of inducing directed base changes in DNA, and it might be of interest to investigate its behaviour and molecular biology not only in bacteria but also in mammalian cell cultures.

Abbreviations: A, adenine; C, cytosine; G, guanine; T, thymine.

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Sex attractants for male *Heliothis armigera* (Hbn.)

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Summary. (Z)-11-Hexadecenal and (Z)-11-tetradecenal were found to be sex attractants of male *Heliothis armigera* (Hbn.).

Heliothis armigera (Hübner) (Lep. Noctuidae) is a polyphagous insect widespread in Asia, Africa, Europe and Australia[4]. Because of its importance as a pest of field crops it was of interest to find attractants that could be used for monitoring its populations.

In evaluating synthetic compounds which cause electroantennogram response in insects[5], we found 3 aldehydes which elicited a significant electroantennogram response in male *H. armigera*. These were (Z)-11-hexadecenal [(Z)-11-HDA], (Z)-11-tetradecenal [(Z)-11-TDA] and (Z)-9-tetradecenal [(Z)-9-TDA]. (Z)-11-HDA and (Z)-9-TDA are known sex pheromone components of *H. virescens* (Fabricius)[6,7] and the former is a component of *H. zea* (Boddie) pheromone[6]. The behavioral reaction of male *H. armigera* to the 3 aldehydes was studied under laboratory and field conditions.

The experimental compounds (> 95% pure by capillary column gas chromatography) absorbed on 1 cm^2 filter paper (Whatman No. 1) were placed on top of a 30 × 30 × 30 cm screen cage containing 100 males. The males were observed for sexual reaction, i.e. exploratory flight towards the odor source and mating attempts. Each compound at 0.1, 1, and 10 µg dose was tested on 3 different cages. For field tests, the compounds were impregnated in 1 × 0.5 × 0.1 cm natural rubber and placed in traps[8] in cotton fields. 1 trap per treatment was used in tests 1-3 and 5 traps per treatment in tests 4 and 5. Traps were inspected daily and rotated from 1 trap location to the next[9].

(Z)-11-HDA and (Z)-11-TDA at 1 and 10 µg caused sexual excitation in caged males, but the latter did not stimulate the males at the lower concentration (table 1). (Z)-9-TDA had no stimulatory effect; moreover, it inhibited sexual

Table 1. Qualitative response* of caged male *Heliothis armigera* to synthetic compounds

Compound**	Amount loaded on filter paper (µg)		
	0.1	1.0	10.1
(Z)-11-HDA	+	+	+
(Z)-11-TDA	−	+	+
(Z)-9-TDA	−	−	−
(Z)-11-HDA + (Z)-11-TDA 10:1		+	
(Z)-11-TDA + (Z)-11-HDA 10:1		+	
(Z)-11-HDA + (Z)-9-TDA 10:1		−	
(Z)-11-TDA + (Z)-9-TDA 10:1		−	

* +, positive sexual reaction, in each replicate, of at least 5% of the males at one time during 1 min exposure to the chemical; −, no sexual response in all replicates. ** HDA-hexadecenal; TDA-tetradecenal.