

Appraisal of Anotox, a new anaerobic atmospheric detoxifying agent for use in anaerobic cabinets

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SUMMARY Using *Clostridium sporogenes* cultures, inhibitory effects of its gaseous metabolic end products on the activity of palladium Deoxo catalyst and on surface growth of some clinically significant anaerobes were demonstrated. A new product "Anotox" was shown to adsorb these metabolites, which enabled uninhibited growth of anaerobes, and prolonged the life of the catalyst.

The surface growth of anaerobes requires the provision of an oxygen-free atmosphere, usually in a jar or cabinet, and for this purpose Deoxo palladinised alumina (Engelhard) is commonly used to remove oxygen by catalysis with hydrogen. Catalyst activity in the anaerobic jar may be monitored manometrically; thus failure to achieve a secondary vacuum after evacuation/replacement occurs if the catalyst is inactivated by products of bacterial metabolism, notably hydrogen sulphide.

During periodic removal of the front panel of an anaerobic cabinet for servicing, it was noted that the cabinet atmosphere was becoming more and more malodorous and toxic, causing irritation to the eyes and respiratory mucus membranes. It seemed likely that this atmospheric toxicity, doubtless attributable to the steady accumulation of volatile fatty acids, alcohols and hydrogen sulphide, might not only cause premature loss of catalyst activity, but also have an adverse effect on the surface growth of anaerobic organisms.

The aim of the present study was to investigate these aspects of cabinet atmospheric pollution. As part of the work evaluations were made of a new product—"Anotox"—a substance which is designed to absorb volatile fatty acids and hydrogen sulphide.

Material and methods

EQUIPMENT, REAGENTS AND MEDIA

Anaerobic jars used were the standard Don Whitley Scientific model of 10 plate capacity. They were operated by a standard evacuation/replacement

technique using an atmosphere of 90% H₂ and 10% CO₂.

Chromatographic analyses were performed on a Pye Unicam series 104 gas liquid chromatograph.

Anotox (Don Whitley Scientific) is a granular material based on alkaline-activated carbon, impregnated with ferric hydroxide which is incorporated to bind hydrogen sulphide chemically and prevent its desorption from the carbon surface. Anotox has a surface area of approximately 1000 m²/g, and under anaerobic conditions adsorbs 5-10% of its own weight of H₂S, in addition to a wide range of volatile fatty acids.

Hydrogen sulphide indicator strips were prepared in the laboratory in the usual way by impregnating filter paper strips with a saturated aqueous solution of lead acetate. H₂S was manufactured by the action of dilute hydrochloric acid on ferrous sulphide, the gas being collected over warm water in an inverted measuring cylinder or directly into a rubber bladder.

Horse blood agar plates (Lab M Ready Poured) were used for all surface culture work including surface viable counts.¹ When comparative experiments were performed plates of the same batch number were used.

Cooked meat medium cultures were grown in 100 ml aliquots of broth containing 4 g meat particles (Lab M).

Brain heart infusion broth (Difco) plus 10% glycerol was used for the preparation of standard suspensions of organisms.

ORGANISMS

Organisms (which were all stock laboratory strains) were *Clostridium sporogenes*, *Cl butyricum*, *Cl sordellii*, *Cl perfringens*, *Cl tetani*, *Bacteroides fragilis*, *B thetaiotaomicron*, *B capillosus*, *Peptostreptococcus anaerobius* and *Veillonella parvula*.

PRODUCTION OF "TOXIC" ANAEROBIC ATMOSPHERE

Experiments designed to determine the effect of volatile products of metabolism on the Deoxo catalyst used a stock strain of *Cl sporogenes* because this proteolytic organism produces both an abundance of H_2S and a variety of alcohols and volatile acids.

Two sets of horse blood agar plates were heavily inoculated with the organism to produce a lawn of growth. These plates were divided between two anaerobic jars, both of which were provided with 3 g active catalyst. One of the jars contained, in addition, 3 g of Anotox held in a wire gauze sachet mounted alongside the catalyst. Lead acetate indicator paper for the qualitative detection of H_2S was included in both jars. After 2 days' incubation at $37^\circ C$ the atmospheres inside the jars were examined for their "toxicity." In addition, the cold catalyst and the Anotox granules were examined for the presence of volatile fatty acids, and the catalyst in each was tested for activity both manometrically, and biologically using surface cultures of *Cl tetani*.

DETECTION OF VOLATILE FATTY ACIDS IN JAR ATMOSPHERE

The atmospheres of anaerobic jars (with and without "Anotox") were examined for the presence of volatile fatty acids and H_2S . The fatty acids were isolated by evacuation of the atmospheres through a wash bottle containing 5 ml 1 N NaOH. This solution was then acidified, extracted and concentrated for gas liquid chromatographic analysis.

The results of these analyses (Figs. 1 and 2) showed that the atmosphere in jars without Anotox contained detectable amounts of acetic, propionic, butyric, isovaleric, valeric and isocaproic acids, whereas the atmospheres from jars incubated with Anotox contained only a small amount of acetic acid. As judged by gross blackening of the lead acetate indicator paper strips which occurred only in jars without Anotox, abundant H_2S was present in these but little H_2S accumulated in jars containing Anotox.

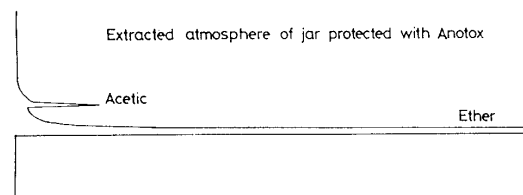


Fig. 1 Chromatogram of atmosphere from jar with Anotox.

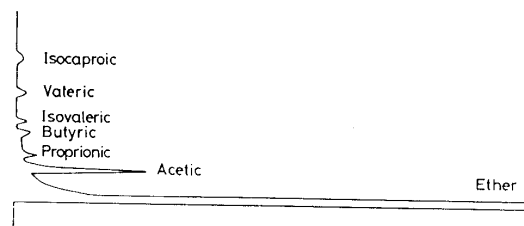


Fig. 2 Chromatogram of atmosphere from jar without Anotox.

POST-INCUBATION ACTIVITY OF COLD CATALYST

The cold catalyst sachet that had been incubated in the presence of Anotox retained its full activity, producing a substantial secondary vacuum in only five minutes, whereas the "unprotected" catalyst was inactive (Fig. 3). Not surprisingly, therefore, the jar containing the active catalyst supported surface growth of *Cl tetani* whereas the one containing the inactive catalyst did not.

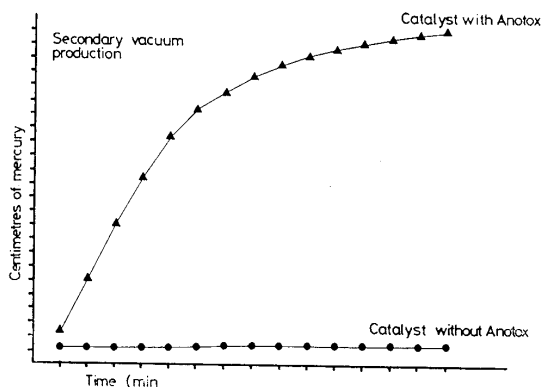


Fig. 3 Comparison of catalyst activity with and without Anotox after incubation for 48 h with *Cl sporogenes* cultures.

DETECTION OF VOLATILE FATTY ACIDS ON COLD CATALYST AND ON ANOTOX

The catalyst and Anotox granules from jars incubated with *Cl sporogenes* were extracted with 50% H_2SO_4 and the extract analysed chromatographically for volatile fatty acids. Extracts of fresh (unused) catalyst and Anotox served as controls.

The acid extract of cold catalyst from jars without Anotox contained a range of fatty acids (Fig. 4), whereas that from the catalyst incubated in the presence of Anotox contained only a little acetic acid. Not surprisingly, the extract obtained from exposed Anotox contained substantial amounts of a

variety of volatile fatty acids (Fig. 5). It is pertinent to note that the pattern of fatty acids present in the extracts from exposed Anotox was the same as that produced by *Cl sporogenes* in fluid culture.

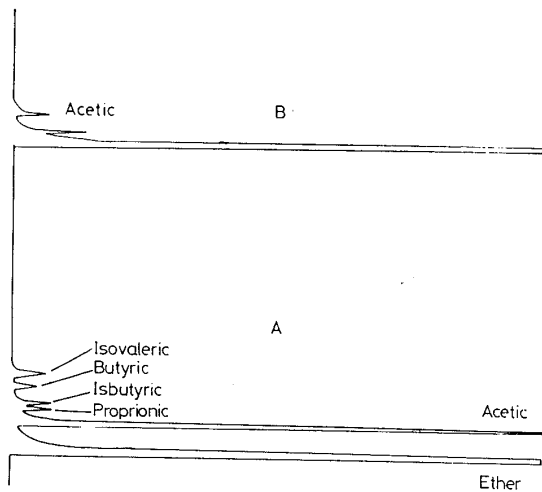


Fig. 4 Chromatogram of volatile fatty acids present on catalyst (A) without and (B) with Anotox.

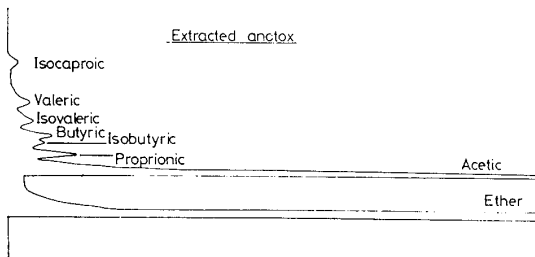


Fig. 5 Chromatogram of Anotox extract after incubation with *Cl sporogenes* cultures.

EFFECT OF HYDROGEN SULPHIDE ON COLD CATALYST, AND ON COLD CATALYST PLUS ANOTOX

A volume of 200 ml of hydrogen sulphide was manufactured as previously described. Half of this volume was added to an anaerobic jar containing 3 g active catalyst; the other half was added to a similar jar containing, additionally, 3 g Anotox. Both jars had previously been "put up" by the evacuation/replacement technique so that the secondary partial vacuum was sufficient to draw in the H_2S . After incubation at $37^\circ C$ for 48 h the activity of both catalysts was determined manometrically; they were then heated at $160^\circ C$ for one hour and retested. There was complete inactivation of cold catalyst

alone, but catalyst incubated with Anotox retained partial activity on direct testing, and this reverted to full activity after heating (Fig. 6).

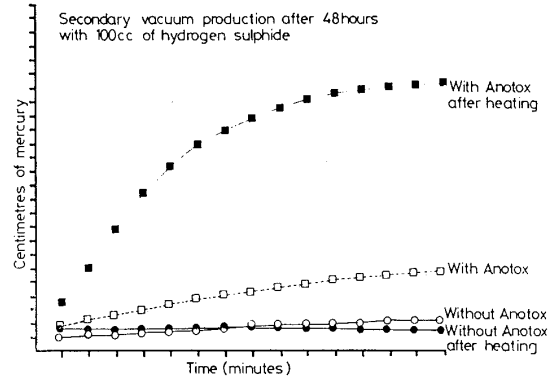


Fig. 6 Comparison of catalyst activity after exposure to 100 ml H_2S with and without Anotox before and after heating.

These findings confirm the manufacturer's claim that Anotox adsorbs over 5% its own weight of H_2S (from Avogadro's Number the calculations showed that 3 g would absorb 99 ml).

The physical nature of hydrogen sulphide poisoning of palladium catalyst was elucidated by Maxted,² and substantiated by Mukhlenov *et al*³ who agreed that palladium adsorbs hydrogen sulphide on to its surface in amounts normally insufficient to suppress completely its occlusive power for hydrogen. After a period at room temperature the H_2S dissociates leaving the sulphur atom attached to the palladium surface. It was suggested that the sulphur combines with the palladium to form Pd_4S , so that one atom of sulphur inactivates four atoms of palladium. However, attachment of the complete H_2S molecule blocks more active sites of catalysis than the dissociated form, and is consequently more inhibitory to the catalysts occlusive power for hydrogen. Dissociation to the less inhibitory form may be accelerated by heating at temperatures above $100^\circ C$. After repeated exposure to H_2S the active sites are progressively blocked with sulphur so that the activity of the catalyst is gradually decreased until a point is reached when, despite heating, the catalyst is irreversibly poisoned.

To test this theory of catalyst poisoning and to determine the protective power of Anotox, an extended experiment was undertaken involving exposure of the catalyst, with and without Anotox, to 10 plate-cultures of *Cl sporogenes* during a series of two-day incubation cycles. At the end of each

cycle the catalyst was tested for its ability to draw a secondary vacuum, heated at 160°C for one hour, and then tested again. This procedure was repeated many times (occasionally with a three-day cycle to accommodate weekends) until irreversible poisoning had occurred. New plate cultures of *Cl sporogenes* were used periodically.

The results of this study, summarised in Fig. 7, showed that after the first cycle, unprotected catalyst was inactive upon immediate testing, but was restored to full activity after heating. The catalyst protected with Anotox was fully active before heating; this was to be expected in the light of previous results. After cycles 2 and 3 it was clear that the protected catalyst had developed an increasing "lag phase" before active catalysis commenced; this delay would correspond to the conversion of H₂S to the less "poisonous" dissociated form with a consequent increase in catalytic activity; after heating full activity was restored. The unprotected catalyst continued to show no activity upon immediate testing, but was restored to full activity after heating.

This state of affairs continued through the following six cycles, when the unprotected catalyst showed evidence of decreased activity even after heating. After a total of 39 days' incubation, the unprotected catalyst remained totally inactive after heating and was thus irreversibly poisoned. The protected catalyst, however, retained activity after heating.

EFFECT OF VOLATILE FATTY ACIDS ON COLD CATALYST

As shown in Fig. 5, Deoxo catalyst incubated in the presence of *Cl sporogenes* adsorbed volatile fatty acids. To determine whether fatty acids alone had any adverse effect upon the activity of the catalyst, it was exposed to standard solutions of individual volatile fatty acid, both at room temperature and at 37°C under aerobic and anaerobic conditions. Even after long periods of exposure the catalyst showed no loss of activity. Interestingly, heating at 160°C for one hour did not greatly reduce the amount of the volatile fatty acids present.

EFFECT OF "TOXIC" ATMOSPHERE ON SURFACE GROWTH OF ANAEROBES

Two cooked meat broths were inoculated with the stock strain of *Cl sporogenes*, and placed in two anaerobic jars. Both jars contained 3 g active catalyst and in addition one contained Anotox. Lead acetate paper was attached to the inside of both jars by means of laboratory autoclave tape, the jars were "put up" and incubated at 37°C for 72 h. After incubation the jar atmospheres were collected by vacuum pump evacuation into a rubber bladder. To control this procedure the same collection method was applied to a jar containing pure 90% H₂-10% CO₂ gas mixture.

Tenfold dilutions (10⁻¹ to 10⁻⁶) of the organisms listed below were prepared in brain heart infusion

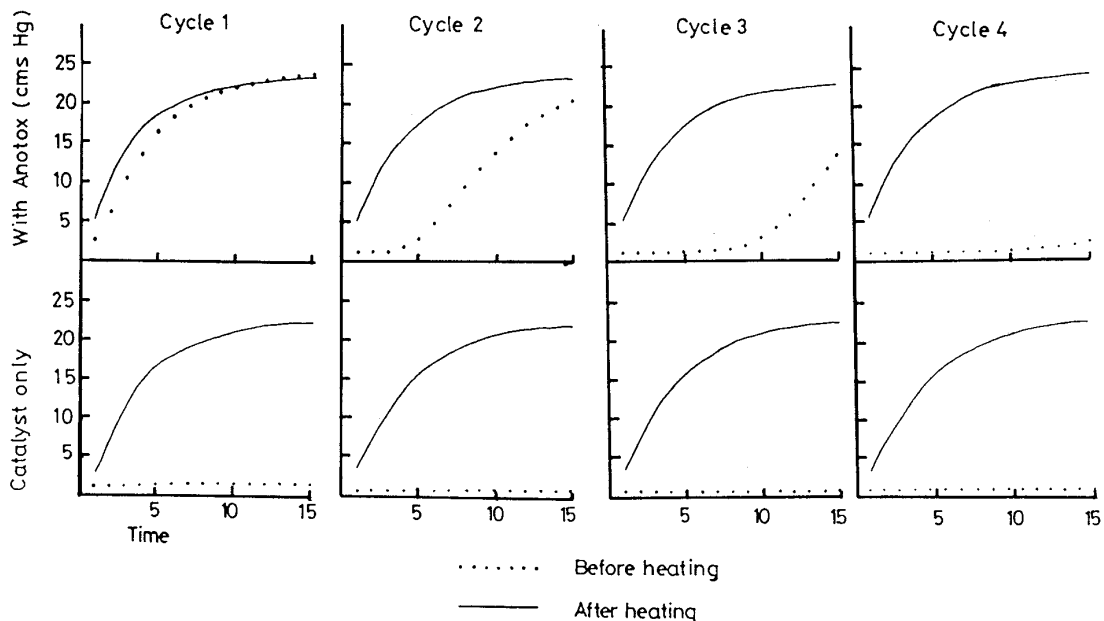


Fig. 7 Comparison of catalyst activity with and without Anotox over extended period of incubation with *Cl sporogenes* cultures.

broth, and a 20 μ l aliquot of each inoculated on to three blood agar plates. The three batches of plates were placed in separate anaerobic jars containing active catalyst and the jars evacuated to -60 cm of mercury. The atmosphere from the jar incubated with *Cl sporogenes* cooked meat cultures without Anotox was introduced into the first jar. The second jar contained the atmosphere from the jar with added Anotox, while the control jar contained the fresh gas mixture collected through the pump. All three jars developed a secondary vacuum of about 10 cm of mercury, and this was replaced with the remainder of the respective gases. The jars were incubated at 37°C for 48 h.

Results

After incubation, surface viable counts were made and the results of the three different systems compared. As is clear from Table 1 there was comparable growth in both the control jar and the jar containing the Anotox-adsorbed "protected" atmosphere. There was, however, a notable failure of organisms to grow in the jar that contained the unabsorbed "sporogenes" atmosphere, although growth of *Cl sordellii* confirmed that anaerobiosis had been achieved.

Cultures that had not grown were tested for viability by incubation in an anaerobic cabinet for 24 h (Table 1).

Thus, it seems that the "sporogenes atmosphere" was inhibitory to the growth of the anaerobes, but not bactericidal, except perhaps, for *Cl tetani* and *Cl butyricum*.

EFFECT OF VOLATILE FATTY ACIDS ON SURFACE GROWTH OF ANAEROBES

Two sets of blood agar plates were inoculated and incubated in separate anaerobic jars at 37°C for

Table 1 Surface viable counts (\log_{10}) in different atmospheres

Organism	Gas from jar with Anotox	Gas from jar without Anotox		Control
		Initially	After further incubation*	
<i>B fragilis</i>	6.54	No growth	5.47	6.30
<i>B thetaiotaomicron</i>	6.23	No growth	6.25	6.27
<i>B capillosus</i>	3.30	No growth	2.39	3.23
<i>P anaerobius</i>	5.04	No growth	2.30	5.11
<i>V parvula</i>	6.69	No growth	3.00	6.69
<i>Cl butyricum</i>	3.00	No growth	No growth	3.00
<i>Cl sordellii</i>	6.11	5.56	5.56	6.17
<i>Cl perfringens</i>	3.30	3.30	3.30	3.30
<i>Cl tetani</i>	Growth	No growth	No growth	Growth

*Same plates after incubation for a further 24 h in an anaerobic cabinet.

24 and 48 h with the same test organisms referred to above. One set was exposed throughout incubation to 5 ml of the standard volatile fatty acid solution which contains acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic and caproic acids.

EFFECT OF HYDROGEN SULPHIDE ON SURFACE GROWTH OF ANAEROBES

Six sets of plates inoculated with the same test organisms were "put up" in six anaerobic jars. When catalysis was complete a partial vacuum was drawn by pump for replacement by the required volume of H₂S. The final concentrations of H₂S in the jar atmospheres were 1%, 2%, 3%, 4% and 5%. The sixth jar was a control without added H₂S. All jars were incubated at 37°C for 48 h.

The results of these experiments involving exposure of freshly inoculated plates of anaerobes to the volatile fatty acid mixture, and to increasing concentrations of H₂S are summarised in Tables 2 and 3.

Table 2 Surface viable counts (\log_{10}) after exposure for 48 h to volatile fatty acids

Organism	Control jar	Test jar
<i>B fragilis</i>	6.47	6.34
<i>B thetaiotaomicron</i>	6.23	6.20
<i>B capillosus</i>	5.00	4.36
<i>P anaerobius</i>	7.23	7.11
<i>V parvula</i>	7.84	7.75
<i>Cl butyricum</i>	4.30	4.47
<i>Cl sordellii</i>	6.30	6.47
<i>Cl perfringens</i>	4.47	4.00
<i>Cl tetani</i>	+++ swarm	± swarm

Table 3 Surface viable (\log_{10}) counts after exposure to concentrations of hydrogen sulphide

Organism	%H ₂ S			
	Control	1	2	3-5
<i>B fragilis</i>	6.49	6.47	6.47	No growth
<i>B thetaiotaomicron</i>	6.27	6.20	6.23	"
<i>B capillosus</i>	3.95	3.95	3.04	"
<i>P anaerobius</i>	7.17	7.11	6.77	"
<i>V parvula</i>	7.84	7.86	7.73	"
<i>Cl butyricum</i>	4.00	3.00	2.30	"
<i>Cl sordellii</i>	6.69	6.30	6.00	"
<i>Cl perfringens</i>	6.00	6.00	4.60	"
<i>Cl tetani</i>	+++ swarm	+++ swarm	+++ swarm	"

Exposure of the cultures to mixed volatile fatty acids was without significant effect on their growth. H₂S, however, inhibited surface growth at concentrations above 2%. It thus seems clear that the growth-inhibiting properties of the *Cl sporogenes* atmosphere is due either to H₂S, to another com-

ponent not yet demonstrated, or to a combination of components. This warrants further study, since the inhibiting concentrations of H₂S represent concentrations of greater than 20 000 ppm which are unlikely to be achieved under normal conditions in an anaerobic jar, but may very well accumulate in an anaerobic cabinet.

Discussion

This study has demonstrated that the accumulation of certain gaseous metabolic end products of bacterial metabolism in the atmosphere of the anaerobic jar may reach levels of toxicity that prevent the surface growth of some clinically significant anaerobes. The atmosphere of anaerobic cabinets is especially prone to the accumulation of these metabolites, and their removal both benefits the surface growth of these anaerobes and prolongs the life of the anaerobic catalyst. The most important intoxicant appears to be hydrogen sulphide. Other methods have been described for removal of H₂S from the atmosphere of anaerobic glove boxes,^{4,5} but their adsorptive power for other anaerobic metabolites has not been investigated. A recently developed commercially available cabinet (Don Whitley Scientific) incorporates Anotox in a sachet alongside the catalyst in the chamber; it also recycles the cabinet atmosphere externally through an aluminium cylinder containing Anotox. The performance of this cabinet has been reported previously.⁶

The present study has demonstrated the need for an atmospheric detoxifying agent when large loads of actively metabolising anaerobes may lead to the accumulation of products (especially H₂S) inhibitory to surface growth. The present findings are in keeping with the theory of H₂S poisoning of the palladium catalyst, and have provided a practical demonstration of its gradual inactivation leading finally to irreversible poisoning. It has also been shown that

Anotox substantially prolongs the active life of the catalyst by adsorbing H₂S, although comparisons of Anotox with plain activated charcoal, which also adsorbs H₂S, have not been made. The study has further demonstrated the presence of volatile fatty acids in the anaerobic atmosphere.

An interesting observation was made concerning the laboratory autoclave tape (3M autoclave tape No 1222). When exposed to the volatile fatty acids present in the atmosphere it developed darkened stripes across it as it does when used for its normal purpose of testing steam penetration in an autoclave.⁷

I am indebted to Dr AT Willis for his interest and guidance, to Mr KD Phillips for his advice on technical aspects, to Mrs M Senjack for her secretarial skills and finally to Mr J Harrison for production of the plates.

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