STUDIES ON THE EFFECT OF THE PHENOLIC ANTIOXIDANT BUTYLATED HYDROXYANISOLE ON *STAPHYLOCOCCUS AUREUS* WOOD 46 ©

by

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A la mémoire de

Samir A. Saheb (1942-1981).

SHORT TITLE:

EFFECT OF BUTYLATED HYDROXYANISOLE ON *STAPHYLOCOCCUS AUREUS*.

ABSTRACT

Ph.D. Richard Degré Microbiology

STUDIES ON THE EFFECT OF THE PHENOLIC ANTIOXIDANT BUTYLATED HYDROXYANISOLE ON STAPHYLOCCOCCUS AUREUS WOOD 46

The antimicrobial activity of the phenolic antioxidant butylated hydroxyanisole (BHA) has been known for several years but the lack of knowledge of its specific effects on bacterial metabolism prompted us to study how the antioxidant affected the food-borne pathogen Staphylococcus aureus Wood 46.

BHA was shown to be possibly mutagenic for S. aureus, leading to the formation of membrane mutants different from the parent strain with respect to beta-toxin and staphylokinase activities; the mutants were also less sensitive to the antibacterial activity of BHA but this property was probably the result of a phenotypic adaptation since it was lost when the cells were grown in the absence of the antioxidant. Subinhibitory concentrations of BHA resulted in growth delay and depressed alpha-hemolytic activity. S. aureus was more sensitive to the antioxidant under anaerobic conditions than under aerobic conditions while Ca^{2+} and EDTA respectively reversed and potentiated the antistaphylococcal activity of BHA. The latter was rapidly adsorbed onto bacterial cells and was shown to be a membrane-active agent on the basis of leakage of intracellular material and lysis of protoplasts. Under aerobic conditions the inhibition of

dehydrogenase and respiratory activities as well as the inhibition of amino acids uptake probably lead to inhibition of growth and of cellular division. Glucose uptake was less sensitive to inhibition. BHA was also shown to stimulate adenosine triphosphatase activity, which possibly lead to ATP depletion and growth inhibition under anaerobic conditions. The results are discussed in view of Mitchell's chemiosmotic hypothesis and of the possible use of BHA as a food preservative.

RESUME

Ph.D. Richard Degré Microbiologie

ETUDE DE L'EFFET DE L'ANTIOXYDANT PHENOLIQUE BUTYLHYDROXYANISOLE CHEZ STAPHYLOCOCCUS AUREUS WOOD 46

L'activité antimicrobienne de l'antioxidant phénolique butylhydroxyanisole (BHA) est connue depuis quelques années déjà mais ses effets sur le métabolisme bactérien demeurent cependant inconnus. Nous avons donc étudier ses effets chez Staphylococcus aureus Wood 46, un microorganisme impliqué dans de nombreuses intoxications alimentaires.

Le BHA s'est avéré possiblement mutagène pour S. aureus, menant à la formation de mutants de membrane différents de la souche parentale en ce qui a trait à la production de l'hémolysine-beta et de la staphylokinase. Les mutants étaient aussi moins sensible à l'activité antibactérienne du BHA mais cette propriété était probablement le résultat d'une adaptation étant donné qu'elle était perdue lorsque S. aureus était cultivé en absence de l'antioxydant. Un ralentissement de la croissance et une diminution de l'activité alpha-hémolytique ont été notés lorsque des concentrations de BHA inférieures à la concentration minimale inhibitrice étaient ajoutées au milieu de culture. S. aureus était aussi plus sensible à l'antioxydant en anaérobiose qu'en aérobiose alors que l'ion Ca^{2+} et l'EDTA renversait et augmentait respectivement l'activité antibactérienne du BHA. Celui-ci était très rapidement adsorbé par

les cellules bactériennes et induisait le relargage du matériel intracellulaire et la lyse des protoplastes; ces deux propriétés sont le propre d'agents agissant au niveau de la membrane cytoplasmique. L'inhibition des activités désydrogénasique et respiratoire ainsi que l'inhibition du transport des acides aminés étaient probablement à l'origine de l'inhibition de la croissance et de la division cellulaire. Le transport du glucose était cependant moins sensible à l'inhibition par le BHA. Nous avons aussi mis en évidence une stimulation de l'enzyme adénosine triphosphatase par le BHA. Cette stimulation conduit probablement à un épuisement des réserves cellulaires d'ATP, ce qui expliquerait l'inhibition marquée de la croissance en anaérobiose. Ces résultats sont discutés en fonction de la théorie chemiosmotique de Mitchell et de l'utilisation du BHA comme préservatif alimentaire.

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CLAIM OF CONTRIBUTION TO KNOWLEDGE

- Staphylococcus aureus Wood 46 was more sensitive to BHA under anaerobic conditions than under aerobic conditions.
- Subinhibitory concentrations of BHA caused growth delay in S. aureus Wood 46 and depressed alpha-hemolytic activity.
- EDTA potentiated and Ca^{2+} ions partially reversed the antistaphylococcal activity of BHA.
- BHA was possibly mutagenic for S. aureus Wood 46, leading to the formation of variants that had gainedthe property of producing the beta-toxin and lost the property of producing the staphylokinase. Colonies of the variant showed double zones of hemolysis.
- The variant was a membrane mutant different from the parent strain with respect to pH and bacitracin sensitivity and osmotic stability.
- The variant was less sensitive to BHA than the parent strain but this relative resistance was lost if the variants were grown in the absence of BHA.
- Adsorption of BHA onto the cells of both parent and variant was rapid, both strains adsorbing approximately the same amount.
- BHA was a membrane-active agent causing leakage of intracellular material and lysis of protoplasts.
- BHA inhibited oxygen consumption in S. aureus Wood 46 with lactate, succinate, NADH, glucose, malate or ascorbate as substrate but at the same time reduced the cytochromes.

- BHA inhibited dehydrogenase activities associated with succinate malate, lactate or glucose as substrate in whole cells of S. aureus Wood 46.
- BHA inhibited uptake of glutamic acid, isoleucine and lysine by whole cells of S. aureus Wood 46. Glucose uptake was less sensitive to inhibition.
- BHA stimulated ATPase activity associated with membrane fractions of S. aureus Wood 46.

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LITERATURE REVIEW

Since they increase the shelf-life of a variety of foods, the food additives are economically vital to food industry. Phenolic antioxidants are among them. This review will consider the regulations on the use of antioxidants, the toxicological data and the antimicrobial activity of phenolic antioxidants. However, we will first describe the mechanism of autoxidation of lipids and those involved in its prevention by antioxidants. The information given on the mechanism of action and biological activities of antioxidants will allow us to define in a final chapter the objectives of this thesis.

1.1 Lipid oxidation mechanism

Autoxidation of oil and fat molecules is that process which occurs spontaneously under relatively mild conditions resulting in rancidity, reversion and other types of off-flavors and -odors (Sims and Fioriti, 1980). The radical "auto" refers to a self-propagating process leading to a chain reaction. The reaction involves the addition of an oxygen molecule to a carbon atom adjacent to an unsaturated C-C bond. This reaction is initiated by a free radical and results in the formation of hydroperoxides (Sherwin, 1976). The overall reaction can be depicted as follow:

Initiation

$$RH + O_2 \longrightarrow R\cdot + \cdot OH$$

Propagation

$$R\cdot + O_2 \longrightarrow ROO\cdot$$

$$ROO\cdot + RH \rightarrow ROOH + R\cdot$$

The reaction is initiated by the generation of a free radical (R.) under the influence of light. The resulting fatty free radical (R.) is highly reactive and reacts with atmospheric or dissolved oxygen to produce peroxides (ROO.) and hydroperoxides (ROOH). Thus the process generates a second free radical which propagates the reaction. The hydroperoxides may undergo further changes producing a wide variety of very odoriferous compounds: aldehydes, ketones, fatty acids, alcohols and hydrocarbons (Loury, 1967). These compounds give the rancid odor and flavor that characterize rancid fats. The last step or termination occurs in one of the following ways:

$$R\cdot + R\cdot \longrightarrow R\text{-}R$$

$$ROO\cdot + R\cdot \longrightarrow ROOR$$

$$ROO\cdot + ROO\cdot \rightarrow ROOR + O_2$$

$$R\cdot + AH \longrightarrow RH + A\cdot$$

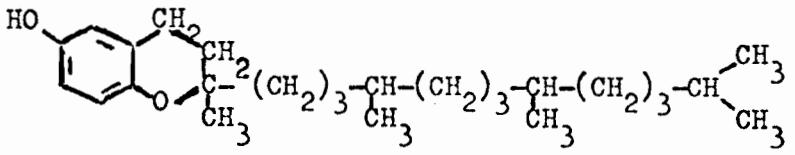
where A. is a stable radical.

Several parameters can interfere with the autoxidation process of fatty acids. These include: the degree of unsaturation of the molecule; the oxygen tension; the temperature; the presence of ultra-violet light. Some pigments and enzymes (chlorophylls, cytochrome C, myoglobin, hemoglobin and hemin) and some metals can accelerate the process (Sherwin, 1976, 1978; Marcuse 1973; Vigneron, 1956). Thus, by lowering the oxygen tension, the temperature and the concentration of catalysts (pigments, enzymes, metals) and by minimizing the action of light, one can expect to significantly reduce the autoxidation reaction. However it is not always feasible and some alternative means must be considered. One of them is to use antioxidants and the next section will explain their mechanism of action.

1.2 Mechanism of antioxidant action

It was known for some time that several vegetable oils containing high levels of unsaturated fatty acids and of tocopherols were less susceptible to autoxidation than others (Sherwin, 1976). The structure of tocopherols, which include vitamine E, is illustrated in Fig. 1. The concentrations of tocopherols in different vegetable oils is variable (Table 1) but a residual level in processed oils is important to ensure oxidative stability of these oils. As postulated by Bolland and Have (1947) the functional structure of tocopherols is the phenolic ring which allow them to give a proton to a fatty acid free radical. Phenolic antioxidants added into processed food for preservation act essentially in the same way. Fig. 2 depicts the mechanism of action of phenolic antio-

Fig. 1. Tocopherols found in vegetable matter.



Tocol

Tocopherols	α	5,7,8-Trimethyltocol*
	β	5,8-Dimethyltocol
	γ	7,8-Dimethyltocol*
	δ	8-Methyltocol*
	ε	5,7-Dimethyltocol
	η	7-Methyltocol

* Predominant in vegetable oils.

Table 1. Concentrations of tocopherols in vegetable oils. (After Sherwin, 1976)

Vegetable oil	Typical tocopherol contents of finished vegetable oils (ppm)
Coconut	83
Palm	560
Olive	30-300
Peanut	480
Rapeseed	580
Cottonseed	870-950
Sesameseed	180
Corn	900
Sunflowerseed	700
Soybean	940-1000
Safflowerseed	800

xidants. The phenolic moiety gives a proton to a free radical and the antioxidant free radical generated is stabilized by the formation of stable resonance hybrids.

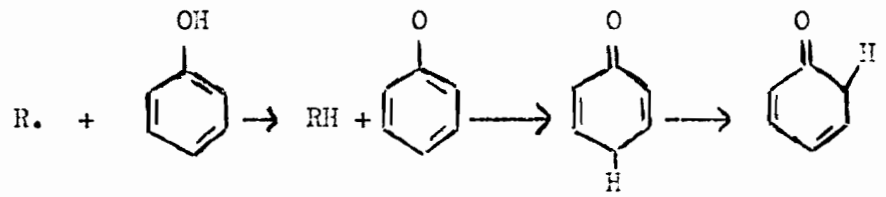
The evidence that phenolic antioxidants could act as reducing agents was provided by the work of Kurechi et al (1980). These workers investigated the hydrogen or electron donating ability of several antioxidants including butylated hydroxyanisole (BHA) and reported that ferric ion Fe^{3+} was reduced to ferrous ion Fe^{2+} by many antioxidants.

Thus, phenolic antioxidant appear to act as hydrogen donors and perform their useful function by preventing the propagating step of the free radical reaction. By doing so they inhibit the formation of rancid fats, a reaction leading to many typical off-flavors detectable only in advanced stages of oxidation. However they are not effective in preventing flavor reversion, a reaction connected with the initiation step and detectable at very early stages of oxidation (Sims and Fioriti, 1980).

Phenolic antioxidants, tocopherols and gum guaiac (a resinous exudate from a West Indies tree) are referred to as primary antioxidants since they inhibit the free radical mechanism of autoxidation. Synergists are substances that are able to enhance the activity of primary antioxidants but do not show any significant antioxidant activity when used alone (Sherwin, 1976). Citric acid, isopropyl citrate, ascorbyl palmitate, tartaric acid and lecithin are part of this group. The mechanisms by which they could interact with primary antioxidants is not known but some mechanisms have been postulated and these include (Sherwin, 1976):

- chelation or deactivation of prooxidant metals in oil,
- regeneration or sparing of primary antioxidants,

Fig. 2. Phenolic antioxidant mechanism in vegetable oil.



Fatty free radical | Phenol | Oil molecule | Antioxidant free radical (Stable resonance hybrids)

- inhibition of peroxide decomposition, thus interrupting the autoxidation process.

1.3 Regulations on the use of antioxidants

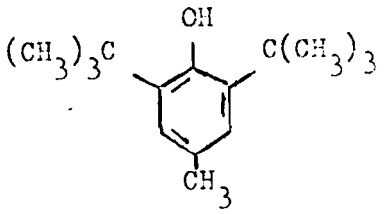
Primary antioxidants used by the food industry for human consumption are divided into two large groups:

- natural antioxidants like the tocopherols and gum guaiac,
- synthetic antioxidants, including butylated hydroxytoluene (BHT), butylated hydroxyanisole(BHA), propyl gallate (PG) and tertiary butylhydroquinone(TBHQ).The structure of these compounds is illustrated in Fig. 3.

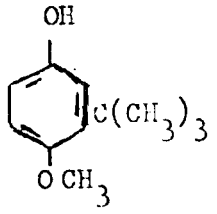
Table 2 presents the primary antioxidants and synergists used in the preservation of vegetable oil in different countries (Sherwin, 1976). According to Table 2 nordihydroguaiaretic acid (NDGA) is the only antioxidant used in Rumania even if this antioxidant is banned in Canada and in the United States, partly on the basis of Canadian feeding studies showing toxicity (Goodman et al, 1970).

In Canada, PG, BHA and BHT are the only synthetic primary antioxidants that are approved as additives in vegetable oil. 2,4,5-trihydroxybutyrophenone (THBP), 4-hydroxymethyl-2, 6-di-tert-butylphenol and tert-butylhydroquinone (TBHQ), which are banned in Canada, have been approved by the U.S.A. Food and Drug Administration (FDA).

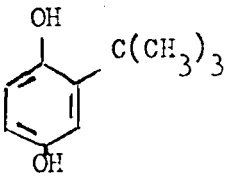
Fig. 3. The structure of phenolic derivatives.



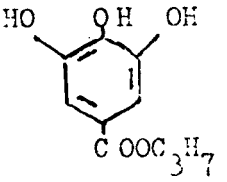
BHT



BHA



TBHQ



PG

Table 2. Primary antioxidants and synergists for vegetable oils in various countries (After Sherwin, 1976)

	Primary antioxidants												Synergists									
Countries	Tocopherols	Gum guaiac	Propyl gallate	Butyl gallate	Octyl gallate	Dodecyl gallate	NDGA	BHA	BHT	THPB	HMDB*	TBHQ	Citric acid	Isopropyl citrate	Phosphoric acid	Thiodipropionic acid	Dilauryl thiodipropionate	Dioctadecyl thiodipropionate	Ascorbic acid	Ascorbyl palmitate	Trataric acid	Lecithin
Australia			X		X	X		X														
Austria	X	X	X	X	X	X	X	X	X													
Belgium	X		X		X	X		X	X											X		
Brazil			X		X	X		X	X			X										
Canada	X	X	X					X	X				X	X					X	X	X	X
Ceylon			X		X	X		X	X													
Czechoslovakia			X																			
Denmark	X	X	X		X	X	X	X	X				X						X	X	X	X
Finland	X		X		X	X	X	X	X				X						X			
France			X		X	X		X	X										X			
Britain			X		X	X		X	X													
Greece			X		X	X																
Haiti			X				X	X	X													
Hong Kong			X		X	X		X	X													
India	X	X	X		X	X	X	X	X				X						X		X	X
Italy	X		X		X	X		X	X										X			
Jamaica			X		X	X		X	X			X										
Japan			X					X	X													
Korea			X					X	X													
Malaysia			X		X	X		X	X													
Mexico	X	X	X				X	X					X			X						
Morocco			X		X	X			X													
Netherlands	X		X		X	X		X	X				X						X	X		X
New Zealand			X		X	X		X	X													
Nicaragua	X	X	X				X	X	X				X		X	X						X
Norway	X	X	X		X	X	X	X	X										X			
Pakistan	X	X	X		X	X	X	X								X	X					
Peru			X		X	X		X	X			X										
Poland	X																		X	X		
South Africa	X	X	X		X	X		X					X						X		X	X
Rumania							X															
Spain			X		X	X		X	X													
Sweden	X	X	X		X	X		X	X													X
Switzerland	X		X		X	X		X					X						X			X
Turkey	X		X		X	X		X	X													
Taiwan			X		X	X		X	X			X										
USA	X	X	X					X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
USSR						X		X	X													
Wales			X		X	X		X	X													
Yugoslavia			X		X	X	X	X					X						X			

* 4-hydroxymethyl-2, 6-di-tert-butylphenol

The amount of antioxidant acceptable in foods is also important to consider. Table 3 lists the maximum levels of the 3 major phenolic antioxidants tolerated as food additives in the United States and Canada nowadays. BHA, BHT and PG can be used alone or in combination but the amount added must stay under a threshold limit which is defined for each type of food. In general the concentration allowed for each individual antioxidant added to food is 0,02% of the fat content of food. As for meat and poultry products, the acceptable level for each individual antioxidant is limited to 0,01%, but they might be used in combination up to a total of 0,02%.

1.4 Biological activities of phenolic antioxidants

Phenolic antioxidants are widely used by the food industry for the preservation of lipid-containing foods and their effects on the shelf-life of those products are so beneficial that it would be very difficult to cope without them. Moreover they are used on a large scale. As a result the daily intake of BHA and BHT in the United States is estimated to be 0,2 mg/kg body weight of each food additive for adults ("Comprehensive Survey of Industry on the Use of Food Chemicals Generally Recognized As Safe". National Academy of Sciences, Washington, D.C. 1972), which is within the limit of 0,5 mg/kg body weight of both BHA and BHT recommended by the Joint Food and Agriculture Organization/ World Health Organization (FAO/WHO) Expert Committee on Food Additives in 1974. Hence, the daily intake of phenolic antioxidants is significant. These figures become

Table 3. Addition limits of antioxidant(s) to various foods in ppm based on total weight of food*.

Food	BHA	BHT	PG	Total permissible
Beverages	2	-	-	2
Cereals	50	50		50
Chewing gum base	1000	1000	1000	1000
Stabilizers for shortenings	200	200	-	200
Fruit, dry glaceed	32	-	-	32
Meats, dried	100	100	100	100
Potato flakes	50	50	-	50
Potato granules	10	10	-	10
Potato shreds, dehydrated	50	50		50
Rice, enriched	-	33	-	33
Sausage, dry	30	30	30	60
Sweet potato flakes	50	50	-	50
Yeast, active dry	1000	-	-	1000

* From Stuckey, B. N., in The Handbook of Food Additives, 2nd ed., Furia, T. E., Ed. CRC Press, Cleveland, 1972, 215.

important in view of the fact that some phenolic antioxidants, as it was shown for BHT, accumulate to a higher concentration in the fatty tissues of man than of the rat (Collings and Sharrat, 1970) in which most of the toxicological studies have been carried out. Moreover, many biological activities have been associated with phenolic antioxidants. Some of these biological effects will be described in this section. Among the biological effects, phenolic antioxidants have been shown to inhibit microbial growth and a complete section will be devoted to this aspect.

1.4.1 Toxicity

The liver is the major target of phenolic antioxidants. BHT (Brown et al, 1959; Pascal and Terroine, 1970; Saheb and Saheb, 1977) and BHA (Gaunt et al, 1965; Gilbert and Goldberg, 1965; Martin and Gilbert, 1968) were shown to cause hepatic hypertrophia in rats, which is accompanied by histological changes. Hence, BHT causes a proliferation of the smooth endoplasmic reticulum and an increase in the mitotic activity and in the size of hepatocytes (Botham et al, 1970; Brown et al, 1959; Lane and Lieber, 1967). BHA has similar effects in monkeys but at lower concentrations (Allen and Engblom, 1972). TBHQ does not have any significant effect in rats nor dogs at concentrations up to 0,5% of the diet (Astill et al, 1975) but PG causes a significant increase in the mitotic activity of liver cells in rats (Feuer et al, 1965). More recently Hirose et al (1981) reported that BHT-treated rats showed in-

creased liver weight and increased blood cholesterol.

Ingestion of BHT also affects different enzymatic systems. It has been shown to induce the activity of a group of liver enzymes called "Processing enzymes" that are involved in the transformation of drugs and this induction is accompanied by an increase in the P_{450} cytochrome content (Gray et al, 1972). However, at the same concentration, BHA stimulates only two microsomal enzymes: BHT-oxidase and biphenyl-4-hydroxylase (Martin and Gilbert, 1968). Curiously in vitro testing has shown that BHT, BHA and PG inhibit liver microsomal monooxygenases (Yang et al, 1974; Torrielli and Slater, 1971; Yang and Strickhart, 1974). Among other enzymes affected by BHT are the glucose-6-phosphatase (EC 3.1.3.9), the acid phosphatase (EC 3.1.3.2) and the mitochondrial cytochrome oxidase (EC 1.9.3.1) activities that are reduced (Feuer et al, 1965; Gaunt et al, 1965; Pascal, 1969; Pascal and Terroine, 1969) and the glucose-6-P-dehydrogenase (G6PD, EC 1.1.1.49) activity that is stimulated (Feuer et al, 1965). The same stimulatory effect on G6PD was reported for BHA and PG (Feuer et al, 1965). It is interesting to note that BHT and PG act as uncouplers of oxidation and phosphorylation in liver mitochondria (Pascal and Terroine, 1975). TBHQ does not seem to affect significantly those enzymatic systems (Astill et al, 1975).

The lung is another major organ affected by BHT. Studies have shown that this antioxidant induces pulmonary injury in mice after intraperitoneal (Marino and Mitchell, 1972; Saheb and Witschi, 1975) or

per os administration (Witschi and Lock, 1978). Omaye et al (1977) reported increased activities of pulmonary glutathione (GSH) peroxidase (EC 1.11.1.9), GSH reductase (EC 1.6.4.2), G6PD and superoxide dismutase (EC 1.15.1.1) while Arany et al (1981) reported enhanced activities of hexokinase (EC 2.7.1.1), phosphofructokinase (EC 2.7.1.11) and pyruvate kinase (EC 2.7.1.40) in the lungs of mice.

Ford et al (1980) reported that the renal function was also affected by this class of food additives. Hence, administration of BHA and BHT adversely affected electrolyte balance in rats and these antioxidants had a specific, depressive effect on organic transport.

A growing awareness of the effects of exposure to drugs and food additives during early life on emotional, intellectual and neurological development have become a matter of great concern in recent years and it has been shown that BHT at doses up to 0,5% of the diet produces marked physical as well as preweaning behavioral toxicity in rats (Vorhees et al, 1981).

1.4.2 Mutagenicity

In view of the high correlation between carcinogenicity and mutagenicity, the possible mutagenicity of a food additive is of great interest. Most of the techniques involved in such testing rely on the hypothesis that most chemical carcinogens are able to interact with DNA or can be processed in the body to do so.

Using Salmonella TA98 and TA1538, which detect frameshift muta-

gens, BHA, BHT, PG and TBHQ were shown to be non-mutagenic in the Ames assay (Bonin and Baker, 1980). However, tests with Saccharomyces D-3 showed a biologically significant increase in the frequency of recombinants (Fabrizio, 1974) and BHA and BHT were also shown to induce sister chromatid exchanges in hamster cells (Abe and Sosaki, 1977). Shelef and Chin (1980) ascertained that BHA, BHT and PG were not mutagenic for Salmonella strains TA98 and TA100, the latter being used for the detection of base substitution mutagens. The same authors (Shelef and Chin, 1980) also reported that BHA and BHT substantially increased aflatoxin B_1-induced mutagenesis in the two tester strains. It was also reported that BHA, BHT and PG potentiated the X-ray-induced chromosome damage in barley and onion (Kaul and Zutshi, 1977; Veena and Kaul, 1979; Kaul, 1979). In association with the chemical mutagen propane sultone, it was observed that BHA, BHT and PG increased chromosome breakage as well as seedling injury (Veena and Kaul, 1979). Ben-Gurion (1979) presented evidences that pyrogallol and purpurogallin, antioxidants used in hydrocarbon fuels or lubricants (Merck index, 9th ed., 1976, p. 1031) were both inducers of colicine E2 as well as mutagenic for Salmonella strains TA1537 and TA100.

1.4.3 Carcinogenicity

Few reports have been published on the carcinogenic effect of the phenolic antioxidants. In 1974 Clapp et al reported that BHT at a dose of 0,75% of the diet, causes after 18 months of administration, an increase in the frequency of pulmonary adenoma in BALB/C mice. However

Hirose et al (1981) could not detect any carcinogenic effect of this antioxidant in Wistar rats. Ito et al (1982) subsequently reported that BHA was carcinogenic in F344 rats, primarily affecting the forestomach, when administered orally in the diet.

1.4.4 Antioxidants and carcinogens metabolism

Although there are some reports suggesting that BHA and BHT might be carcinogens it is interesting to note that there are many reports in the literature of antioxidants preventing the carcinogenic effect of several carcinogens. Wattenberg (1980) has reviewed these effects that are summarized in Table 4. The most extensive work was carried out with BHA, BHT and ethoxyquin (ETO), the latter being a phenolic antioxidant used in commercial animal diets.

It is now generally accepted that most carcinogens are not active per se but need metabolic activation by different enzymatic systems present in the mammalian cell. It is beyond the scope of this review to discuss the different mechanisms proposed for the activation of procarcinogens into potential carcinogens but the most accepted hypotheses include formation of alkylating groups, formation of carbonium ions from primary or secondary oxides and formation of nitrenium ions (Kahl, 1982). Hence, it has been proposed that phenolic antioxidants could interfere with the formation of procarcinogens by interacting with the enzymes responsible for their activation and thus, could lead to the formation of metabolites of detoxication (Lam and Wattenberg,

Table 4. Inhibition of carcinogen-induced neoplasia by BHA, BHT and ETO*.

Carcinogen	Antioxidant	Species	Site of neoplasm inhibited	Ref.
BP[1]	BHA	Mouse	Lung	Wattenberg, 1973
BP	BHA, BHT	Mouse	Forestomach	Wattenberg, 1972a
DMBA[2]	BHA, ETO	Mouse	Forestomach	Wattenberg, 1972a
DMBA	BHA, BHT	Mouse	Skin	Slaga and Bracken, 1977
DMBA	BHA, BHT, ETO	Rat	Breast	Wattenberg, 1972a
DMBA	BHA	Mouse	Lung	Wattenberg, 1973
7-HMB[3] anthracene	BHA	Mouse	Lung	Wattenberg, 1973
DB[4] anthracene	BHA	Mouse	Lung	Wattenberg, 1973
DENA[5]	BHA, ETO	Mouse	Lung	Wattenberg, 1972b
4-NQ-oxide[6]	BHA, ETO	Mouse	Lung	Wattenberg, 1972b
Uric mustard	BHA	Mouse	Lung	Wattenberg, 1973
Urethane	BHA	Mouse	Lung	Wattenberg, 1973
FAA[7]	BHT	Rat	Liver	Ulland et al, 1973
N-OH-FAA[8]	BHT	Rat	Liver, breast	Ulland et al, 1973
Azoxy-methane	BHT	Rat	Large intestine	Zedeck et al, 1972

* From Wattenberg, L. W., in Carcinogenesis, a Comprehensive Survey. Vol. 5. Slaga, T. J., Ed. Raven Press, New York, 1980, 87.

1 Benzo(a)pyrene

2 7,12-dimethylbenz(a)anthracene

3 7-Hydroxymethyl-12-methylbenz(a)anthracene

4 Dibenz(a,h)anthracene

5 Diethylnitrosamine

6 4-Nitroquinoline-N-oxide

7 N-2-fluorenylacetamide

8 N-hydroxy-N-2-fluorenylacetamide

1977; Lam et al, 1980). However the possibility of a direct chemical reaction between the inhibitor and the active metabolite should not be overlooked as well as an inhibition of in vivo activation processes (Calle and Sullivan, 1982).

1.5 Antimicrobial activity of phenolic antioxidants

Among the various biological activities that have been associated with phenolic antioxidants, antimicrobial activity is one of the most interesting. Hence the use of these additives as preservatives alone or in combination with other chemicals might be very attractive for the industry. First, it is obvious that additives with dual function, i.e. antioxidative and antimicrobial activities, will be particularly beneficial in the future since it would tend to limit the amount of chemicals added to foods. Moreover, new applications could originate with respect to this antimicrobial activity. For example, BHA has already been included, on an experimental basis, in the composition of mouthwashes to enhance their poor cidal action (Kabara, 1980); BHT was used to treat herpes virus cutaneous infection in mice (Keith et al, 1982).

This section will review the nature and properties of the antimicrobial activity of phenolic antioxidants against different groups of microorganisms, namely, viruses, protozoa, fungi and bacteria.

1.5.1 Activity against viruses

Snipes et al (1975) were the first to report on the antiviral activity of phenolic antioxidants. They showed that three lipid-containing viruses, the herpes-simplex virus (HSV) and two phages, ϕ6 and PM2, were inactivated by 50% when exposed 30 minutes to 10^{-4} to 10^{-5} M of BHT. However non-lipid-containing viruses such as polioviruses were not affected. The effect of BHT on bacteriophage PM2, specific for a marine pseudomonad, were further investigated by Cupp et al (1975) who reported that phage killing occured rapidly, with the majority of the killing taking place during the first 5 minutes of exposure. They also showed that the phages were disrupted in the presence of 0,2 M BHT. However the degree of inactivation was dependent upon the original phage titer and the number of bacterial cells.

Wanda et al (1976) showed that bacteriophage ϕ6 was totally inactivated by 3×10^{-5} M BHT and 10^{-4} M BHA. The phage envelope was not removed by BHT treament and BHT-treated phages were morphologically indistinguishable from controls but were unable to attach to the host cell, Pseudomonas phaseolicola. It was also found that a drastic drop in the degree of inactivation by 3×10^{-5} M BHT occurred when the temperature was lowered from 20 to 15° C. Calcium, barium and strontium but not magnesium were also effective in enhancing the activity of BHT.

Most interesting is the finding that chickens fed on diets containing BHT (100-200 ppm) did not die when exposed to virulent Newcastle disease virus (NDV), also a lipid containing virus. Also, chi-

ckens fed on a BHT-containing diet did not show any appreciable humoral immune response when exposed to avirulent NDV (Snipes et al, 1975; Brugh, 1977). Among other pathogenic viruses investigated with respect to their susceptibility to BHT, Kim et al (1978) showed that human and murine cytomegaloviruses (CMV) were inactivated by more that 90% with 40 ug of BHT/ml and Semliki Forest virus was inactivated by about 75% after incubation for one hour at 37°C with the same concentration of the antioxidant. They proposed that the interaction of BHT with lipid-containing viral envelopes somehow disturbed the proper function of the viral envelope during adsorption of viral particles to host cell membrane. Although vaccinia virus is also one of the lipid-containing viruses, for an unknown reason BHT had only a slight inactivating effect.

More recently Keith et al (1982) reported that hairless mice with cutaneous infections of herpes simplex virus type 1 (HSV-1), were successfully treated with BHT. Hence the agent was found to be effective in reducing the clearance time of HSV-1 lesions when applied topically to the infected area.

1.5.2 Activity against protozoa

The effect of BHA on Tetrahymena pyriformis has been extensively studied by Surak and his collaborators. In a first report Surak et al (1976a) showed that 20 ppm of BHA in the medium inhibited the cell growth by 50% and the synthesis of DNA, RNA and proteins also by 50%. The protein and nucleic acid synthesis inhibition occurred within 10

minutes after the addition of the antioxidant in the medium. Glucose transport and lipid synthesis were not significantly affected. Microscopic examination did not reveal any membrane alteration like it was previously reported for BHT (Surak et al, 1976b). Contrary to their previous report (Surak et al, 1976a), they later showed that BHA altered lipid synthesis of T. pyriformis (Surak, 1980). Among the major changes noted within 3 hours of exposure were a decrease in the relative incorporation of radioactive acetate into tetrahymenol and an increased incorporation of the label into triglycerides. After 24 hours of exposure to increasing levels of BHA (3,12 to 12,5 ug/ml) there was a decrease in the relative synthesis of polar lipids and an increase in the relative synthesis of triglycerides, tetrahymenol, squalene and free fatty acids.

Surak and Singh (1980) brought more precisions on the kinds of alterations caused by BHA in polar lipid synthesis. Hence, they showed that BHA at concentrations up to 12,5 ug/ml inhibited the synthesis of cardiolipin and the incorporation of labelled acetate into lysophosphatidylcholine, 2-aminoethyl-phospholipids and an unknown polar lipid while increasing the incorporation of radiolabel into phosphatidylethanolamine and another unknown polar lipid. The authors proposed that the striking differences between these results and others according to which lipid synthesis and membrane were not affected may be attributed to the carrier used to administer the antioxidant, since dimethylsulfoxide was used in the previous study (Surak et al, 1976a) and ethanol in the others (Surak, 1980; Surak and Singh, 1980).

1.5.3 Activity against fungi

One of the first reports on the antifungal activity of phenolic antioxidants was published by Chang and Branen (1975). They showed that growth and aflatoxin production by Aspergillus parasiticus mycelium was inhibited in a glucose/salts medium containing 250 ug BHA/ml while 1000 ug/ml was necessary to inhibit spore germination.

Fung et al (1977) studied the effect of BHT and BHA on A. flavus growth and toxin production. The antioxidants were added at 0,005 to 0,02 g per plate of solid media (60 or 100 mm in diameter). While BHT was ineffective in inhibiting A. flavus growth and toxin production, BHA inhibited the growth under the conditions mentioned above. Moreover, using a 60 mm diameter petri dish containing 0,01 g of BHA, they showed that the production of aflatoxin B_2, G_1 and G_2 was completely inhibited while that of aflatoxin B_1 was only partially inhibited.

Turcotte and Saheb (1978) reported on the antifungal activity of three antioxidants, namely BHA, BHT and ETO. They showed that the minimal inhibitory concentrations (mic) for BHA and BHT against Saccharomyces cerevisiae were 100 and 25 ug/ml respectively. ETO did not inhibit significantly the growth of this microorganism. The mic of BHA against Candida albicans was 100 ug/ml. At the same concentration BHT and ETO inhibited only partially the growth of this yeast.

Beggs et al (1978) showed that BHA, PG and NDGA enhance amphotericin B activity against two strains of C. albicans and one of C.

parapsilosis. In fact it was found that subinhibitory concentrations of any one of three different antioxidants in combination with fungistatic or weakly fungicidal levels of amphotericin B were highly lethal to those three strains. Although synergism was not seen in tests with a strain of Torulopsis glabrata, antioxidants prolonged the inhibitory action of this antibiotic. On the basis of these findings the authors proposed that antioxidants prevented autoxidation of the drug, a polyene macrolide with seven conjugated double bonds, thus stabilizing its activity. In addition the antioxidants had their own antifungal spectra of activity.

Ahmad (1979) reported that Geotrichum, Penicillium and Aspergillus were inhibited by 200 ug BHA/ml in glucose/salts medium and that BHA did not have synergistic antimicrobial activity with sorbates against A. flavus.

1.5.4 Activity against bacteria

Microorganisms involved in food poisoning are those that have been the most extensively tested for their susceptibility to antioxidants.

The first study dealing with the antibacterial activity of BHT was carried out by Ward and Ward (1967). They reported only partial inhibition of Salmonella senftenberg grown on brilliant green agar containing 1% antioxidant. Growth of S. typhimurium however was totally inhibited at 37° C in nutrient broth containing 400 ug BHA/ml (Chang and Branen, 1975). Turcotte and Saheb (1978) reported that BHT did not

significantly affect the growth of S. typhimurium and S. montevideo when 100 ug/ml was added to brain heart infusion broth.

At the same concentrations BHA and ETO inhibited the growth of S. typhimurium by 33% and 26% respectively but had no significant effect on the growth of S. video. Other members of the Enterobacteriaceae such as Escherichia coli, Proteus mirabilis, P. vulgaris and Enterobacter cloacae were also relatively resistant to the action of those three antioxidants in the same conditions. Turcotte and Saheb (1978) also reported that the association of ETO with BHT increased the inhibitory activity. This observation plus the fact that the sensitivity of E. coli cells was increased after an osmotic shock treatment led these authors to postulate that the cell wall structure of Enterobacteriaceae might play a role in the resistance of this group of microorganisms to BHT. These results substantiated those of Shih and Harris (1977) according to which BHA at concentrations up to 200 ug/ml did not affect E. coli growth significantly. However both PG and NDGA at 400 ug/ml were strong inhibitors of E. coli growth. These studies were carried out in trypticase soy broth (TSB) at 35°C. With another strain of S. typhimurium, Davidson et al (1979) were able to totally inhibit the cell growth by adding 150 ug BHA/ml in TSB at 32° C. It is thus possible that there might be differences in sensitivity to BHA between strains of the same species. However since the conditions used in the reports mentioned above were different we should be cautious in our conclusion.

Davidson et al (1981) reported that combinations of potassium

sorbate and BHA inhibited synergistically by 50% to 80% the final growth of S. typhimurium. These authors estimated growth by turbidity measurements after 72 hours of incubation of 32° C in TSB with the following levels of sorbate and BHA: 500 ppm sorbate/50 ppm BHA, 500 ppm sorbate/100 ppm BHA, 1000 ppm sorbate/50 ppm BHA and 1000 ppm sorbate/100 ppm BHA.

Pierson et al (1980) showed that 400 ug BHA/ml caused a decrease followed by an increase in viable cell counts of S. typhimurium. However the culture reached only 10^7 cells/ml after 48 hours of incubation in TSB at 35° using an inoculum size of 10^5 cells/ml. Combination of 150 ug/ml of BHA and of 150 ug/ml of propylparaben showed the same result.

Davidson et al (1981) reported that TBHQ had little or no effect on growth of S. typhimurium.

Although there are some discrepancies on the mic's of antioxidants using Salmonella as target, most of the reports agree that the mic for Staphylococcus aureus grown in nutrient broth or TSB is 150 ug/ml (Chang and Branen, 1975; Davidson et al 1979; Turcotte and Saheb, 1978). Shih and Harris (1977) is the only exception as they found a mic of 400 ug/ml. These results tend to demonstrate that phenolic antioxidants are more active against Gram-positive bacteria than against Gram-negative bacteria belonging to the Enterobacteriaceae. We cannot however extend these considerations to all other Gram-negative bacteria since Turcotte and Saheb (1978) showed that the growth of Bacteroides species was significantly affected by 6,25 ug/ml, 100 ug/ml and 100 ug/ml of BHT, BHA and ETO respectively.

They suggested that the greater sensitivity of Bacteroides to antioxidants reflects the differences in the structure of the lipopolysaccharides of Bacteroides species which do not contain 2-keto-3-deoxyoctonate, nor heptoses (Hofstad, 1974).

Ayaz et al (1980) reported complete inhibition of S. aureus growth in BHI broth containing 202 ug BHA/ml or 154 ug BHT/ml as well as with 45 ug/ml of both BHA and BHT. Growth was evaluated by turbidity measurements in BHI broth during a period of 24 hours. When concentrations greater than 151 ug BHA/ml or than 103 ug BHT/ml were added to the culture, growth was inhibited to the extent that enterotoxin A could not be detected after 24 hours of incubation.

Pierson et al (1980) showed that a combination of 50 ug/ml of BHA-propylparaben was needed to produce a gradual reduction in viable cells of S. aureus, a concentration much lower than those needed with these two agents added separately. Davidson et al (1981) also showed that combinations of potassium sorbate, BHA and TBHQ were effective in inhibiting synergistically S. aureus growth. Hence combinations of 500 ppm sorbate/50 ppm BHA and 1000 ppm sorbate/ 10 ppm TBHQ inhibited the final growth of S. aureus by 40 and 55% respectively.

Growth was evaluated by turbidity measurements after 72 hours of incubation in TSB at 32° C. Robach and Stateler (1980) also reported that combinations of sorbate, TBHQ, sorbate and BHA resulted in synergistic inhibition of growth of this microorganism. Finally Lahellec et al (1981) reported that potassium sorbate at 1, 3, and 5% levels in combination with BHA, BHT and PG (50 and 100 ppm) showed greater bactericidal or bacteriostatic effects on S. aureus strains at pH 5 than at pH 7 and that 50 ppm of TBHQ with or without the addition of sorbate was highly inhibitory to S. aureus strains.

BHA was also tested as inhibitor of Vibrio parahaemolyticus, which is an etiological agent in foodborne illness arising from ingestion of contaminated seafood (Robach et al, 1977). It was shown that this microorganism, when grown in TSB supplemented with 2,5% (w/v) NaCl, was inhibited by 50 ug/ml but that 400 ug/ml were needed in a crab meat homogenate. However 200 ug/ml caused a 90% decrease in the 10^5 cells/ml inoculum before growth was initiated. In both media growth was measured at 35°C.

Robach and Pierson (1979) tested BHA, BHT and PG against Clostridium botulinum in prereduced thiotone yeast-extract glucose medium at 37°C. Spore growth was prevented by addition of 50 ug BHA/ml and 200 ug BHT/ml for at least 21 days but when exposed to 200 ug PG/ml spore growth resumed after only two days. The similarity of structure between BHA and esters of parahydroxybenzoic acid led these authors to speculate that phenolic antioxidant could possibly act at the membrane level or as reducing agent. Hence they would compete with the electron donor

systems within the cells and thereby inhibit growth. However they had no experimental evidence to support these hypotheses. Reddy et al (1982) reported that 200 ug/ml of BHA, BHT, NDGA and TBHQ inhibited C. botulinum growth and toxin production for 7, 7, 7 and 1 day(s) respectively in thiotone yeast-extract glucose medium.

Clostridium perfringens was completely inhibited by 150 ug BHA/ml in Fluid Thioglycollate Medium at 37°C but the strains tested were more resistant at 45°C (Klindworth et al, 1979). These authors also reported that BHA was bactericidal for non-growing cell suspensions at 100 ug/ml.

Vardaman et al (1978) showed that 10 ug BHT/ml totally inhibited Mycoplasma synoviae growth at 37° C.

Saheb et al (1978) reported that the combination of BHT and lauric acid were synergistic against S. aureus and BHT and palmitoleic acid against S. aureus and Bacteroides fragilis ss. fragilis.

Most of the work reported in this section was carried out in the last 15 years. However few attempts were made to elucidate the mechanism of action of phenolic antioxidants. The most extensive study of the antimicrobial activity of these agents were carried out on a protozoan, T. pyriformis (Surak et al, 1976a, 1976b; Surak, 1980;Surak and Singh, 1980) but the mechanism of antibacterial activity has never been studied. Hence, all the studies reported in this section were dealing with the determination of the mic's under different conditions. The similarity of structure of BHA and the esters of parahydroxybenzoic acid and phenols is striking and the mechanism of action could also be closely

related. According to this, BHA could possibly act at the membrane level and affect systems associated with the membrane: specific transport mechanisms, electron transport and oxidative phosphorylation, protein and DNA synthesis (Hamilton, 1971).

Phenolic antioxidants may play an important role in the preservation of foods in the future since they possess antimicrobial and antioxidative activities. On the basis of these 2 properties Branen et al (1980) mentioned that BHA appears to be the most useful antioxidant. In our study we considered the effect of BHA on S. aureus, a microorganism that has been shown to be very sensitive to the antioxidant, like other Gram-positive bacteria. BHT was not selected because of its high toxicity and the recommendation of the U.S. F.D.A. to restrict the use of BHT (Federal Register, pg. 27603 to 27609; May 31, 1972).

The elucidation of the antibacterial activity against S. aureus might give some clues on the resistance of Gram-negative organisms and even on the possible action on eukaryotic cells. It may also help to find out whether the presence of BHA in foods may lead to the selection of a resistant population.

S. aureus is also a very important microorganism involved in both contamination of food supplies and in food poisoning and therefore, a major target for any antimicrobial added to food. It is also a widely studied microorganism whose physiology is reasonably well understood.

Staphylococcus aureus is a typical Gram-positive coccus 0,8 to 1 um in diameter forming smooth, convex colonies with an entire edge.

The cell wall is composed of peptidoglycan, ribitol teichoic acid and protein A, which is a group specific precipitinogen. It is a facultative anaerobe obtaining its energy via the glycolysis, pentose and citric acid cycles and forming mainly lactate anaerobically and acetate aerobically. Like other facultative anaerobes, the glucose catabolite repression is an important characteristic of their metabolism. Hence, glucose represses the enzymes of the tricarboxylic acid cycle succinic dehydrogenase and fumarase and the cytochromes. In this organism, the uptake of amino acids is powered by the proton motive force (PMF) generated across the cytoplasmic membrane while the phosphotransferase system is responsible for the transport of sugars (Saier, 1977). S. aureus also produces several exoenzymes which include hemolysins (alpha, beta, delta and epsilon), enterotoxins (A,B,C,D,E), coagulase, protease, staphylokinase, nuclease and lipase.

MATERIALS AND METHODS

2.1 Strains

The strain of Staphylococcus aureus Wood 46 used throughout this study was obtained from J. DeRepentigny (University of Montreal) while strains of Streptococcus agalactiae and Corynebacterium pseudotuberculosis, used for the determination of hemolysins, were kindly supplied by R. Higgins from the same institution. Six other strains of S. aureus were from our own collection.

2.1.1 Stock cultures

In order to minimize any changes that could occur upon serial transfers, stock cultures of S. aureus Wood 46 were prepared. Cultures inoculated from a lyophylized culture or a slant kept at 4°C were grown overnight in one of the 4 media listed in para. 2.2. Then they were frozen at -70°C in 2 ml volumes. When required, the appropriate number of tubes were thawed rapidly in a water bath kept at 37°C and used to inoculate the corresponding medium.

2.2 Media

The strains were maintained on nutrient agar slants with periodic transfers every 4 weeks. The slants were incubated at 37°C for 24

hours and kept at 4°C. In most experiments the liquid culture medium used was the Brain Heart Infusion (BHI, Difco). This medium was solidified by adding 1.5% agar (Difco). Blood agar medium was obtained by adding 5% citrated sheep blood to the BHI agar or Columbia agar (Institut Armand-Frappier Production, appendix 1). Bacitracin agar was prepared by adding bacitracin to a final concentration of 50 ug/ml (2,8 U/ml, Sigma) to BHI agar just before pouring. Other media used in specific experiments will be described in the appropriate section.

2.3 Bacterial susceptibility to BHA

The minimal inhibitory concentration (mic) of BHA towards S. aureus was determined by adding serial twofold dilutions of this food additive in broth. The mic was defined as the lowest concentration of BHA preventing visible growth of the bacteria after 6 hours of incubation at 37°C with inocula ranging from 2 to 5 x 10^6 colony forming units (CFU)/ml. The mic was determined in the following 4 media: brain heart infusion (BHI, Difco and Carr Scarborough), tryptic soy broth (TSB, Difco), nutrient broth (Gibco) and in the special medium of Collings and Lascelles (1963, Appendix 2). BHA was added as an ethanolic solution and the volumes added were such that the final concentration of ethanol in the medium was 2% (v/v). Controls containing 2% (v/v) ethanol were always run in parallel.

BHA was purchased from Sigma (St-Louis, Mo.), ICN (Cleveland, O.) and BDH (Montreal, Que.).

2.4 Effects of BHA on growing and non-growing cell suspensions of S. aureus

A mid-log phase culture was used to inoculate BHI broth containing either BHA added as an ethanolic solution or ethanol as a control. The flasks were shaken (250 rpm) at 37°C and the CFU determined at intervals by dilutions and plating on Columbia blood agar and tryptic soy agar (TSA, Difco) supplemented with 0,5% sodium pyruvate, the latter being used as the optimal medium for counting injured and uninjured cells (Hurst and Hughes, 1981). At intervals microscopic examinations were carried out to ensure that clumping of the cells was not responsible for eventual drop, in viable counts.

A similar procedure was used with non-growing cell suspensions using cells harvested by centrifugation at 4°C (11,000 x g, 10 minutes) at late log phase, washed twice in phosphate buffer (0,05 M, pH 7,0) and resuspended at a concentration of 10^8 CFU/ml in the same buffer. Incubation was carried out at 30°C.

2.4.1 Effect of BHA on cellular morphology

S. aureus Wood 46 (10^7 CFU/ml) was incubated for 6 hours in BHI broth containing 0,28 mM BHA at 37°C. Samples from the inoculum and taken at 3 and 6 hours were centrifuged (1,000 x g, 10 minutes) and fixed with glutaraldehyde and osmic acid. They were then coated with Vestopal (Polysciences, Warrington, Pa), cut into thin sections

with an ultramicrotome and examined under the electron microscope. Technical assistance for the preparation of the thin sections was provided by the Centre de recherches en virologie of the Institut Armand-Frappier.

2.4.2 Reversal and potentiation of inhibition caused by BHA

The effects of different agents, added alone or in combination with BHA, on the growth of S. aureus were determined after 24 hours of incubation in the same conditions as those described for mic studies (para. 2.3). The effect of $CaCl_2$, $MgCl_2$ and EDTA were determined in BHI broth. For the effect of glycerol, the medium of Hugo and Stretton (1966) was used (appendix 3) and S. aureus was subcultured 10 times in this medium before its susceptibility towards BHA was tested.

2.4.3 Determination of lipase activity

Lipase activity was determined according to the method of Lawrence et al (1967) with supernatants of both parent and variant strains grown for 18 hours in BHI broth. Ten ml of a 1% emulsion of tributyrin prepared in distilled water was added to 90 ml of a hot solution of agar (1,2% purified agar, Oxoid) in 0,05 M phosphate buffer (pH 8). One ml of this preparation was spread over a microscope slide and a hole punched in the agar. Aliquots of the supernatants were added into

the well and the slides incubated for 24 hours at 37°C. Clarification around the hole was considered as an indication of tributyrin hydrolysis.

2.5 Characterization of the BHA resistant variant

2.5.1 Hemolytic activity

2.5.1.1 Qualitative determination of the hemolytic pattern

A preliminary characterization of the hemolytic pattern was carried out by the method of Elek and Levy (1950) according to which the alpha-hemolysin produces a zone of complete hemolysis on sheep blood agar while the beta-hemolysin produces a zone of discoloration, also called partial hemolysis, and forms lines within the zone of discoloration.

2.5.1.2 Reverse CAMP test

Further characterization of the hemolytic pattern was carried out by the one-plate method, also called the reverse CAMP test, described by Skalka et al (1979). This test makes use of Streptococcus agalactiae and Corynebacterium pseudotuberculosis, whose exoproducts are known respectively to detect small amounts of beta-hemolysin by completion of partial hemolysis and to inhibit staphylococcal alpha- and

beta-toxins. S. aureus, S. agalactiae and C. pseudotuberculosis were streaked on blood agar plates along single lines perpendicular to each other but not touching each other. These plates were then incubated overnight at 37°C. Completion of partial hemolysis of S. aureus by S. agalactiae exoproducts was interpreted as an indication of the presence of beta-toxin.

2.5.1.3 Electrophoretic localization

Alpha- and beta-toxins were detected by the electrophoretical method of Haque (1967). The supernatants from 18 hours old cultures of the bacterial strain grown in BHI at 37°C were centrifuged at 4°C (11,000 x g, 10 minutes) and filtered through a 0,20 um membrane filter. The last preparation was kept at -70°C until used.

The hemolysins present in these preparations were separated from each other by electrophoresis in a 1% agar gel (purified agar, Oxoid) using barbital buffer (pH 8,4). The melted agar was poured onto a glass slide (9 x 11 cm). Wells were then punched in the solidified agar and these were filled with the culture filtrates. Electrophoresis was run for 2 hours at a constant current supply of 45 ma per plate. After this time, the agar gel was covered with a suspension of rabbit or sheep red blood cells in melted 1% agar held at 45°C. The plates were then incubated for 1 hour at 37°C under 100% humidity. In the case of the beta-toxin, the first incubation was followed by a second incubation of 30 minutes at 4°C. According to this method, the

alpha-toxin migrates 18 mm towards the cathode and lyses rabbit erythrocytes while the beta-toxin migrates 36 mm towards the cathode and lyses sheep erythrocytes.

2.5.1.4 Tube assay

The hemolytic titer was determined with BHI supernatants as prepared in para. 2.5.1.3 and the alpha- and beta-toxin activities were estimated with rabbit and sheep erythrocytes respectively. The supernatants were diluted in 0,05 M phosphate buffered saline (PBS, pH 7,0) containing 0,1% bovine serum albumin as stabilizing agent. To 1 ml of a series of dilutions was added 1 ml of a 3 times washed suspension of erythrocytes which was standardized so that a sample, after hemolysis with saponin and adding an equal volume of diluent, gave an absorbance of 1,0 at 545 nm. The mixtures of red blood cells and supernatants were incubated in a 37°C water bath for 60 minutes and then centrifuged briefly and the absorbance of the supernatant read at 545 nm. One unit of hemolysin (H.U.) was defined as the amount which releases half the hemoglobin of the red blood cell suspension under the conditions mentioned above (Bernheimer and Schwartz, 1963). It can be calculated as follow:

$$1\ \text{H.U.} = \frac{1/\text{dilution} \times \text{O.D.}^{*}\ \text{of the sample}}{\text{O.D}_{50}\ \text{of the standardized erythrocytes suspension.}}$$

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* Optical density.

For the measurement of the beta-toxin activity, also known as a hot-cold hemolysin, the incubation at 37°C was followed by a 30 minutes incubation at 4°C (Bernheimer et al, 1974).

2.5.1.5 Cell-associated beta-toxin

Cell-associated beta-toxin was determined in cell lysates obtained by an enzymatic treatment (McNiven and Arbuthnott, 1972). Cells from 25 ml of culture in BHI were harvested at the onset of the stationary phase (11,000 x g, 10 minutes), washed twice in PBS (0,05 M, pH 7,0) and resuspended in 2 ml of 0,05 M tris (hydroxy) aminomethane-HCl (Tris) buffer (pH 7,5) containing 0,145 M NaCl and 55 U of lysostaphin (Sigma). After the addition of 0,05 ml saline containing 150 ug of deoxyribonuclease (Sigma), the volume was adjusted to 2,5 ml. The reaction mixture was incubated for 60 minutes at 37°C and then centrifuged at 13,000 x g for 15 minutes at 4°C. Hemolytic activity in the cell lysate was determined as described previously (para. 2.5.1.4) using sheep erythrocytes.

2.5.2 Staphylokinase activity

Qualitative determination of staphylokinase was done according to the method of Lack and Wailling (1954). A plasma-agar mixture was heated at 56°C for 20 minutes before pouring. This produced an opaque medium and fibrinolysis was judged by the zone of clearing around

colonies after 18 to 48 hours of incubation at 37°C. To ascertain that the fibrinolysis was due to plasmin (staphylokinase), we also incorporated soybean trypsin inhibitor into the medium, which inhibits fibrinolysis by plasmin but not by staphylococcal protease.

2.5.3 Phage typing

The analysis was performed by the Laboratoire de Santé publique du Québec (Ste-Anne de Bellevue, Québec) using the technique described by Blair and Williams (1961)

2.5.4 Susceptibility to bacitracin

Bacitracin mic against S. aureus was determined in BHI according to the technique described for BHA mic determination (para. 2.3.).

2.5.5 pH sensitivity

Sensitivity of S. aureus to pH was determined according to the method of Kent and Lennarz (1972) for S. aureus. Colonies of S. aureus grown on blood agar were selected on the basis of their hemolytic pattern on sheep blood agar and streaked onto the pH media (appendix 4) adjusted to pH 5,2 and 7,0. The plates were then incubated overnight at 37°C. According to Kent and Lennarz (1972), cells that are unable to grow at

pH 5,2 but grow normally at pH 7,0 are membrane mutants.

2.5.6 Osmotic stability

Osmotic stability was determined according to the method of Altenbern (1975) for S. aureus. S. aureus was grown overnight at 37°C with aeration (250 rpm). Cells from 2 ml culture in BHI were harvested at 4°C (11,000 x g, 10 minutes) and resuspended in 5 ml of 0,05 M phosphate buffer, pH 7,5, containing concentrations of NaCl ranging from 0,85 to 12%. The optical density was measured at 600 nm and then lysostaphin was added to give a final concentration of 20 ug/ml. Subsequently the tubes were incubated in a 37°C water bath for 30 minutes and the optical density was again determined. The optical density remaining after the incubation was used for direct comparison of osmotic stability of the strains.

2.5.7 Effect of BHA on protoplasts

Protoplasts were produced according to the technique of Huber and Schuhard (1970). S. aureus was grown in BHI broth overnight at 37°C. Cells were harvested at 4°C (11,000 x g, 10 minutes), resuspended in Tris buffer and diluted 1:9 (v/v) in a sufficient volume of 30% (w/v) NaCl solution at 37°C. The test preparations received 1 ml of lysostaphin (10 U/ml) and were incubated for 60 minutes at 37°C in a water bath. Periodic examinations by phase contrast microscopy ensured that proto-

plasts were formed. After this period of incubation the protoplast preparations were transfered to an ice bath and allowed to stabilize for 10 minutes. Then BHA was added and the optical density at 500 nm was monitored at intervals against 24% (v/v) NaCl solution as a blank. Controls receiving the same amount of ethanol (2%, v/v) were run in parallel.

2.6 Testing for the mutagenicity of BHA

To demonstrate that BHA was possibly causing genetic alterations to the Wood 46 strain and that it was not merely acting as a selecting agent for the variant strains, the following experiment was carried out. Five ml of BHI broth containing 0,14 and 0,07 mM BHA were dispensed in 23 tubes and a small inoculum (3×10^3 CFU/tube) was added at zero time. These tubes were then incubated at 37°C with agitation. Growth was stopped at approximately mid-exponential phase or at 7 days (whichever came first) and the whole content of each tube was plated on either bacitracin agar for further replica plating on blood agar, or directly on blood agar. Controls were set as follow: 28 tubes containing 5 ml of medium plus 2% (v/v) ethanol, which was used as a solubilizing agent for BHA, were divided in two groups. The whole content of 14 of these tubes was plated as described earlier immediately after the addition of the inoculum to check for the possibility that resistant bacteria were already present in the inocula. The other 14 tubes were incubated until mid-exponential phase and their content plated

as described previously.

2.7 Gas chromatographic analysis to determine the fate of BHA in S. aureus culture

These experiments were carried out in order to determine whether S. aureus was able to metabolize BHA during an incubation of 30 hours at 37°C in BHI broth containing 0,28 mM BHA. BHI broth containing 0,28 mM BHA was used as a control at zero time. Cultures and controls were acidified or made basic and extracted 3 times with 1 volume of ethyl acetate. These extracts were dried on magnesium sulfate, evaporated to dryness and the residues solubilized in 2 ml of methanol. Five ul of these methanolic preparations were injected the column. The column used was a 183 cm x 2 mm I.D. glass column packed with 3% SE 30 on 80/100 Supelcopac from Supelco Inc (Bellafonte, Pa). The chromatograph was a Tracor model 220 GLC and the oven was programmed between 100 and 180°C at a rate of 3°/minute. BHA was identified by comparison with the retention time of a BHA standard.

2.8 Treatment with mutagenic and curing agents

2.8.1 Treatment with ethylmethane-sulfonate (EMS)

Treatment with EMS was carried according to the method of Forsgren et al (1971) with S. aureus. S. aureus Wood 46 was grown

with agitation (250 rpm) overnight in BHI broth and 2 ml samples were subsequently mixed with 0,4 ml of EMS (Sigma). The mixture was incubated for 10 minutes at 37°C after which the cells were washed twice in phosphate buffer (para. 2.4) and then resuspended in 5 ml of BHI broth and incubated for 6 hours at 37°C. The suspension was then diluted and plated on sheep blood agar in order to detect the hemolytic pattern.

2.8.2 Treatments with sodium dodecyl sulfate (SDS), guanidine hydrochloride (GuHCl) and acridine orange (AO).

Treatments with SDS (0,002%, 0,004% and 0,008%, final concentrations) GuHCl (2 mg/ml, final concentration) and AO (25 ug/ml, final concentration) were carried out according to the methods of Sonstein and Baldwin (1972), Costa et al (1980) and Forsgren et al (1971) respectively. BHI culture media containing the appropriate concentrations of the curing agents were inoculated with S. aureus Wood 46 (10^5 CFU/ml) and incubated with agitation (250 rpm) at 37°C for 48 hours in the case of AO and for 18 hours with the 2 other agents. After that period of time the suspensions were diluted and plated as described above (para. 2.8.1).

2.9 Adsorption of BHA onto bacterial cells

Cells from mid-log phase cultures were washed and resuspended (para. 2.4) in BHA containing pH 7,0, 0,05 M phosphate buffer to give a final concentration of 3×10^9 CFU/ml. Since BHA was added as an ethanolic solution, controls containing the same amount of ethanol but no BHA were also run in parallel. Incubation was at 30°C with agitation (250 rpm). At intervals cells were removed by centrifugation (1000 x g , 10 minutes) and the supernatant clarified by centrifugation at 4°C (10,000 x g, 20 minutes). The supernatant was extracted with one volume of ether. BHA was recovered in the organic phase and estimated by absorption at 292 nm with reference to a standardized curve, according to the method of Alicino et al (1963). Preliminary studies showed that no traces of BHA were detected in the aqueous phase as estimated spectrophotometrically. Adsorption of BHA was calculated by difference with respect to the inoculated control containing 2% ethanol only.

The adsorption isotherm was determined as follows: preliminary experiments showed that adsorption was rapid and completed within 15 minutes; hence, cell suspensions prepared as mentioned above were incubated with different concentration of BHA for 15 minutes, after which time the adsorption was measured as described above.

2.10 Leakage of nucleotides

Cell suspensions were prepared as mentioned previously (para.

2.9). Incubation was at 4°C in order to avoid leakage of nucleotides resulting from autolytic enzyme activity. Suspensions were lightly agitated by means of magnetic stirrers and treated for 2 hours with different concentrations of BHA. After this period, BHA was removed as in para. 2.9 and the material absorbing at 260 nm was estimated in the aqueous phase against a blank consisting of phosphate buffer.

2.11 Effect of BHA on oxygen consumption

2.11.1 Preparation of whole cell suspensions and cell-free extracts

Cells from 18 hours old cultures in nutrient broth were harvested by centrifugation at 10,000 x g for 15 minutes at 4°C in a Beckman J-21-B centrifuge. The cells were washed twice with 0,05 M potassium phosphate buffer, pH 7,0 and resuspended in an appropriate volume of the same buffer. Dry weights were determined by drying 1 ml samples of cell suspensions to a constant dry weight in an air oven at 105°C.

To prepare cell-free extracts, cell suspensions were passed three times through the cold French Pressure Cell at 18,000 psi (1 psi= 703.069 kg/m^2). After centrifugation at 20,000 x g for 30 minutes the resulting supernatants were used as the cell-free extracts. Protein concentration in the cell-free extractswere determined by the method of Lowry et al (1951) using crystalline bovine serum albumin as standard.

2.11.2 Measurement of oxidation

To study the effect of BHA and other inhibitors on the oxidation of different substrates by cell-free extracts or cell suspensions of S. aureus, oxidase activities were assayed by determining the the rate of O_2 uptake using the Clark oxygen electrode (Model 53, Yellow Spring Instrument Co.). All assays were carried out at 30°C in 0,05 M potassium phosphate buffer, pH 7,0 in a total volume of 3 ml. The reaction mixture containing buffer and cell suspensions or cell-free extracts was incubated for 3 minutes in the presence or absence of BHA or other inhibitors and the endogenous O_2 uptake was recorded. Then substrate (7 mM) was added and the final rate of O_2 uptake was measured. The experiments were repeated three times.

2.11.3 Inhibitors

The inhibitors rotenone, antimycin A, 2-n-nonyl-4-hydroxyquinoline-N-oxide (HQNO) as well as BHA were dissolved in 95% ethanol. Atabrine and cyanide were aqueous solutions. Where indicated preparations were incubated during equilibration period with appropriate concentrations of inhibitors. This volume of ethanol did not affect oxidase activity. All chemicals were purchased from Sigma Chemical Co. (St-Louis, Mo.)

2.11.4 Effect of BHA on proton translocation

Proton translocation was measured according to the method of Boyd and Beveridge (1981). Cells were harvested at mid-exponential phase (para. 2.4), washed twice in pH 7,0, 0,05 M phosphate buffer and resuspended at a concentration of 3×10^9 CFU/ml in 2 mM glycylglycine buffer (pH 7,3). Prior to measurements of proton translocation, cells were treated with BHA for 15 minutes at 30°C. Controls were treated with 2% ethanol (v/v) since BHA was added as an ethanolic solution. Pretreated cell suspensions were pulsed with 0,01 M HCl in order to lower the pH to 3,8 and the subsequent rise in pH monitored during 10 minutes at 30°C by means of a glass electrode connected to a chart recorder.

2.12 Effect of BHA on the reduction of cytochromes

2.12.1 Difference spectra

The reduced minus oxidized difference spectra were obtained at room temperature using a Cary Model 118C dual-beam recording spectrophotometer. Aerobic versus anaerobic difference spectra were obtained by comparison of the sample made anaerobic by evacuation in Thunberg-type cuvettes with samples exposed to air for 3 to 5 minutes.

2.13 Effect of BHA on dehydrogenase activity

Cells of S. aureus from a late log-phase culture in nutrient broth were harvested by centrifugation (para. 2.4), washed twice with 0,10 M phosphate buffer, (pH 7,6) and resuspended in the same buffer at a concentration of 3×10^9 CFU/ml. The suspension was incubated at 30°C for 2 hours with agitation (300 rpm) to reduce as much as possible the concentration of endogenous substrates. Dehydrogenase activity was determined by measuring the reduction of triphenyl tetrazolium chloride (TTC) using the method of Hugo and Longworth (1966). Prior to incubation with TTC cell suspensions were treated for 15 minutes at 30°C with BHA. To 2,5 ml of the treated cell suspension were added 1,0 ml of TTC (250 ug/ml), 1,4 ml of phosphate buffer and 0,1 ml of substrate (0,02 M) or 1 ml of TTC plus 1,5 ml phosphate buffer, the latter to monitor reduction of TTC by residual endogeneous substrates. Heat-killed cells (10 minutes exposure to 100°C) were used as a negative control. Other controls included the system lacking either TTC or cells. The mixture was incubated at 37°C for 45 minutes. After this period the colored formazan produced by the reduction of TTC was extracted with 5 ml of acetone. The cells were discarded by centrifugation (5,000 x g, 10 minutes) and the absorbance of the supernatant was measured spectrophotometrically at 485 nm against a blank containing heat-killed cells treated in the same way. BHA did not absorb at this wavelength.

2.14 Effect of BHA on the uptake of nutrients

Cells from mid-log phase cultures of S. aureus grown in the special medium of Collings and Lascelles (1963, appendix 2) were harvested (para. 2.4) and resuspended in 0,05 M phosphate buffer (pH 7,0) to a final concentration of 3×10^9 CFU/ml. Prior to the measurements of nutrient uptake, cells were treated for 15 minutes at 30°C with BHA or with 2% (v/v) ethanol as a control. The effect of BHA on the uptake of amino acids (0,5 uCi/ml) was examined in the presence of 0,5 mM glucose at 30°C and with agitation (250 rpm). Duplicate 1 ml samples were removed at intervals and filtered (0,45 um filters) under vacuum with a manifold filtration apparatus. Bacteria collected on the filters were washed with 5 x 1 ml of ice cold 0,05 M phosphate buffer (pH 7,0) and the filters counted for radioactivity.

In the case of glucose uptake (1 uCi/ml), cells were treated with BHA in the presence of cold glucose (0,2 mM).

2.14.1 Measurement of radioactivity

Uniformly (^{14}C) labelled L-glutamic acid (271 mCi/mmole), L-isoleucine (347 mCi/mmole), L-lysine (338 mCi/mmole) and glucose (4 mCi/mmole) were obtained from New England Nuclear (NEN, Boston, MA) Radioactivity was measured with a Beckman CPM 100 liquid scintillation system for either 10 minutes or 1% error using 10 ml of Bray's solution

(NEN). Counts were corrected for quenching by means of internal standardization using ^{14}C-toluene (NEN). The efficiency of counting was about 65%.

2.15 Effect of BHA on adenosine triphosphatase (ATPase) activity

2.15.1 Membrane isolation

Membranes were isolated according to the method of Kubak and Yotis (1981). Cells were grown in TSB at 37°C with shaking (250 rpm) and harvested at mid-exponential phase (para. 2.4). They were washed twice in 0,05 M phosphate buffer and resuspended in hypertonic buffer (appendix 5): 5 g (wet weight) of cells in 20 ml of buffer. This preparation was treated with lysostaphin (375 ug) and DNase (500 ug) for 1 hour at 37°C. After this period an additional 50 ug of lysostaphin was added and incubation continued for 15 minutes. The protoplasts were removed by centrifugation at 4°C (10,000 x g, 30 minutes), resuspended in 10 ml of hypotonic buffer (appendix 5) and allowed to equilibrate on ice for 20 minutes. Then the mixture was charged with DNase (500 ug) and RNase (500 ug, Sigma) and incubated at 37°C for 20 minutes. The lysate was centrifuged (2,000 x g, 10 minutes, 4°C) and the supernatants containing the membrane fraction centrifuged again in the same conditions. This crude preparation was finally centrifuged at 4°C (35,000 x g, 25 minutes) and the membrane pellet was washed once in the

hypertonic buffer and then washed five times in the hypotonic buffer. The membranes were stored in small volumes at -70°C. Protein determination was carried out by the method of Lowry et al (1951).

2.15.2 ATPase assay

Membrane-associated ATPase activity was determined according to the technique of Kubak and Yotis (1981). The ATPase reaction mixture (appendix 6) (final volume: 1 ml) containing various concentrations of BHA was loaded with 20 ul of the membrane preparation and incubated for 30 minutes at 37°C. Since BHA was added as an ethanolic solution, the reaction mixtures in all cases, including the controls without BHA, were brought to a final ethanolic concentration of 1% (v/v). After the 30 minutes incubation period 0,5 ml perchloric acid (1,5 M) were added to stop the reaction and the mixture was centrifuged at 4°C (2,000 x g, 15 minutes). ATPase activity was measured by estimating the release of inorganic phosphorus (P_i) in the supernatant using the method of Chen et al (1956). According to this method the concentration of P_i released is measured at 820 nm with reference to a standard curve using P_i obtained from Sigma. BHA does not absorb at this wavelength.

The controls included the reaction mixture in which the membrane preparation was added after perchloric acid treatment. Care was taken to use a vanadate-free ATP preparation (Na-ATP, Sigma) since vanadate has been shown to inhibit the enzyme activity (Cantley et al, (1977).

One unit of enzyme activity is expressed as the amount of enzyme releasing 1 umole of P_i in a 30 minute period; specific activity is expressed as units per mg of membrane protein.

2.15.3 Effect of Ca^{2+}

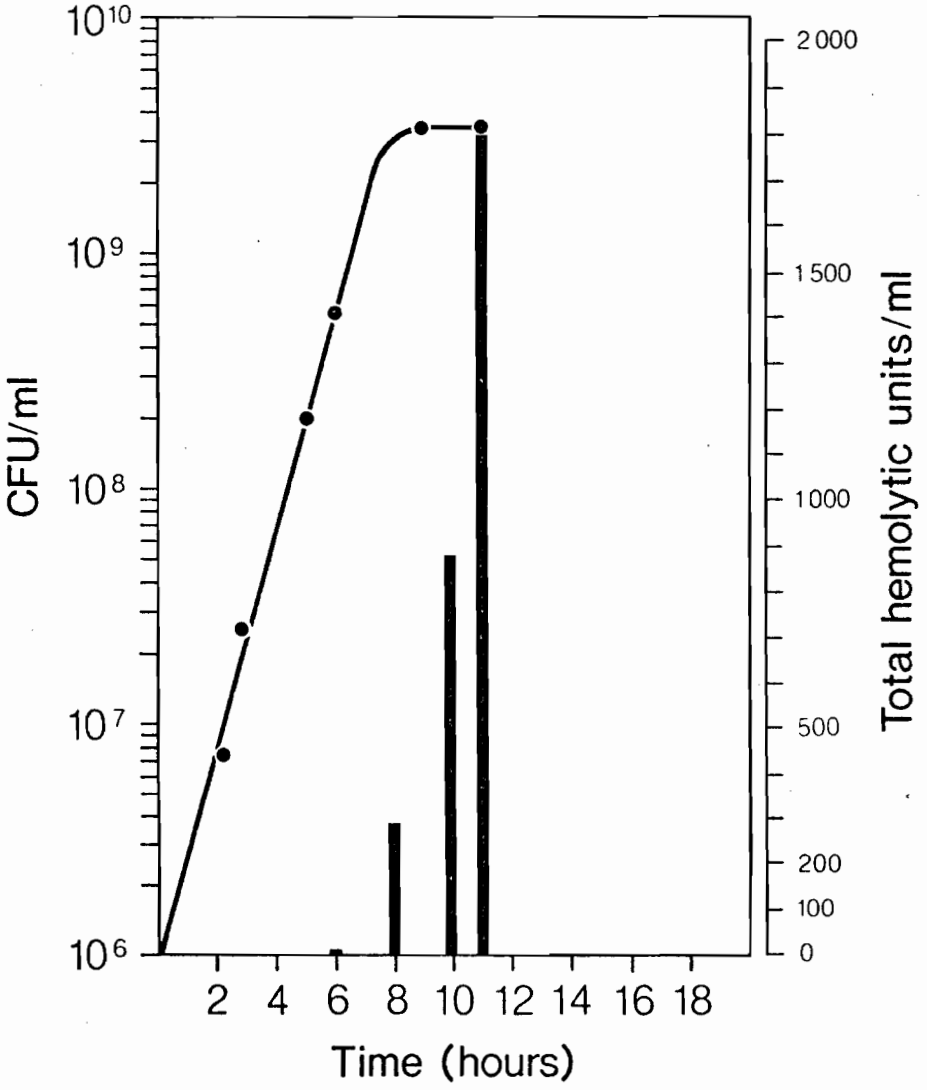
The effect of Ca^{2+} on ATPase activity was estimated by adding different Ca^{2+} concentrations to the ATPase reaction mixture.

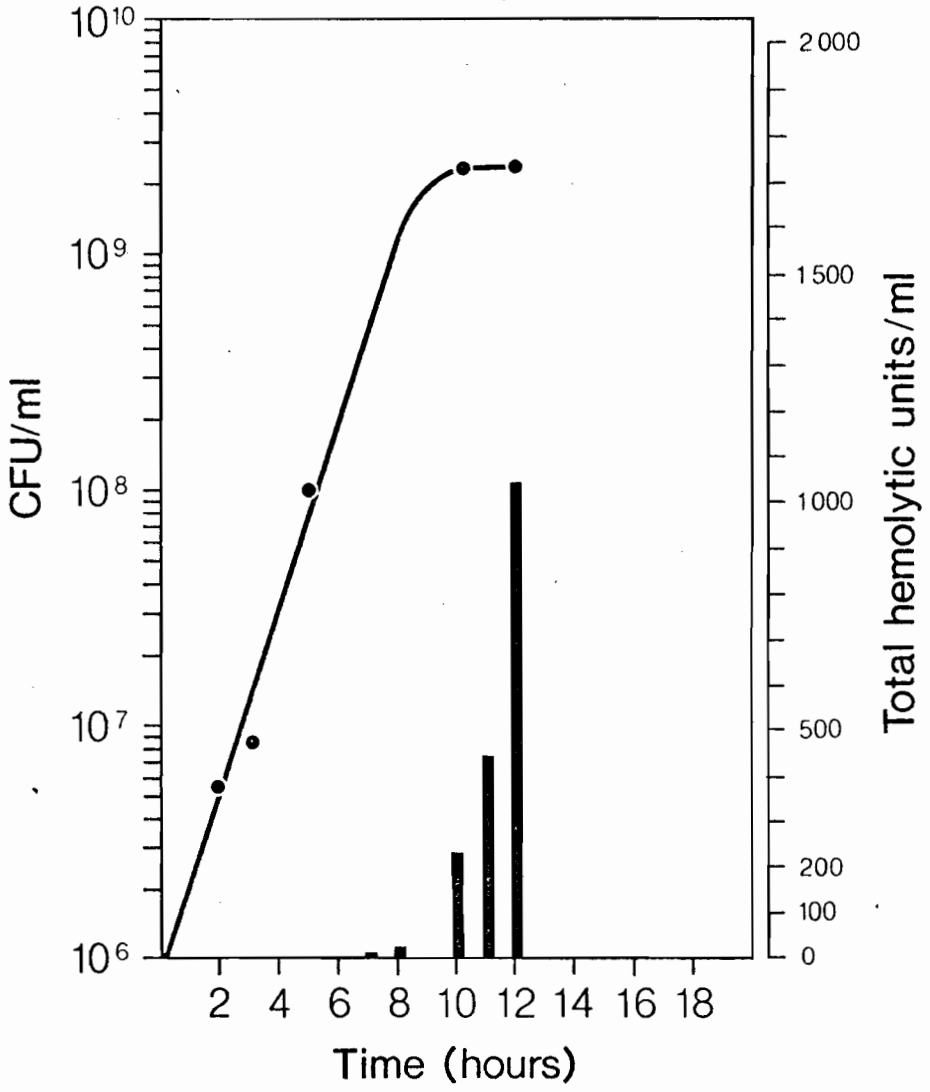
RESULTS

3.1 Minimal inhibitory concentrations

Mic value of BHA against S. aureus Wood 46, as determined after 6 hours under aerobic conditions, was 0,28 mM in BHI broth, TSB, nutrient broth and in the special medium of Collings and Lascelles (1963). Mic was also determined under anaerobic conditions in BHI broth. This particular strain of S. aureus was found to be more sensitive to BHA under anaerobic conditions since in those conditions, 0,14 mM was sufficient to inhibit the growth of the organism for 24 hours. Prior to testing in anaerobic conditions, S. aureus was subcultured 10 times in the absence of oxygen to ensure that it was fully adapted to these conditions of growth.

In aerobic conditions a concentration of 0,07 mM had no effect on the visible growth of S. aureus Wood 46 as compared to that of a control containing a final concentration of 2% (v/v) ethanol. This volume of ethanol was essential to ensure solubilization of BHA and had only a slight inhibitory effect on the growth rate of this strain as compared to the medium without ethanol (Fig. 4 and 5). One hundred ug/ml totally inhibited visible growth and no viable cells were recovered after 24 hours of incubation as determined by plating on blood agar and on optimal medium (Hurst and Hughes, 1981). Plating on these two different media ascertained that cells were killed and that decrease in viability was not merely the result of injured cells that were unable


10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
CFU/ml
2 000
1 500
1 000
500
200
100
0
Total hemolytic units/ml
2 4 6 8 10 12 14 16 18
Time (hours)


10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
CFU/ml
2 000
1 500
1000
500
200
100
0
Total hemolytic units/ml
2 4 6 8 10 12 14 16 18
Time (hours)

to grow on blood agar. The results indicate that a concentration of 0,56 mM was bactericidal for S. aureus Wood 46.

3.2 Effect of BHA on growing cell suspensions

3.2.1 Effect on viability

To further evaluate the effect of BHA on growing cell suspensions, S. aureus Wood 46 was grown aerobically in BHI broth containing 0,14 and 0,28 mM and the CFU monitored at intervals. Controls with ethanol have already been described and indicated that the presence of 2% (v/v) ethanol in the growth medium only slightly delayed the growth (Fig. 4 and 5).

In the presence of 0,14 mM BHA the overall growth rate was decreased but growth was mainly affected during the first 4 hours of incubation (Fig. 6). After this period growth resumed but the growth rate was slightly lower than that of the control.

Addition of 0,28 mM BHA to the medium resulted in a growth curve characterized by a bactericidal phase followed by a phase of active growth (Fig. 7). Hence, there was a significant decrease of the CFU during the first 10 hours. Microscopic examinations revealed that clumping was not responsible for the drop in CFU. Moreover, plating on both blood agar and optimal medium ascertained that injured cells were not responsible for this decrease in the viability of the cell suspension.

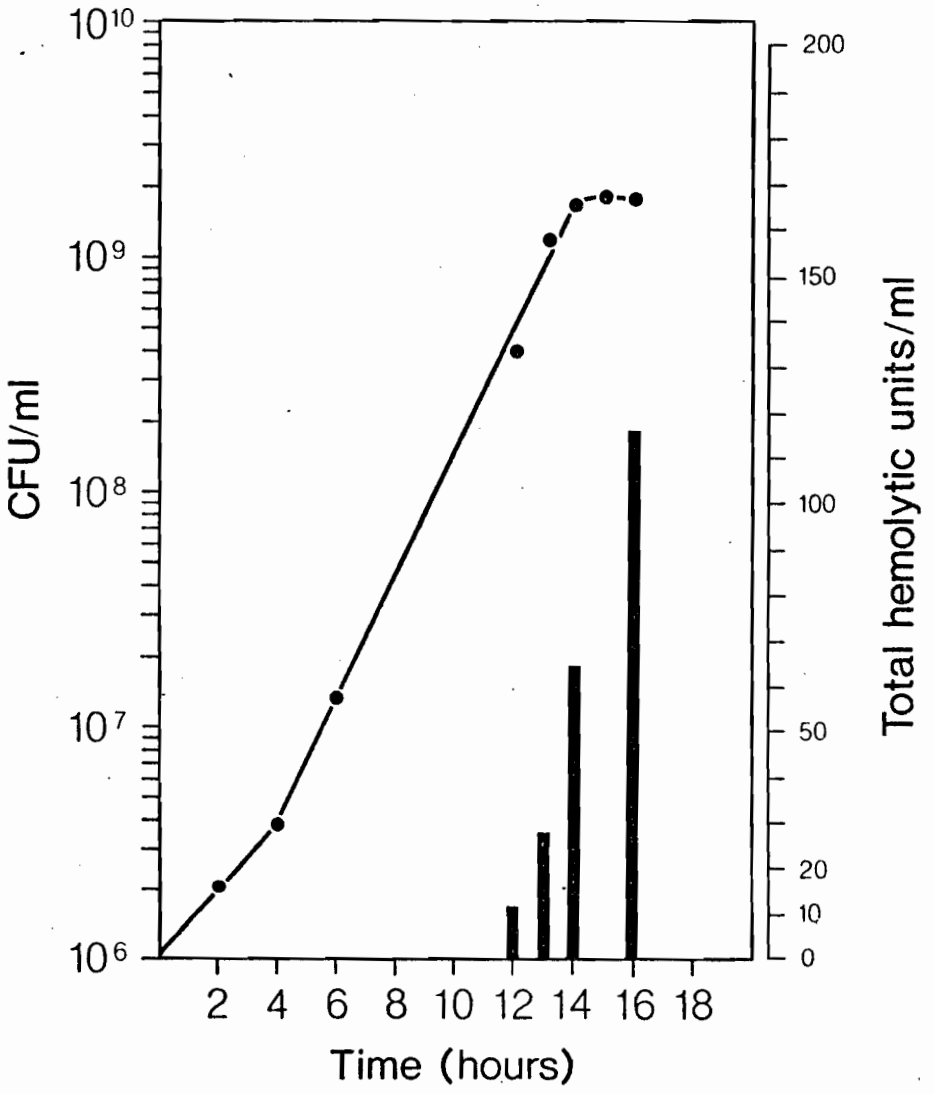
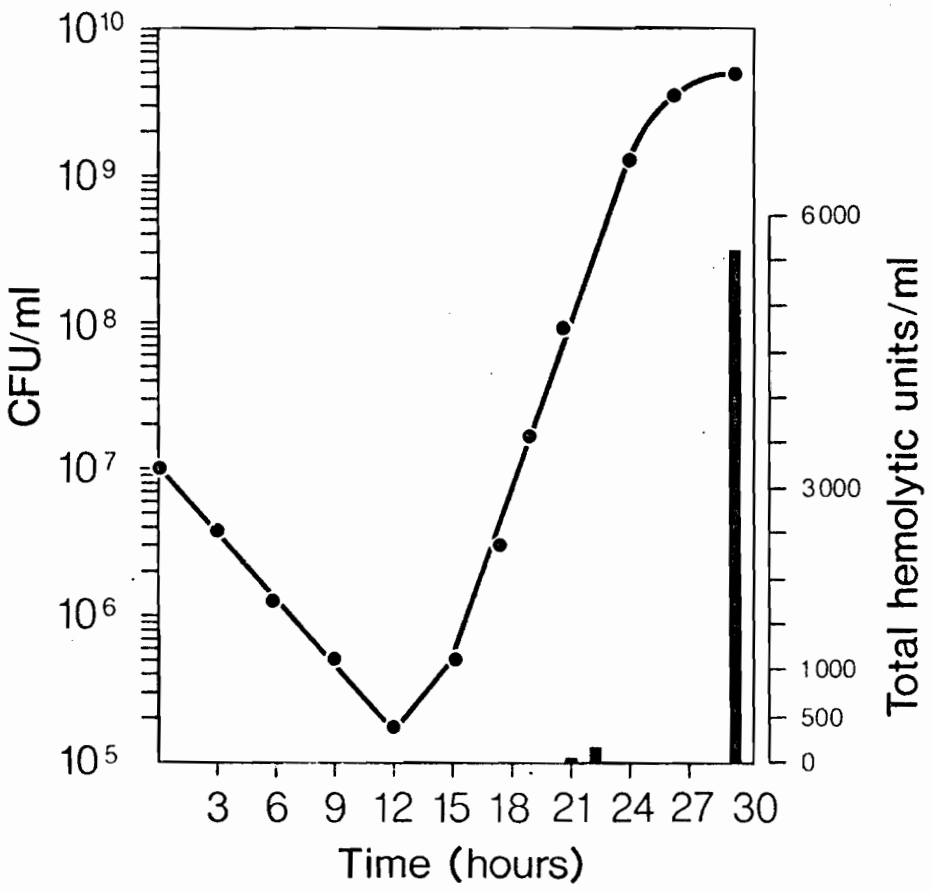

10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
CFU/ml
200
150
100
50
20
10
0
Total hemolytic units/ml
2 4 6 8 10 12 14 16 18
Time (hours)

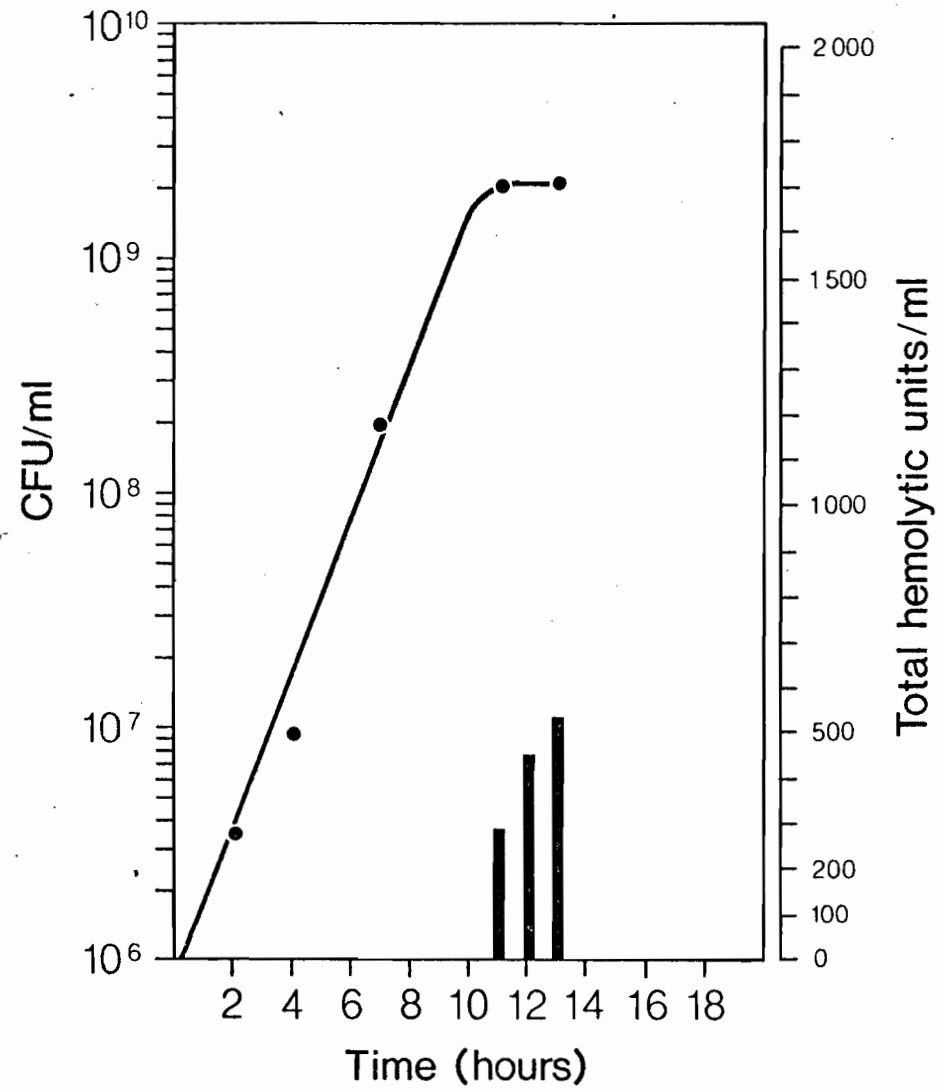
Fig. 7. Growth of S. aureus Wood 46 parent strain at 37°C in BHI broth containing 0,28 mM BHA and associated alpha-hemolytic activity.


10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
CFU/ml
6000
3000
1000
500
0
Total hemolytic units/ml
3 6 9 12 15 18 21 24 27 30
Time (hours)

S. aureus Wood 46 usually produced medium size colonies with zones of complete hemolysis (beta-hemolysis) around the colony. It is interesting to note that colonies different from those of the inoculum were observed throughout the first 10 hours of contact with 0,28 mM BHA . They were either dwarf or normal size colonies with or without a zone of hemolysis surrounding the colony; hemolysis was either complete (beta-hemolysis) or incomplete (alpha-hemolysis). At this point, it is important to recall that on sheep blood agar the alpha-toxin is known to produce a zone of beta-hemolysis while the beta-toxin is known to produce a zone of alpha-hemolysis (Elek and Levy, 1950). Those modified colonies gradually increased in number up to 11 to 12 hours. At this stage, each and every colony that developed on blood agar was morphologically and/or hemolytically different from those arising from the inoculum. These modified colonies were however unstable and reverted to the original type when subcultured once on blood agar and had also restored their sensitivity towards 0,28 mM BHA . However, by the end of the bactericidal phase and for the next 12 hours, a new type of modified colony, different from those just described, and characterized by alternatively hemolytic and non-hemolytic rings around the colonies, was observed on blood agar. This distinct hemolytic pattern will be further characterized in another section.

It is interesting to note that colonies isolated after 26 hours had became fully resistant to 0,28 mM BHA . This new isolate was able to grow in BHI broth containing 0,28 mM BHA (Fig. 8) although in those conditions this variant was growing slightly slower than the

Fig. 8. Growth of S. aureus Wood 46 variant strain at 37°C in BHI broth containing 0.28 mM BHA and associated alpha-hemolytic activity.


10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
CFU/ml
2 000
1 500
1000
500
200
100
0
Total hemolytic units/ml
2 4 6 8 10 12 14 16 18
Time (hours)

parent strain in 2% ethanol (Fig. 5). This resistance was only relative since mic studies conducted in aerobic conditions, in BHI broth, showed that growth was inhibited by 0,56 mM BHA.

When the parent strain was grown overnight in BHI broth without BHA, and the culture left undisturbed, it was noticed that the cells settled down very readily to the bottom of the tube, forming a well defined button. The variant, when grown in the same medium with or without BHA, rather formed a smaller, irregular button while the medium remained hazy, even when the culture was left undisturbed for several hours. Except for the hemolytic pattern, both cultures yielded the same type of colonies.

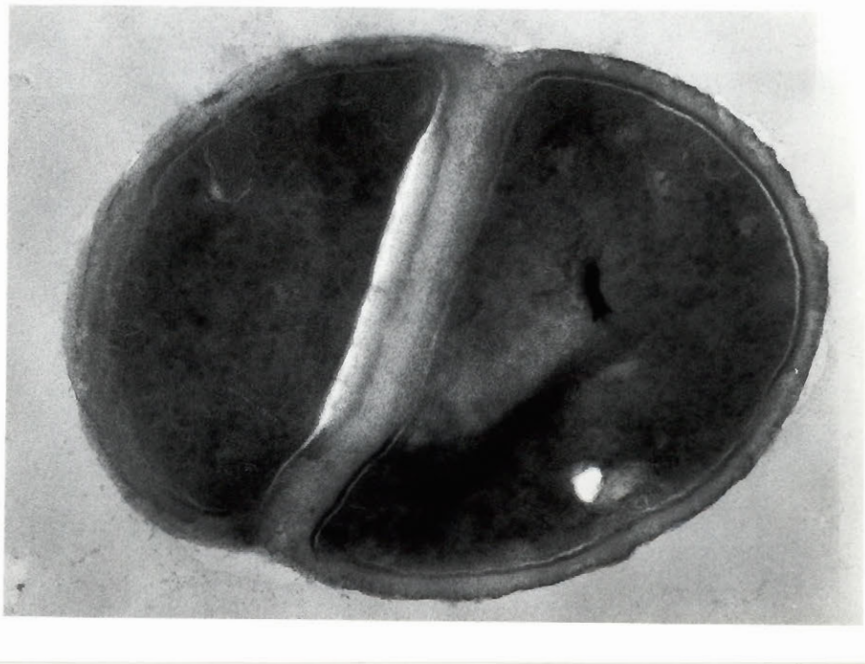
3.2.2 Effect of BHA on cellular morphology

In order to gain some preliminary information on the antibacterial activity of BHA and to determine whether the antioxidant affected the morphology of the cell, electron microscopic examinations were carried out. A suspension of 10^7 CFU/ml of S. aureus Wood 46 was incubated with 0,28 mM BHA at 37°C for 6 hours in BHI broth; after that period the number of CFU had dropped to 10^5 CFU/ml. The inoculum and samples taken at 3 and 6 hours were examined. No significant differences in cellular morphology between the treated suspension and the inoculum were noted (Fig. 9 and 10).

The morphology of the parent and the variant strain was also analyzed after they were grown in BHI without BHA; electron microscopy

Fig. 9. Thin section of S. aureus Wood 46 parent strain grown for 6 hours in BHI broth. 75,600 X.

Fig. 10. Thin section of S. aureus Wood 46 parent strain grown for 6 hours in BHI broth containing 0.28 mM BHA. 75,600 X.



did not reveal any differences between these two strains.

3.2.3 Reversal and potentiation of the inhibition caused by BHA

Growth of S. aureus in a medium containing 3% glycerol, devised to increase its fat content, had no effect on its susceptibility towards BHA since 0,56 mM were necessary to inhibit growth for 24 hours. Addition of $MgCl_2$, up to 4 mM, in combination with BHA, had no effect either. However, the tubes containing 4 mM $CaCl_2$ in combination with 0,56 mM BHA showed a weak turbidity, indicative of growth, after 24 hours. Turbidity increased slightly over the next 24 hours but remained weak. Plating on blood agar did not show colonies with the distinct hemolytic pattern of the variant strain but rather dwarf or normal size colonies, with or without hemolysis.

The mic of EDTA towards S. aureus Wood 46 was 125 ug/ml. A concentration of 62,5 ug/ml had no effect on visible growth after 24 hours. When 0,14 mM BHA and 31,25 ug EDTA/ml were added in combination, growth was inhibited to the point that no viable cells were recovered after 24 hours.

3.3 Effect of BHA on hemolytic activity

The Wood 46 strain of S. aureus has been known for a number of years for its production of alpha-hemolysin (Madoff and Weinstein, 1962; Watanabe and Kato, 1974). According to Abbas-Ali and Coleman

(1977) alpha-toxin is produced at the end of the exponential phase and during the post-exponential phase of growth and, as a result, is considered a secondary metabolite. Because of these previous observations, in our experiments the alpha-hemolytic activity was analyzed at the end of the exponential phase and at the onset of the stationary phase. This activity was measured by the method described in para. 2.5.1.4, with rabbit erythrocytes. The intervals selected correspond to Fig. 4 to 8.

When S. aureus Wood 46 was grown in BHI broth without ethanol, hemolytic activity was detectable after 6 hours at a cellular concentration of about 5×10^8 CFU/ml (Fig. 4). After 10 and 11 hours total activities reached 900 and 1800 H.U./ml respectively; these values correspond to 26 and 53 H.U./10^8 CFU. In the presence of ethanol (Fig. 5), hemolytic activity appeared in the growth medium of 7 hours old cultures and total hemolytic activities were of 400 (18 H.U./10^8 CFU) and 1000 H.U./ml (43 H.U./10^8 CFU) after 11 and 12 hours respectively. Concomitantly to the slower growth rate noticed after addition of 0,14 mM BHA (Fig. 6), hemolytic activity was detected after 12 hours of incubation and reached only 111 H.U./ml (7 H.U./10^8 CFU) after 16 hours.

In the presence of 0,28 mM BHA (Fig 7), hemolytic activity was detectable after 21 hours and reached 5500 H.U./ml (114 H.U./10^8 CFU) after 29 hours of incubation. It is of interest to note that colonies isolated at that time were different from those of the ino-

culum and those isolated with 0,14 mM BHA . The hemolytic activity of the variant strain resistant to BHA that was isolated from cultures containing 0,28 mM BHA was also measured at different intervals. In this particular case the activity reached only 500 H.U./ml (18 H.U./ 10^8 CFU) after 13 hours (Fig. 8).

3.4 Effect of BHA on non-growing cell suspensions

Previous results showed that in actively growing cultures a concentration of 0,28 mM BHA was a threshold concentration inhibiting growth and killing the cells during the first 6 hours of contact but leading to the formation or the selection of variants characterized by a distinct hemolytic pattern. To check whether this concentration of BHA had the same effect on the hemolytic pattern of non-growing suspensions, cells (2×10^8 CFU/ml) of the parent strain were incubated at 30°C with 0,28 mM BHA in phosphate buffer and CFU's monitored over a period of 6 hours and after 24 hours. It was found that this concentration of BHA had no effect on the viability of the cell suspension. The hemolytic pattern of the colonies isolated from the control suspensions after 24 hours was the same as that of controls at zero time. However, in the presence of BHA, dwarf colonies with or without hemolysis were isolated after 5, 6 and 24 hours. The colonies were not stable since they reverted to the original type after one subculture on blood agar. It is noteworthy that no colonies like those shown in Fig. 11 and 12 and characterizing the variant strain were isolated.

Fig. 11. Appearance of staphylococcal colonies (variant strain) on sheep blood agar and showing double zones of hemolysis.

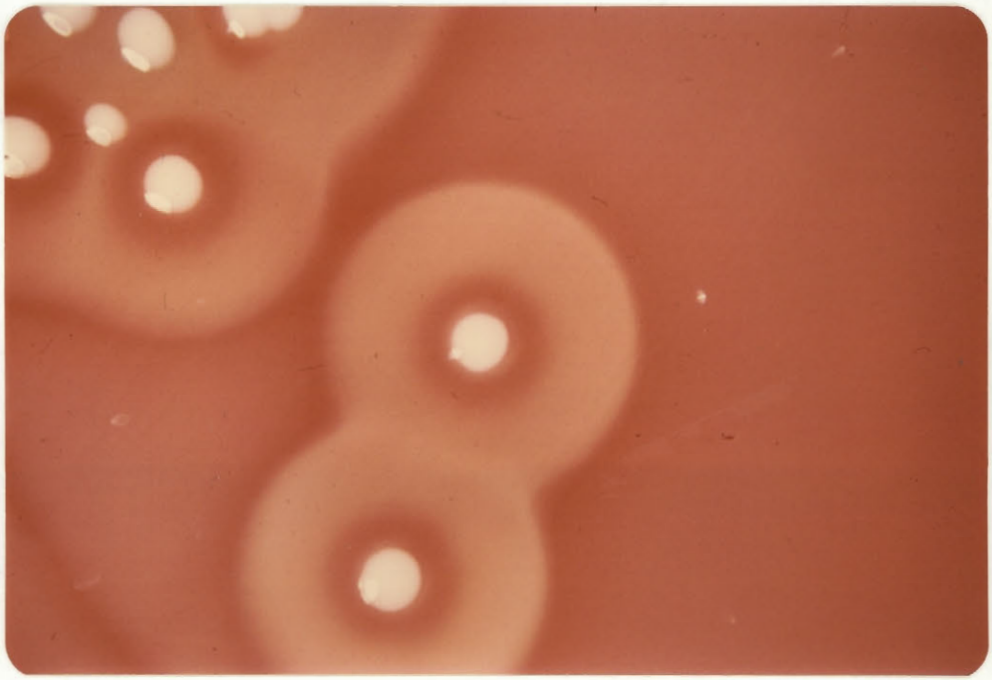
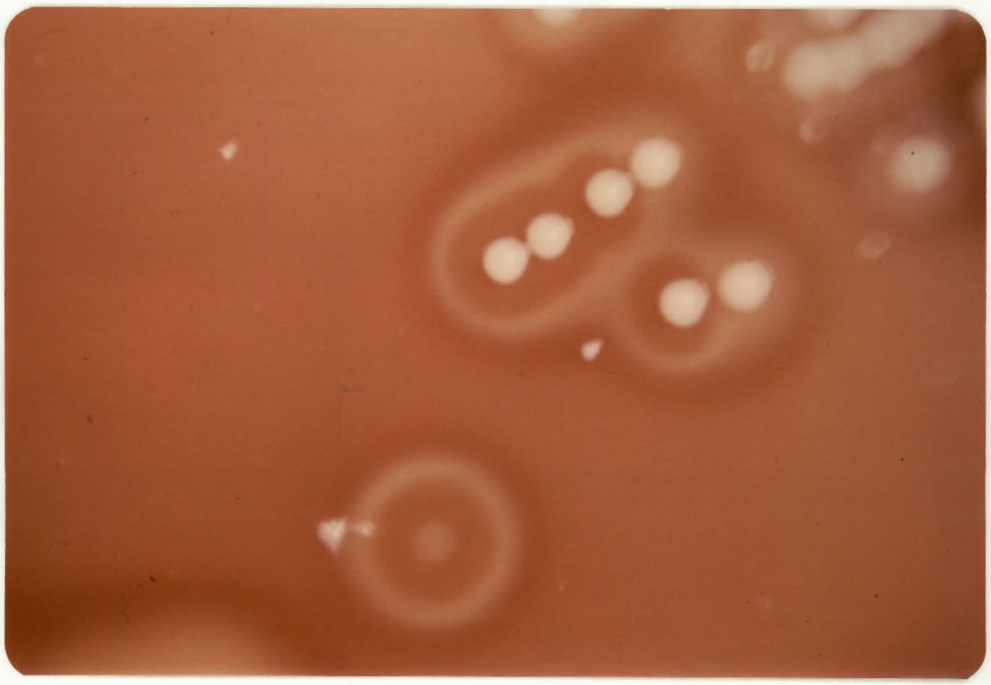


Fig. 12. Appearance of staphylococcal colonies (variant strain) on sheep blood agar and showing a zone of hemolysis underneath the colony.



3.5 Isolation and characterization of variants

3.5.1 Isolation

When a suspension of S. aureus Wood 46 was exposed to 0,28 mM BHA in BHI broth, colonies characterized by hemolytic and non-hemolytic rings around the colony were isolated on blood agar (Fig. 11). Another characteristic of this hemolytic pattern was that, although there was no hemolysis next to the colony, hemolysis was clearly visible underneath the colony when it was removed (Fig. 12). It is noteworthy that this hemolytic pattern was associated with isolated colonies only. That is to say, when a heavy line of growth occured on a petri dish, there was a zone of complete hemolysis (beta-hemolysis) next to it which, in turn, was followed by a zone of incomplete hemolysis (alpha-hemolysis).

Fifty freshly isolated variants were tested in regard of their sensitivity to BHA and mic studies showed that all of them were resistant to 0,28 mM BHA.

3.5.2 Stability of the variants

The distinct hemolytic pattern associated with colonies of the variant was stable after many subcultures on blood agar and after being stored for 2 months on nutrient agar slant without BHA at 4°C. However, prolonged storage on nutrient agar at 4°C resulted in the

loss of resistance to BHA although the distinct hemolytic pattern was unaffected.

3.5.3 Hemolytic activity

Preliminary results indicated that 0,14 mM BHA delayed the growth of S. aureus Wood 46 (para. 3.2.1). Addition of 0,28 mM BHA prior to inoculation resulted in the formation of variants with a hemolytic pattern different from that of the parent strain, as detected on sheep blood agar. To further evaluate the possible differences in the hemolytic properties of both parent and variant strains the following experiment was carried out: supernatants from both strains taken after 12 hours of growth in BHI without BHA were analyzed with respect to total alpha- and beta-hemolytic activities evaluated respectively with rabbit and sheep erythrocytes by the tube assay (Table 5). Although alpha-hemolytic activity was quite the same, there was a significant increase in the beta-hemolytic activity of the variant strain over that of the parent strain. Moreover, the presence of beta-hemolytic activity was ascertained by the fact that an hemolytic active substance, whose titer increased when incubation at 37°C was followed by an incubation at 4°C, was detected in the supernatant of the variant only.

To confirm these results, hemolytic activities of both parent and variant strains were analyzed by means of the reverse CAMP test performed on sheep blood agar. This test is based on the fact that exoproducts of S. agalactiae can potentiate the beta-toxin incomplete

Table 5. Hemolytic titers of S. aureus parent and variant strains after 12 hours of incubation at 37°C in BHI broth.

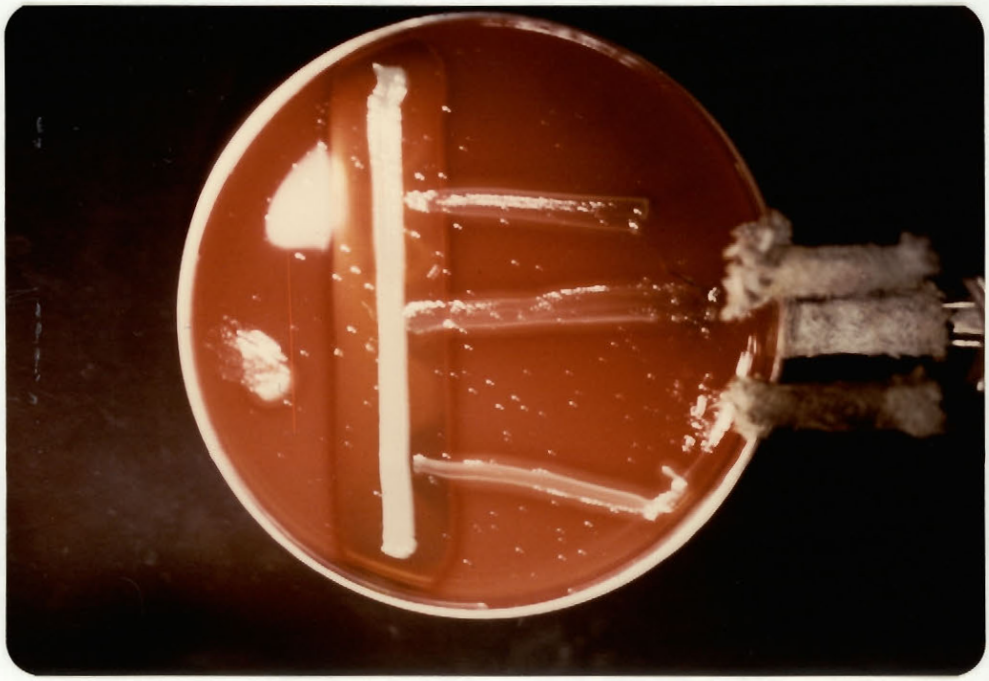
	Erythrocytes	
Strain	Rabbit	Sheep
Parent	432	28
Variant	453	237

hemolysis and turn it into a complete hemolysis. C. pseudotuberculosis exoproducts on the other hand are known to inhibit S. aureus alpha- and beta-hemolytic activities. In this particular case S. agalactiae exoproducts potentiated the incomplete hemolysis of the variant strain (Fig. 13) and had no effect on the complete hemolysis of the parent strain. C. pseudotuberculosis almost completely inhibited the hemolysis of the parent and variant strains. Fifty colonies of the variant were assayed by this method and all of them showed the same results indicating the presence of alpha- and beta-hemolytic activities associated with the variant strain and of alpha-hemolytic activity associated with the parent strain.

Unequivocal evidence for the presence of alpha- and beta-toxins in the variant and of the former in the parent strain were given by isolation of the toxins. It was done by the electrophoretic technique of Haque (1967). According to this method, the alpha-toxin migrates 18 mm towards the cathode and lyses rabbit erythrocytes while the beta-toxin migrates 36 mm towards the cathode and lyses sheep erythrocytes. Supernatants of 5 variant strains taken after 18 hours and of the corresponding parent strain were analyzed and the presence of beta-toxin was detected only in the variant strains. Both strains were alpha-toxin producers.

To eliminate the possibility that the beta-toxin although produced by the parent strain was not released, cell-associated beta-toxin was measured in both strains as described in para. 2.5.1.5 and detected only in the variant strain.

Fig. 13. Effect of S. agalactiae exoproducts (drawn horizontally) on the hemolytic pattern of S. aureus Wood 46 variant strain (drawn vertically).



3.5.4 Staphylokinase and phage typing

According to the report of Winkler et al (1965), staphylococci may produce beta-toxin and staphylokinase, the latter being a proteolytic enzyme. However, most strains that are beta-toxin producers lack the ability to produce the staphylokinase, the inverse being true. Parent and variant strains were studied in this regard and the finding of Winkler et al (1965) corroborated; therefore staphylokinase activity was detected in the parent strain only. These authors also postulated that lysogenization may be responsible for such a phenomenon since they noticed a change of typing pattern among their strains. In our study, however, both strains were of the same typing pattern, 81-83A. However a phage concentration 10 times greater than that used with the parent strain was required to type the variant strain.

3.5.5 Mechanism of resistance to BHA

The experiments that are described in this section were devised in order to have some preliminary clues on the mechanism allowing the resistant variant strains to grow in the presence of 0,28 mM BHA.

3.5.5.1 BHA inactivation

The relative resistance of the variant strain raised the question whether this new isolate could transform the molecule of

BHA into less toxic products. Gas-chromatographic analysis, performed as described in para. 2.7 with acid and basic extracts taken at zero time and after 30 hours of incubation in the presence of 0,28 mM BHA, are shown in Fig. 14 and 15. Both extracts showed the same results. Any modifications of the molecule would have led to a decrease or disappearance of the BHA peak with the possible apparition of new peaks representing new metabolites. Since the amount of BHA detected by GLC after 30 hours of incubation was the same as that added to the medium at zero time, it thus appears that BHA was fully recovered at the end of the treatment and that it was not metabolized by the cells.

3.5.5.2 Membrane mutants

Altenbern (1975) isolated mutants of S. aureus that could be differentiated on the basis of exoprotein secretion; they showed that enterotoxin B and alpha-hemolytic activity were mainly affected. In addition, these mutants displayed pronounced differences in osmotic stability and bacitracin sensitivity. These results suggested that those mutants were membrane mutants. Kent and Lennarz (1972) also reported that membrane mutants of S. aureus could be isolated by a test based on pH sensitivity. Since our previous results indicated that parent and variant strains of S. aureus Wood 46 could be differentiated on the basis of beta-hemolytic and staphylokinase activities, which are exoproteins, we submitted these two strains to the screening procedure of Altenbern (1975) and that of Kent and Lennarz (1972) in order to determine whether

Fig. 14. Gas-chromatogram of the acidified (pH 2) BHI broth extract containing 0,28 mM BHA taken at zero time.

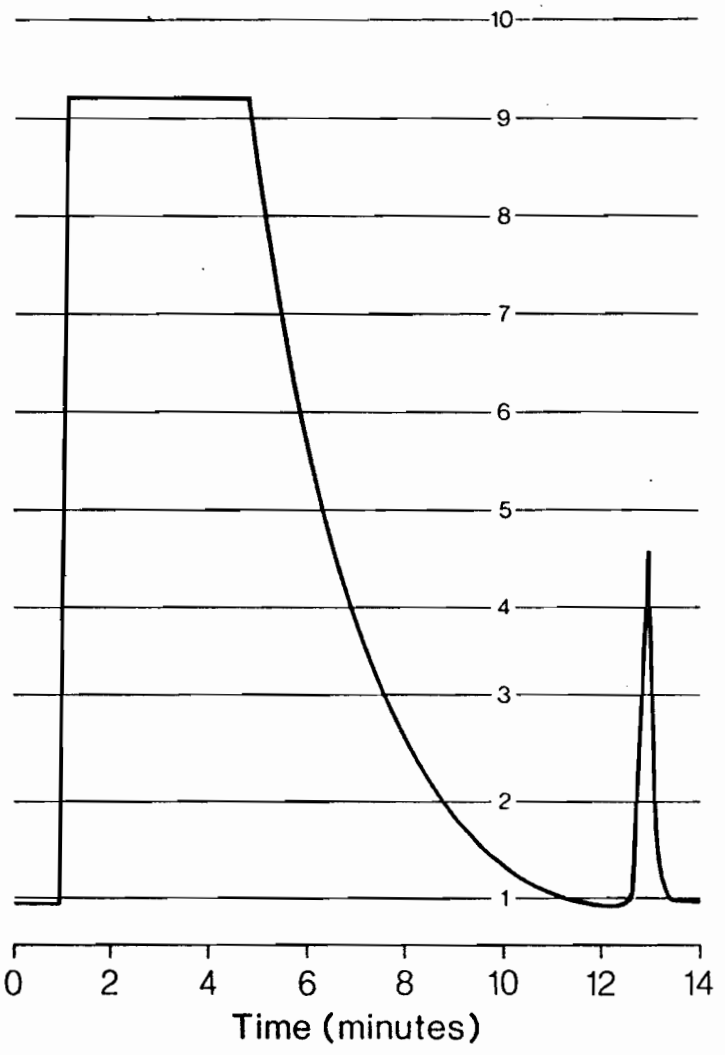
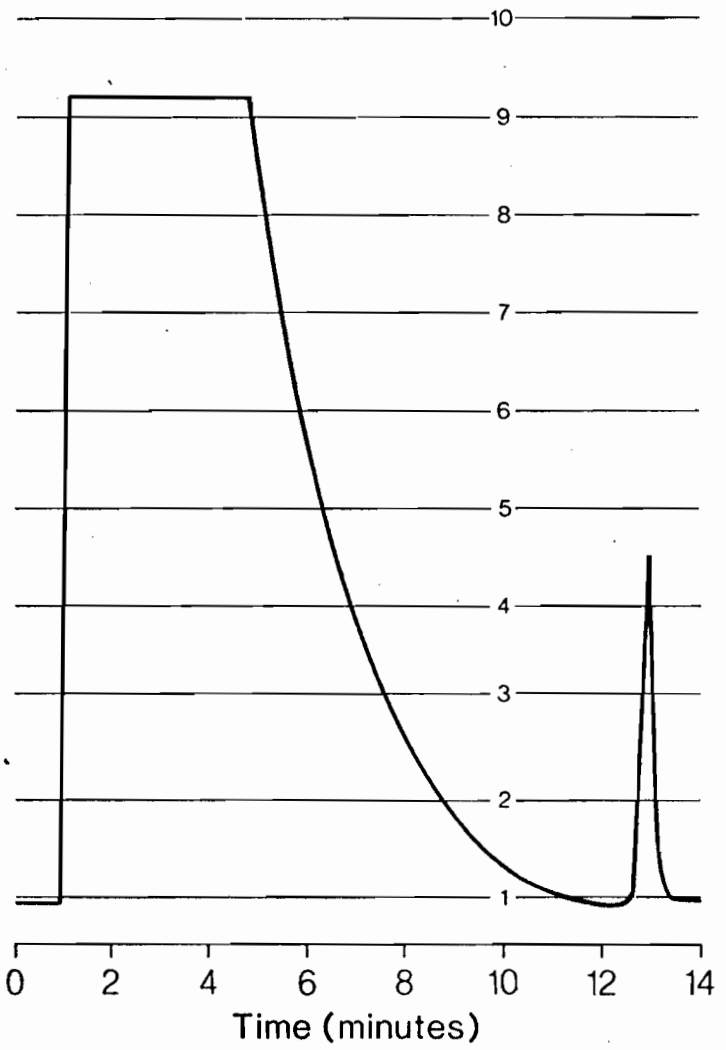

10
9
8
7
6
5
4
3
2
1
0
2
4
6
8
10
12
14
Time (minutes)

Fig. 15. Gas-chromatogram of the acidified (pH 2) S. aureus Wood 46 parent strain-BHI broth extract containing 0,28 mM BHA taken after 30 hours of incubation at 37°C.


10
9
8
7
6
5
4
3
2
1
0
2
4
6
8
10
12
14
Time (minutes)

they had properties suggesting that they were membrane mutants.

Osmotic stability was analyzed as described in Materials and Methods (para. 2.5.6) by exposing cell suspensions to lysostaphin and measuring the stability of the protoplasts at different concentrations of NaCl after 30 minutes of exposure. The slope of the curve in Fig. 16 indicates that, for NaCl concentrations above 4%, the variant strain was more resistant to lysis by lysostaphin than the parent strain. There was no indication that these strains were resistant to lysostaphin since lysis was complete in both cases in the presence of low concentrations of NaCl.

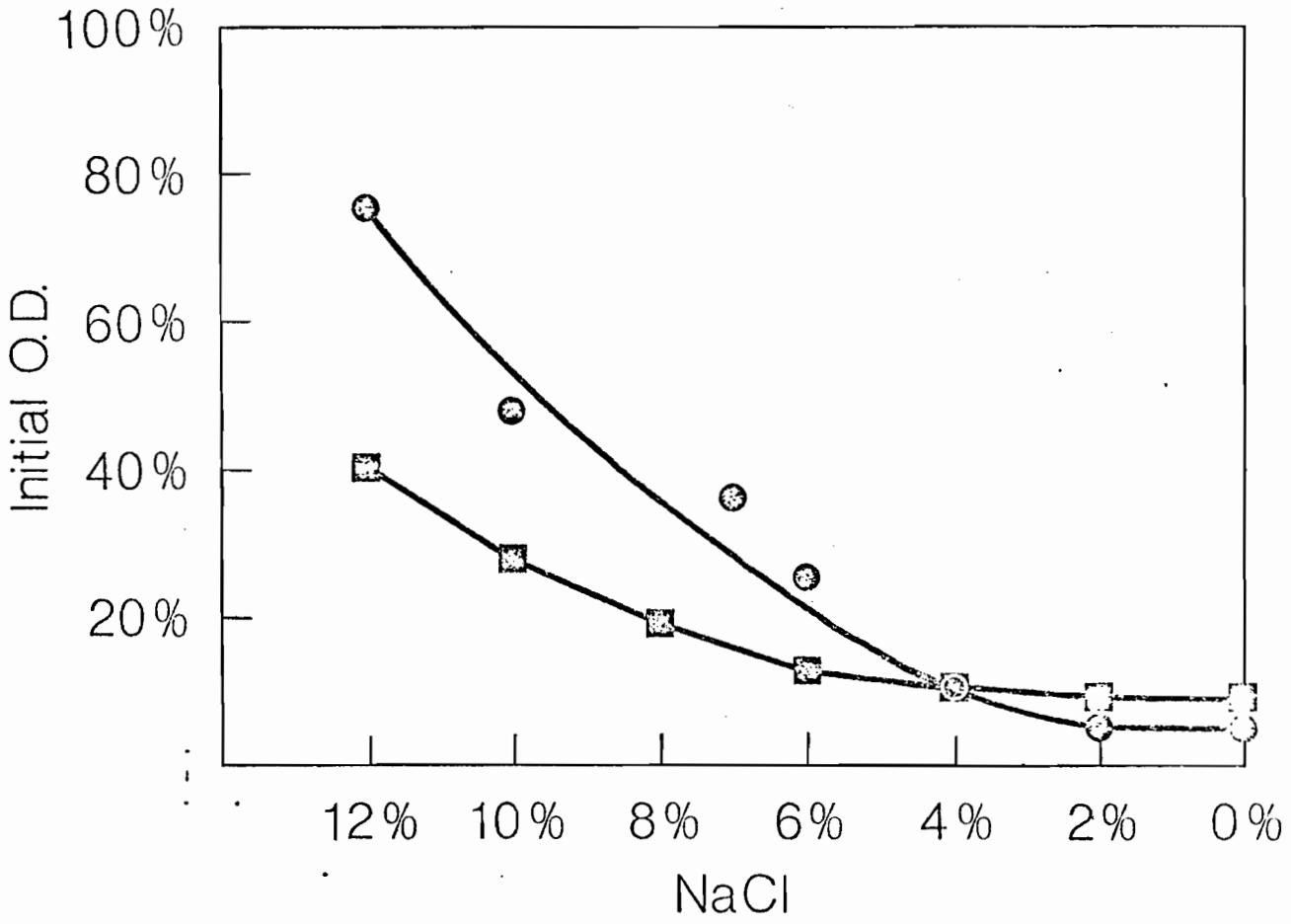
The possibility that the variant was a membrane mutant was strengthened by the fact that, in BHI broth, the variant strain grew in the presence of 44 U./ml of bacitracin but the parent strain did not. Growth of the variant was however inhibited by 88 U./ml of bacitracin. Moreover, only the variant strain grew, after an overnight incubation, on pH medium adjusted to pH 5,2. Colonies were small though. Both strains grew well at pH 7.

3.6 BHA as a mutagenic agent

3.6.1 Reproducibility of the effect of BHA

The production of variants and of the hemolytic pattern associated with it was a highly reproducible phenomenon that occurred indifferently with 4 lots of BHA from 3 different companies listed under Mate-

Fig. 16. Osmotic stability of protoplasts from S. aureus Wood 46 parent (■) and variant (●) strains.


100%
80%
60%
40%
20%
Initial O.D.
12%
10%
8%
6%
4%
2%
0%
NaCl

rials and Methods (para. 2.3) and with no restrictions at all with respect to the 4 liquid media tested.

Six other strains of S. aureus were analyzed for the effect of BHA on their hemolytic pattern. Only one of them responded in the same way. That particular strain was however a proteolytic mutant of strain Wood 46 obtained by mutagenesis with nitrosoguanidine (S.A. Saheb, Ph.D. thesis, University of Montreal, 1974).

3.6.2 Mutagenicity assay

The fact that the variant strain was likely to be a membrane mutant with enzymatic properties different from those of the parent strain raised the question whether BHA was causing genetic alterations to the Wood 46 strain or was merely acting as a selecting agent for variants already present in the inoculum. The experiment described in para. 2.6 was set up to answer this question; it took advantage of the fact that freshly isolated variants were resistant to bacitracin. Hence, plating on bacitracin agar would allow the selection of mutants and further replica plating on blood agar would ascertain that the isolates were stable and had the right hemolytic pattern. Preliminary experiments indicated that variants with the distinct hemolytic pattern could be obtained by treatment with different concentrations of BHA and not only with 0,28 mM . In order to do so, the cellular concentration had to be decreased when the concentration of BHA decreased and vice-versa.

In this case very small inocula (600 CFU/ml) were used in order to avoid as much as possible the presence of spontaneous mutants in the inocula and as a consequence, low concentrations of BHA were also used (0,07 and 0,14 mM). None of the 14 control tubes taken at zero time gave rise to colonies with a hemolytic pattern resembling that of the variant. The same result was obtained with the other controls incubated until early exponential phase. However, after a week of incubation, growth had occurredin 22 of the 23 tubes containing BHA and colonies similar to those of the variant were detected in 16 of the 22 tubes in such a large amount that a loopful inoculum was enough to isolate many colonies with the variant hemolytic pattern. Both concentrations of BHA gave similar results.

The different properties of the parent and the variant strains are summarized in Table 6.

3.6.3 Effect of other mutagens

The possible mutagenicity of BHA and the relative ease with which hemolytic mutants could be detected on blood agar prompted us to determine whether known mutagenic agents could induce the formation of similar mutants. Since many properties of S. aureus are plasmid-dependent (Lacey and Chopra, 1975) while others are more likely to be determined by the bacterial genome, the cells were treated with 3 plasmid-curing agents and an alkylating agent, ethyl methanesulfonate (EMS). The 3 plasmid-curing agents used were acridine orange which

Table 6. Preliminary characterization of S. aureus parent and variant strains.

Character	Parent	Variant
Alpha-toxin	+	-
Beta-toxin	-	+
Staphylokinase	+	-
Phage type	81, 83A	81, 83A
Bacitracin sensitivity	+++[1]	+
pH sensitivity	+++[1]	+
Osmotic stability	+	+++[2]
BHA sensitivity	+++[1]	+

1 Very sensitive, more than the variant strain.

2 Very stable, more than the parent strain.

interferes with plasmid replication, sodium dodecyl sulfate which interferes with membrane attachment sites and guanidine hydrochloride whose action is still a matter of speculation.

Approximately 10,000 colonies were analyzed after treatment with each of the 4 mutagens. 40,000 colonies from nonmutagenized cell suspensions were plated and none of them showed the specific hemolytic pattern obtained by BHA-treatment. All 3 plasmid-curing agents were also ineffective in this regard. However, mutants with the distinct hemolytic pattern were obtained at a frequency of $2,3 \times 10^{-3}$ by treatment with EMS. Five of these mutants were tested for their ability to grow in a medium containing 0,28 mM BHA and all of them were sensitive to that concentration of BHA.

3.7 Effect on the cytoplasmic membrane

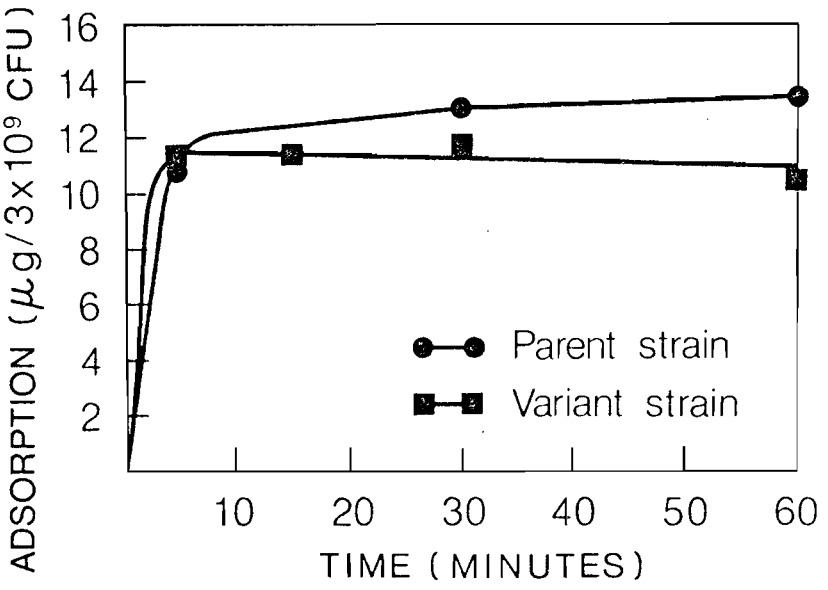
The molecule of BHA is structurally related to that of phenols and phenolic compounds have been shown to act at the membrane level (Becket et al, 1959; Judis, 1962), causing its disruption. As a result, experimentation was oriented towards assays that would ascertained whether BHA is a membrane-active agent. Leakage of low molecular weight metabolites from the metabolic pool and the lysis of protoplasts were selected to assess membrane damage. Nevertheless, adsorption of BHA to the cells of S. aureus was previously studied and is described in the next section.

3.7.1 Adsorption of BHA onto the cells of S. aureus

The first event that occurs when a bacterial cell is exposed to an antimicrobial agent is its adsorption onto the cell ; this process has a direct influence on the susceptibility of the microorganism to that antimicrobial agent. Hence, the resistance of some bacteria towards different drugs can be explained by the fact that they adsorb less drug than the sensitive strains. On the other hand sensitive and resistant strains may adsorb the same amount of drug but a physical or a biochemical barrier may prevent the antimicrobial from reaching its target. In the former case, an increased proportion of lipids associated with the cell wall has been reported to increase the resistance of S. aureus to penicillin (Hugo and Stretton, 1966) while, in the latter, S. aureus has been shown to produce beta-lactamase (Barber, 1949). Since BHA was not metabolized by S. aureus Wood 46 (para. 3.5.5.1), we were able to compare the adsorption pattern of BHA onto both the parent and the resistant strain as described in para. 2.9 and we also determined the nature of the adsorption isotherm. The latter can be used to explain the adsorptive mechanism from the Giles et al (1960) models of adsorption.

To obtain sufficient adsorption of BHA, it was necessary to use cell suspensions of 3×10^9 CFU/ml. In both parent and variant strains the uptake, as measured by the method described in section 2.9, was rapid, much of the adsorption taking place in the first 5 minutes (Fig. 17). Although the technique used, i.e. 2 centrifugation steps,

Fig. 17. Rate of adsorption of BHA (0,28 mM) by suspensions of S. aureus Wood 46 parent strain (3×10^9 CFU/ml) in 0,05 M phosphate buffer, pH7,0.


ADSORPTION (μg/3x10⁹ CFU)
16
14
12
10
8
6
4
2
10
20
30
40
50
60
TIME (MINUTES)
Parent strain
Variant strain

did not allow us to study the precise rate of uptake during the first 5 minutes of contact, it nonetheless brought an indication that the adsorption process and the amount of BHA adsorbed were similar in both strains.

The isotherm obtained with BHA corresponds to the C-type as classified by Giles et al (1960) or constant partition (Fig. 18). This isotherm is a straight line up to 0,84 mM.

3.7.2 Leakage of intracellular material

Membrane-active agents causing a generalized loss of membrane function will generally induce leakage of intracellular material. Thus, experiments were designed in order to ascertain whether BHA could do the same.

Preliminary experiments showed (para. 3.4) that treatment at 30°C with 0,28 mM BHA had no effect on the viability of cell suspensions of the parent strain. Actually, treatment of cell suspensions, for 2 hour periods at 4°C and at concentrations up to 0,56 mM BHA had no effect on the viability of the cells nor on the leakage of cellular material absorbing at 260 nm (Fig. 19). This low temperature was chosen to prevent autolytic enzyme activity. Optical density registered at this wavelength for buffer containing 0,56 mM BHA and less was not different from that of controls containing no BHA. When the concentration was raised to 1,12 mM BHA under the same conditions, there was a significant decrease in the viability of the sus-

Fig. 18. Adsorption of BHA by suspensions of S. aureus Wood 46 parent strain (3×10^9 CFU/ml) in 0,05 M phosphate buffer, pH 7,0.

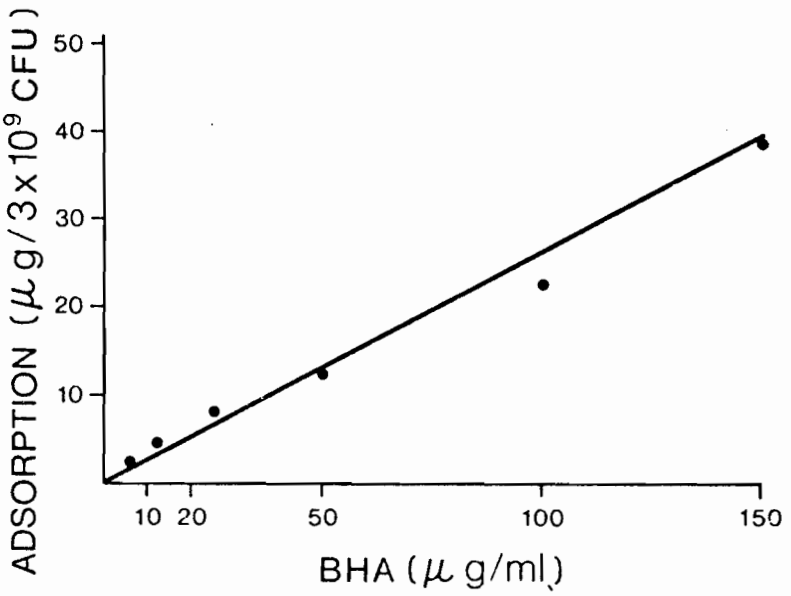
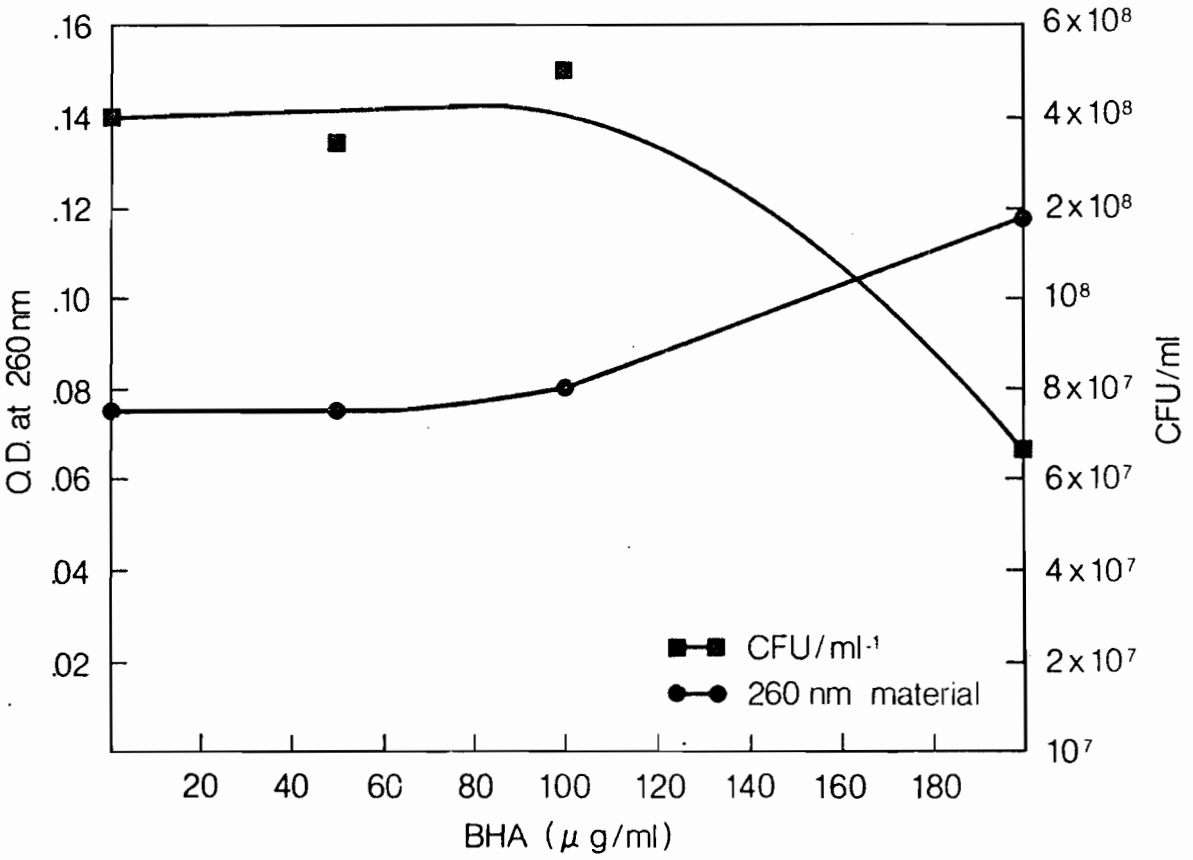

ADSORPTION (μg/3x10⁹ CFU)
50
40
30
20
10
10 20
50
100
150
BHA (μg/ml)

Fig. 19. Effect of BHA on the viability of S. aureus Wood 46 parent strain (3×10^8 CFU/ml) and on the leakage of intracellular material in 0,05 M phosphate buffer, pH 7,0, maintained for 2 hours at 4°C.


.16
.14
.12
.10
.08
.06
.04
.02
O.D. at 260nm
6x10⁸
4x10⁸
2x10⁸
10⁸
8x10⁷
6x10⁷
4x10⁷
2x10⁷
10⁷
CFU/ml
CFU/ml⁻¹
260 nm material
20
40
60
80
100
120
140
160
180
BHA (μg/ml)

pension, which was accompanied by a release of cell constituents absorbing at 260 nm. Plating on blood agar and optimal medium ascertained that this drop of the CFU after treatment with 1,12 mM BHA was not caused by injured cells but by cell death.

3.7.3 Osmotic stability of protoplasts exposed to BHA

Protoplasts of the parental strain were treated with BHA at 4°C. The 3 concentrations tested caused a rapid lysis of protoplasts as measured by the decrease of the optical density at 500 nm (Fig. 20). It is interesting to note that the extent of lysis was the same over a period of 24 hours but the rate of lysis was greater when the concentration of BHA was raised. Protoplasts from the variant strain resistant to BHA were less susceptible to lysis than those of the parent strain (Fig. 21).

3.8 Effect of BHA on the electron transport system of S. aureus

Phenols, like other membrane active agents, can be either bacteriostatic or bactericidal depending only on concentration (Hamilton, 1971). Actually, in a first experiment we noted that in non-growing cell suspensions of 3×10^9 CFU/ml, death was related to leakage of intracytoplasmic material and occurred at a concentration of 1,12 mM BHA. The next experiments will deal with the effects of BHA on non-

Fig. 20. Effect of BHA on protoplast suspensions of S. aureus Wood 46 parent strain in 24% NaCl, 0,05 M phosphate buffer, pH 7,0, maintained at 4°C.

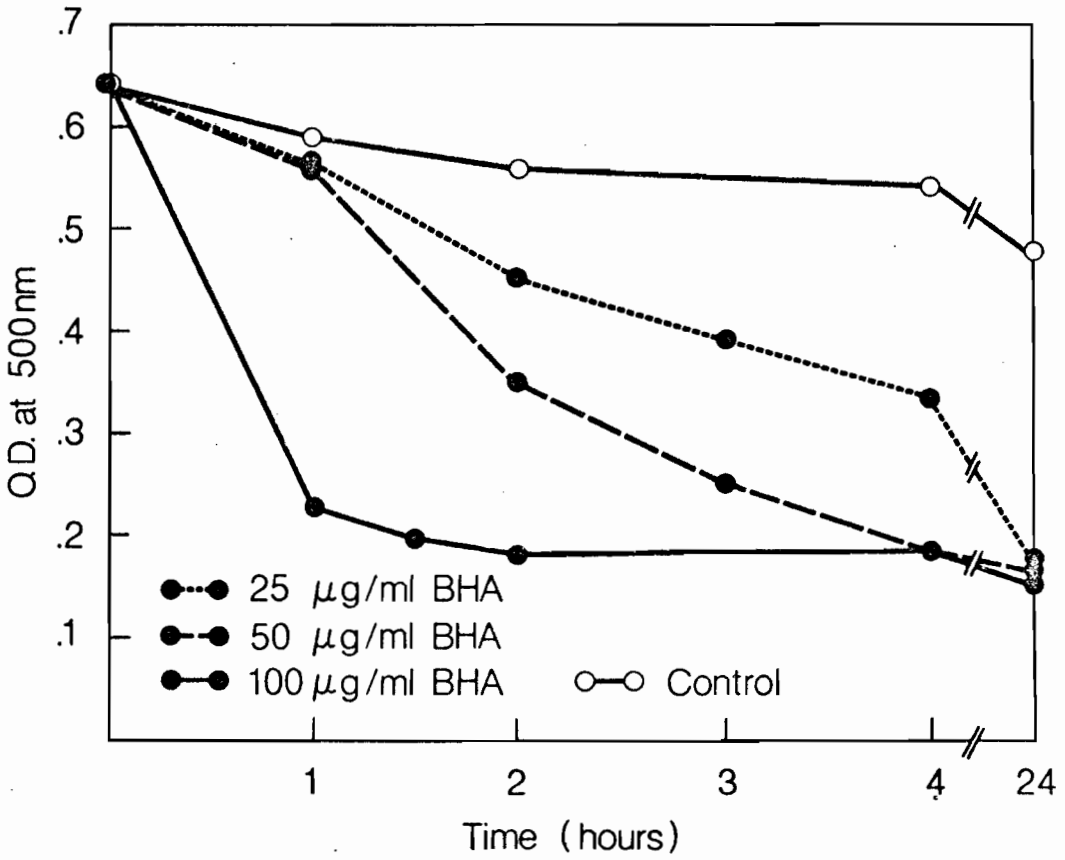
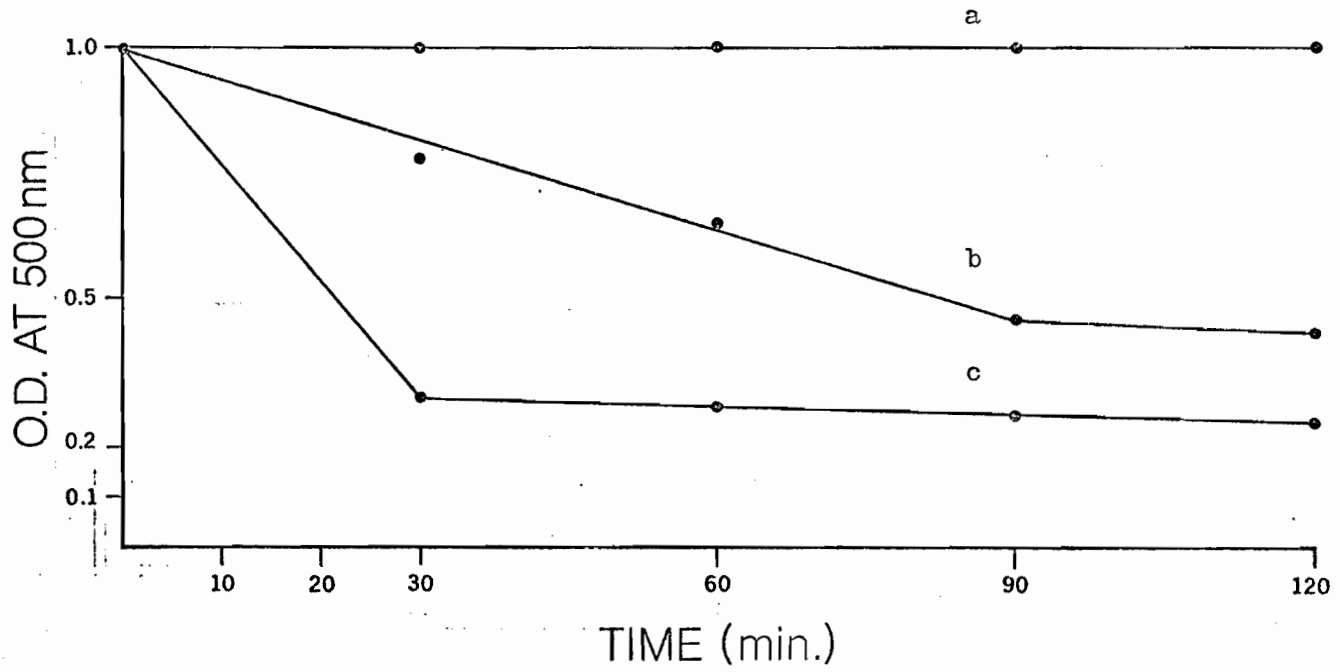

.7
.6
.5
.4
.3
.2
.1
O.D. at 500nm
25 μg/ml BHA
50 μg/ml BHA
100 μg/ml BHA
Control
1
2
3
4
24
Time (hours)

Fig. 21. Effect of BHA (0,28 mM) on protoplast suspensions of S. aureus Wood 46 parent (c) and variant (b) strains in 24% NaCl, 0,05 M phosphate buffer, pH 7,0, maintained at 4°C. Trace a: control suspensions.


a
b
c
1.0
0.5
0.2
0.1
O.D. AT 500nm
10
20
30
60
90
120
TIME (min.)

growing cell suspensions of 3 X 10^9 CFU/ml with concentrations of BHA of 0,56 mM and less. Since previous results (para. 3.7) indicated that BHA was possibly a membrane-active agent, these experiments were devised in order to study the effect of BHA on different membrane functions. Since electron transport is a vital function carried out by the membrane in S. aureus (White and Freeman, 1967) we considered the effect of BHA on this system.

3.8.1 The electron transport system of S. aureus Wood 46

The reduced minus oxidized difference spectra of cell-free extracts of S. aureus are shown in fig. 22. Cell-free extracts treated with dithionite (trace A) revealed distinct Soret peaks at 442 nm, representative of the gamma peaks of cytochrome a + a_3 (Asano and Brodie, 1964) and at 428 nm indicative of the gamma peak of cytochrome b. The peaks at 562 and 530 nm, respectively, indicated the presence of alpha and beta peaks of b type cytochrome while an absorption peak typical of the alpha peak of cytochromes a + a_3 at 605 nm was quite evident (Watson and Smith, 1968). The difference spectrum gave no evidence for the presence of cytochrome c in S. aureus. The trough at 465 nm is caused by the reduction of flavoprotein (Chance and William, 1955).

The reduced CO-treated minus reduced spectrum is shown in Fig. 23. Peaks at 570, 534 and 418 nm indicated that cytochrome o

Fig. 22. Difference absorption spectra of S. aureus Wood 46 parent strain cell-free extracts reduced with (A) dithionite, (B) NADH, and (C) BHA. The reaction mixture in a total volume of 2,0 ml contained 16 mg protein and 100 umoles of potassium phosphate buffer (pH 7,0).

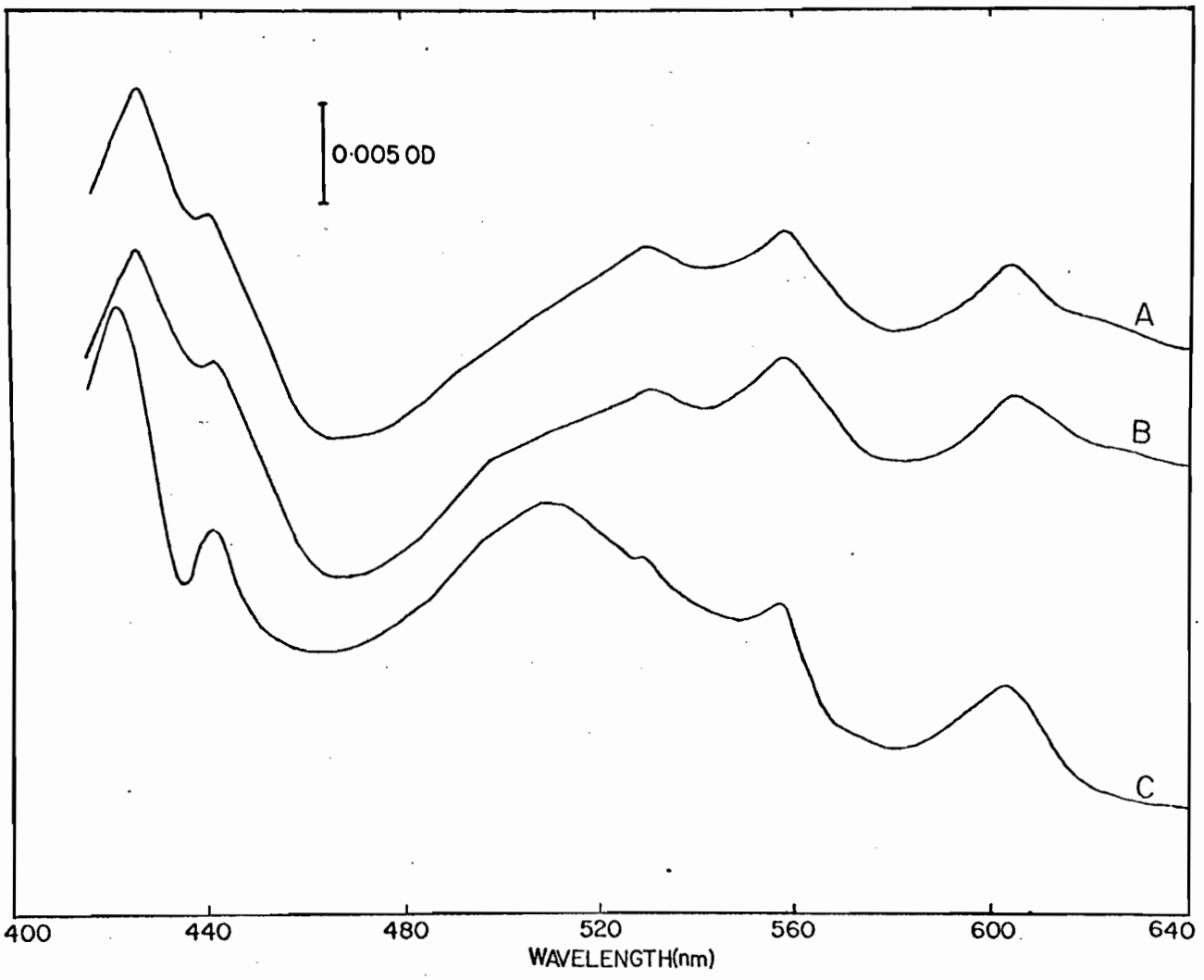
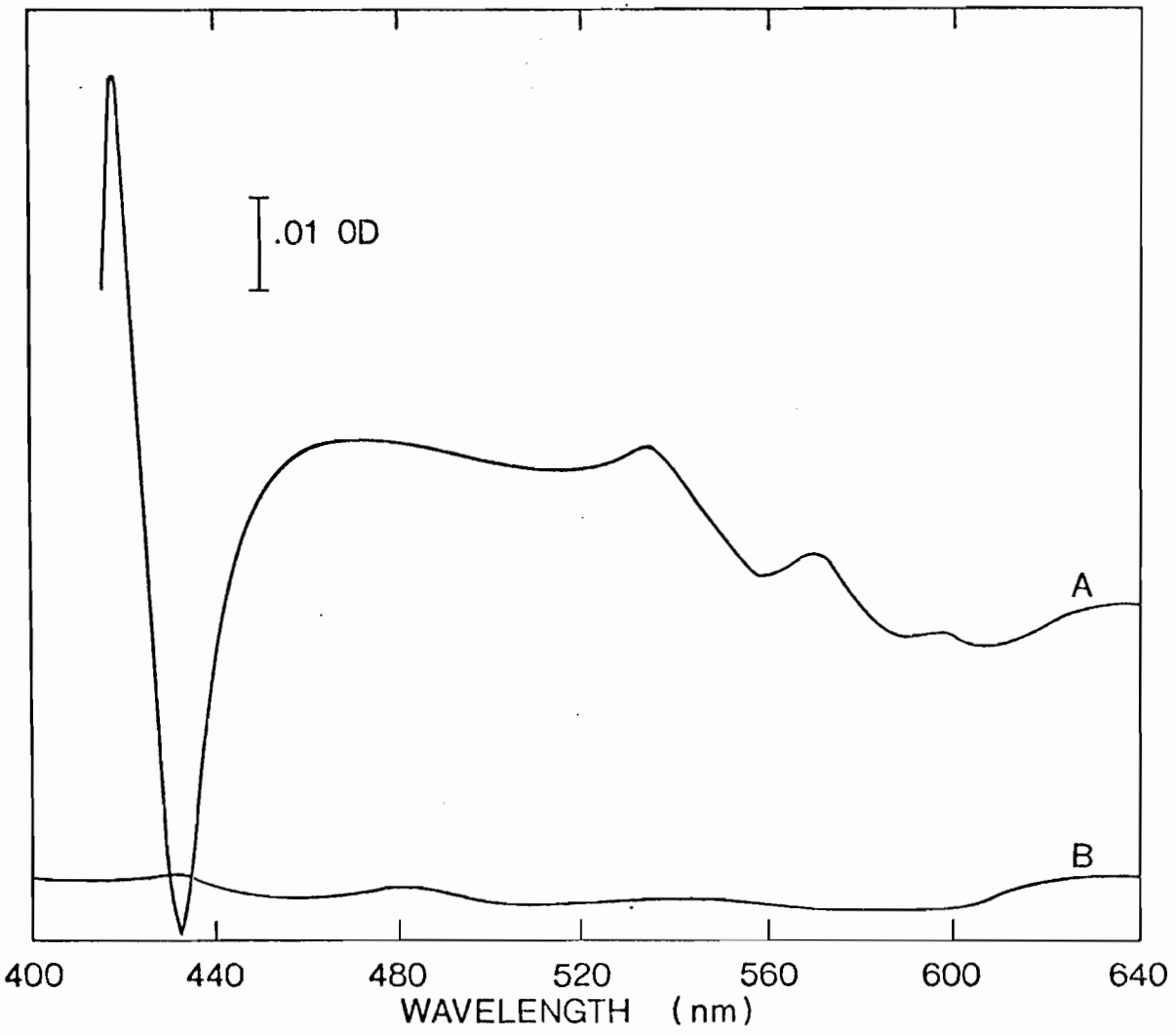

0·005 OD
A
B
C
400
440
480
520
560
600
640
WAVELENGTH(nm)

Fig. 23. Reduced CO minus reduced difference spectrum of S. aureus Wood 46 parent strain cell-free extracts (Trace A); base line: trace B. Reduction was with sodium dithionite. The reaction mixture in a total volume of 2,0 ml contained 14 mg protein and 100 umoles of potassium phosphate buffer (pH 7,0).


.01 OD
A
B
400
440
480
520
560
600
640
WAVELENGTH (nm)

was the terminal oxidase (Smith, 1978).

Addition of NADH to cell-free preparations resulted in a difference spectrum (Fig. 22, trace B) which was similar to that observed upon reduction with dithionite.

3.8.2 Effect of BHA on the electron transport system

When BHA was used as the electron donor, to avoid any reduction of cytochromes by residual substrate, small amount of ferricyanide was added to cell-free extracts. When such cell-free extracts in the reference cuvette were exposed to air and the sample cuvette was made anaerobic, the spectrum showed no reduction of cytochromes. However, addition of 0,4 mM BHA in the sample cuvette caused the appearance of absorption spectrum shown in Fig. 22, trace C. The position of peaks in the BHA-reduced minus oxidized difference spectrum for cytochrome $\underline{a} + \underline{a}_3$ (605 and 442 nm) as well as for alpha and beta peaks of $\underline{b}$ type cytochrome (562 and 530 nm) was identical to that obtained with NADH or dithionite. However, the gamma peak of $\underline{b}$ type cytochrome shifted from 428 to 424 nm. A broad band at 510 nm always occured in the spectrum which could not be related to any known cytochrome. These results thus clearly show that similar to NADH as substrate, BHA also served as an electron donor and caused reduction of cytochromes.

In order to confirm that BHA can indeed serve as an electron donor, authentic mammalian cytochrome $\underline{c}$ was used as the electron

acceptor. Fig. 24 trace A shows that the addition of small amount of dithionite in the sample cuvette containing mammalian cytochrome c caused appearance of peaks of c-type cytochrome at 550, 523 and 420 nm. Likewise, the addition of 0,4 mM BHA to the sample cuvette caused the reduction of cytochrome c (Fig. 24,trace B). The position of alpha and beta peaks (550 and 523 nm) remained the same as observed with dithionite but they were not reduced to the same extent by BHA. However, gamma peak in the Soret region in the presence of BHA was reduced to the same extent as by dithionite but it shifted from 420 to 414 nm. The spectrophotometric observations suggest that electrons are donated by BHA to the electron transport system of S. aureus and as a result cytochromes are reduced.

Although our results showed that BHA acted as a reducing agent like NADH our preliminary results indicated that BHA did not stimulate endogenous respiration of S. aureus like other electron donors such as NADH and succinate but rather inhibited the process. We thus investigated the effects of BHA and other classical inhibitors of the respiratory chain in the presence of NADH and succinate as substrates and the results are shown in Tables 7 and 8. It is seen that although NADH oxidation by cell-free extracts of S. aureus was strongly inhibited by flavoprotein inhibitors such as rotenone and atabrine (Table 7), succinate oxidation by whole cell suspensions was not affected by these inhibitors (Table 8). Oxidation of both NADH and succinate was completely inhibited by 0,03 mM HQNO and 0,10 mM

Fig. 24. Difference absorption spectra of mammalian cytochrome c dissolved in 0,05 M phosphate buffer (pH7,0). Traces A and B represent difference spectra obtained after the addition of dithionite and BHA, respectively, in the sample cuvette.

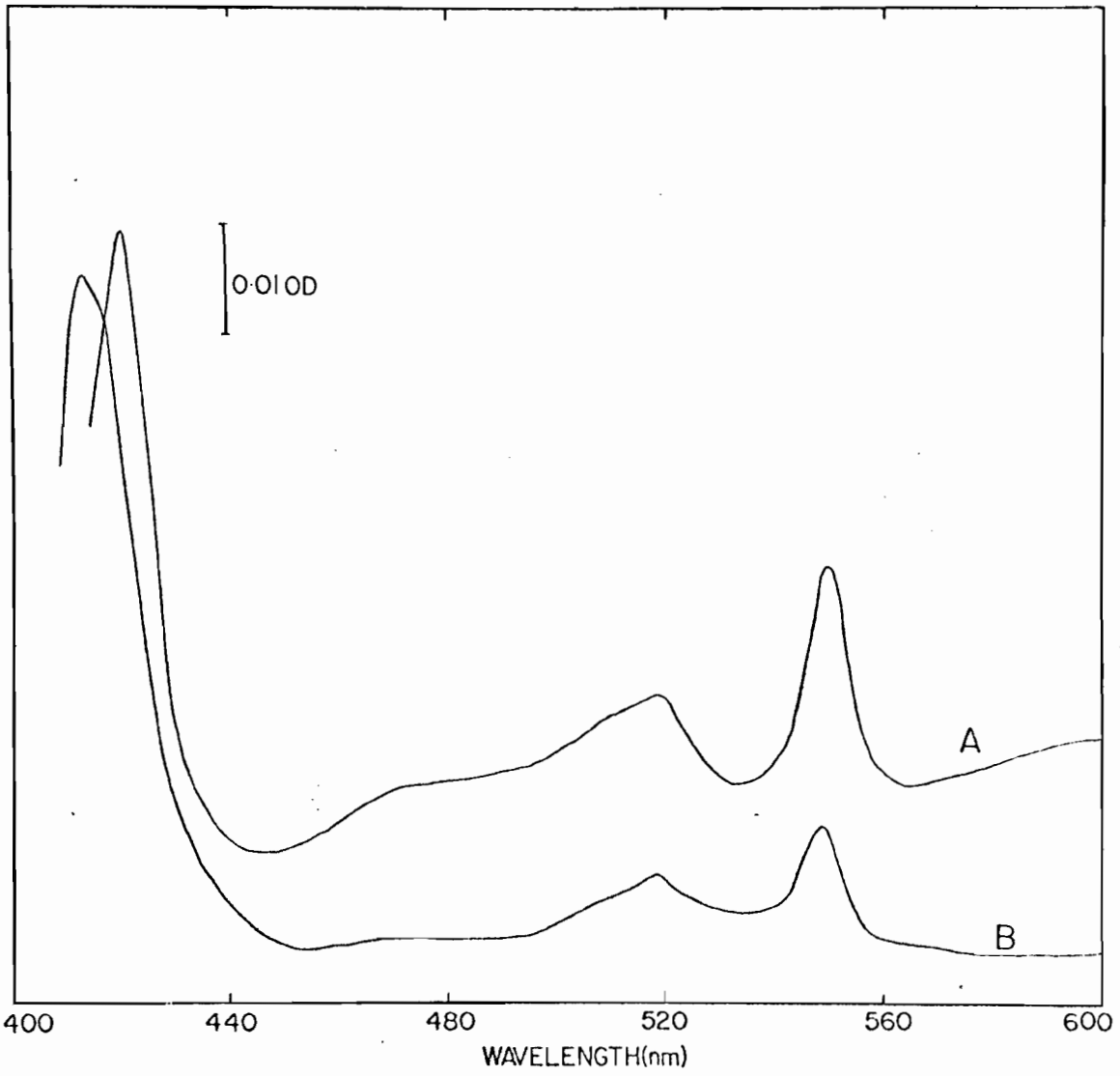

0·01 OD
A
B
400
440
480
520
560
600
WAVELENGTH(nm)

Table 7. Effect of inhibitors on NADH oxidation by cell-free extracts of S. aureus.

Inhibitor	Concn (mM)	O_2 Consumed/10 min (nanoatoms)	% Inhibition
-	-	660	0
Rotenone	0,01	0	100
Atabrine	0,10	99	85
Antimycin A	0,10	231	65
HQNO	0,03	0	100
Cyanide	1,00	0	100
BHA	0,40	264	60

Table 8. Effect of inhibitors on succinate oxidation by whole cell suspensions of S. aureus.

Inhibitor	Concn (mM)	O_2 Consumed/10 min (nanoatoms)	% Inhibition
-	-	396	0
Rotenone	0,01	364	8
Atabrine	0,10	396	0
Antimycin A	0,10	119	70
HQNO	0,03	0	100
Cyanide	1,0	36	91
BHA	0,40	71	82

antimycin A caused 65 and 75% inhibition respectively of NADH and succinate oxidation. The process was markedly sensitive to cyanide when NADH and succinate served as the electron donors. A similar effect of cyanide was observed (not shown) when ascorbate was used as a substrate. It is of interest to note that BHA (0,4 mM) caused 60 and 82% inhibition of NADH and succinate oxidation respectively. The extreme sensitivity of NADH oxidation but not succinate, to rotenone and atabrine is a clear evidence of a functional first site i.e. between pyridine nucleotide and cytochrome b. Antimycin A and HQNO, specific inhibitors of second site and cyanide, specific for the third site, are all very effective inhibitors of both NADH and succinate oxidation by S. aureus.

BHA also inhibited by 60% the oxidation of glucose, malate, lactate and ascorbate. In order to rule out the possibility that BHA might have interfered with the penetration of the substrates into whole cells, cell suspensions were frozen and thawed rapidly and oxidation of the substrates monitored again but with the same results.

The oxidation of lactate, NADH, succinate and glucose were also inhibited in the variant strain but to a lesser extent: for the same concentration of BHA and when the results were corrected for the same amount of protein/ml or bacterial dry weight/ml, inhibition was of 28, 30, 36 and 30% respectively with the substrates listed above.

The volume of ethanol used to solubilize the antioxidant and the inhibitors had no effect on the reduction of cytochromes, nor on oxygen consumption.

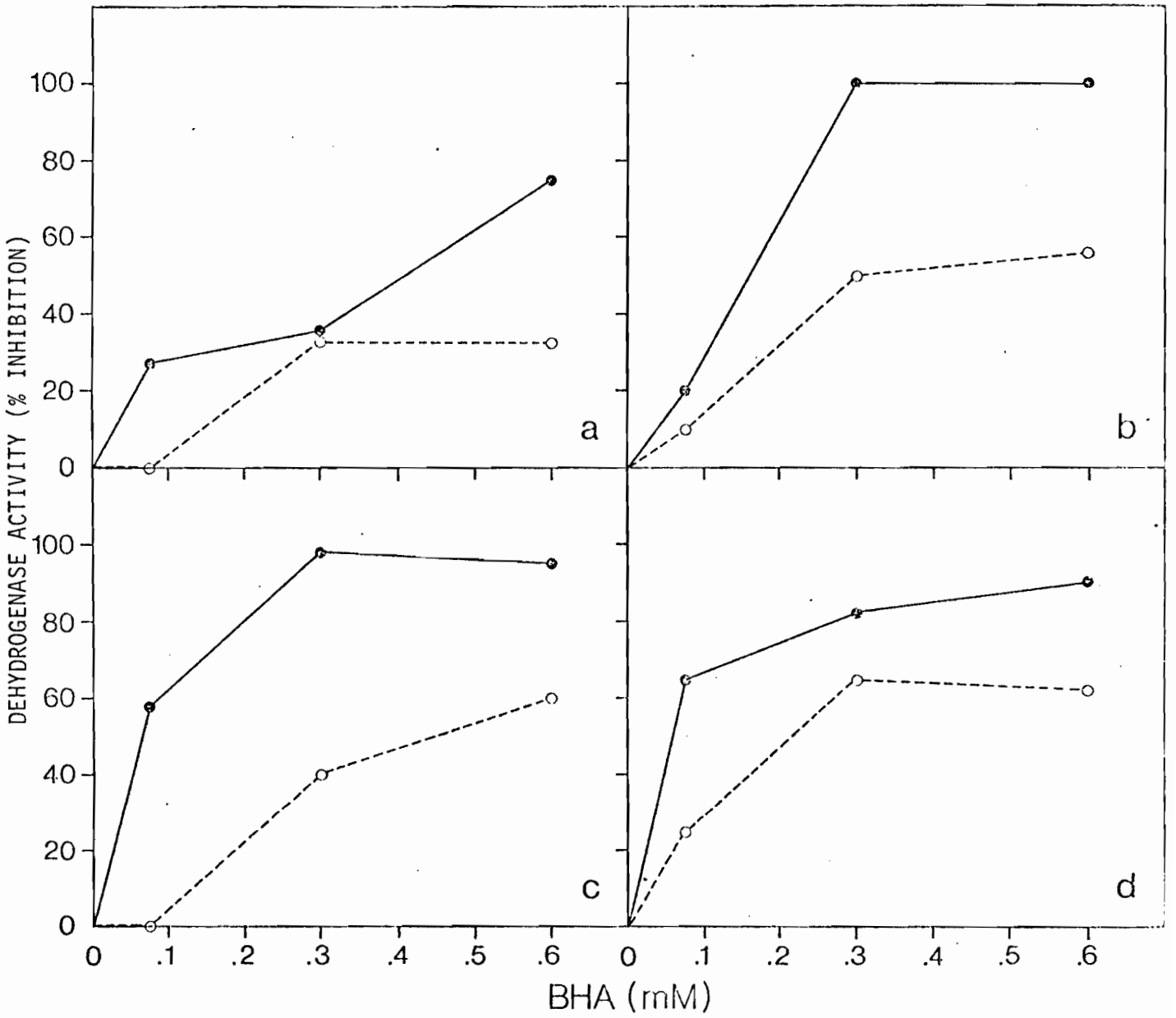
Cells used for spectrophotometric and for oxygen consumption studies were grown in nutrient broth, a glucose-free medium, since growth in a glucose-containing medium was shown to reduce by 40% the cytochrome content in S. aureus (Strasters and Winkler, 1963). It is noteworthy though that BHA showed the same degree of inhibition of oxygen consumption when S. aureus was grown in BHI broth, a glucose-containing medium.

3.9. Effect of BHA on dehydrogenase activity

Experiments carried out to determine the effect of BHA on the respiratory chain showed that the antioxidant inhibited oxygen consumption but at the same time reduced the cytochromes. Associated to the respiratory chain are different dehydrogenases that are responsible for passing electrons to the cytochromes. In order to determine whether the effect of BHA was specific to the respiratory chain or rather caused a general malfunction of the membrane and of the enzymes associated with it, dehydrogenase activities associated with whole cells of both parent and variant strains were monitored with 4 substrates: lactate, glucose, succinate and malate.

Using a cellular concentration of 6 X 10^8 CFU/ml, dehydrogenase activities associated with lactate was inhibited by 27% with 0,075 mM BHA (12,5 ug/ml) but this concentration of BHA did not affect the resistant strain (Fig. 25, trace a). In the presence of 0,6 mM BHA (100 ug/ml) activities were inhibited by 76 and 33% in the parent and variant strain respectively. Activities associated with glucose

Fig. 25. Effect of BHA on S. aureus Wood 46 parent (———) and variant (- - -) strains dehydrogenase activity with a: lactate; b: glucose; c: succinate and d: malate as substrate.


DEHYDROGENASE ACTIVITY (% INHIBITION)
100
80
60
40
20
0
a
b
c
d
0
.1
.2
.3
.4
.5
.6
BHA (mM)

were completely abolished by 0,28 mM (50 ug/ml) and 0,56mM BHA in the parent strain while those of the variant were inhibited by 50 and 56% (Fig. 25, trace b). It is noteworthy that with a cell concentration of 3 X 10^9 CFU/ml, there was no significant inhibition of these dehydrogenase activities.

However, succinate and malate dehydrogenase activities were very sensitive to inhibition by BHA even for a cell concentration of 3 X 10^9 CFU/ml. It is seen that activities associated with succinate and malate in the parent strain were inhibited by 98 and 82% in the presence of 0,3 mM BHA while those of the variant were inhibited by 40 and 67% respectively (Fig. 25, traces c and d). An increase in the concentration of BHA up to 0,6 mM with succinate led to an inhibition of 60% of the activity of the variant strain while that associated with malate was comparable to the inhibition obtained with 0,3 mM BHA.

3.10 Effect of BHA on nutrient uptake

According to Mitchell's chemiosmotic hypothesis (Mitchell, 1963) the extrusion of protons across the cytoplasmic membrane is responsible for the formation of the PMF which is comprised of a pH gradient and of a membrane potential. In the facultative anaerobe S. aureus, proton extrusion is likely to be generated by the respiratory chain in aerobic conditions and by the reversible ATPase in anaerobic conditions (Kashket, 1981).

Since BHA has been shown to inhibit respiratory chain activity (Degré et al, 1983), it was of interest to determine whether amino acid uptake would be affected since this process is driven by the PMF generated by the respiratory chain. We studied glutamic acid, isoleucine and lysine uptake, which are respectively acidic, neutral and basic amino acids. Glucose uptake, which is energized by a high energy phosphate bond in the form of phosphoenolpyruvate, was also monitored. In comparing the effects of BHA on two different transport systems with respect to their energization, we intended to determine whether BHA is inhibitory to some specific transport system, causes a generalized malfunction of the membrane or does not affect at all those systems.

Uptake was defined as the total cellular-associated counts after the cells were separated from the labeled substrate in the incubation medium. Uptake was monitored in the presence and in the absence of BHA at various time intervals starting at 2,5 minutes after the addition of the substrate up to 60 minutes.

3.10.1 Effect of BHA on nutrient uptake in the parent strain

Fig. 26 shows that glutamic acid initial rate of uptake was significantly affected by 0,28 and 0,56 mM BHA. Hence after the initial 10 minutes of exposure total uptake reached 16 and 7% of maximum uptake as compared to 78% in the control.

Lysine uptake followed a pattern different from that of glutamic acid but, again, initial uptake was significantly affected (Fig. 27). After 10 minutes, uptake was 15 and 10% of that of the control with 0,28 and 0,56 mM BHA respectively. Maximum uptake, as measured after 60 minutes, was abolished by 55 and 83%.

Isoleucine, whose uptake is driven by both components of the PMF, was less sensitive to the process (Fig. 28). Hence initial uptake in the presence of 0,28 mM BHA was close to that of the control after 5 minutes of exposure since it reached 68% of that of the control suspension. However, 0,56 mM BHA more seriously inhibited initial uptake which was only 12% of that of the control after 5 minutes. But it increased to 24% of total uptake after 40 minutes.

Fig. 29 shows that glucose uptake in both control and treated cell suspensions (0,56 mM ○) paralleled one another, indicating that glucose uptake was relatively resistant to inhibition by BHA. Hence, after 5 minutes of exposure, uptake corresponded to 61% of that of the control. A concentration of 0,28 mM BHA had no effect on glucose uptake.

Fig. 26. Effect of BHA on S. aureus Wood 46 parent strain glutamic acid uptake; ● : control; ■ : 0,28 mM ; ○ : 0,56 mM .

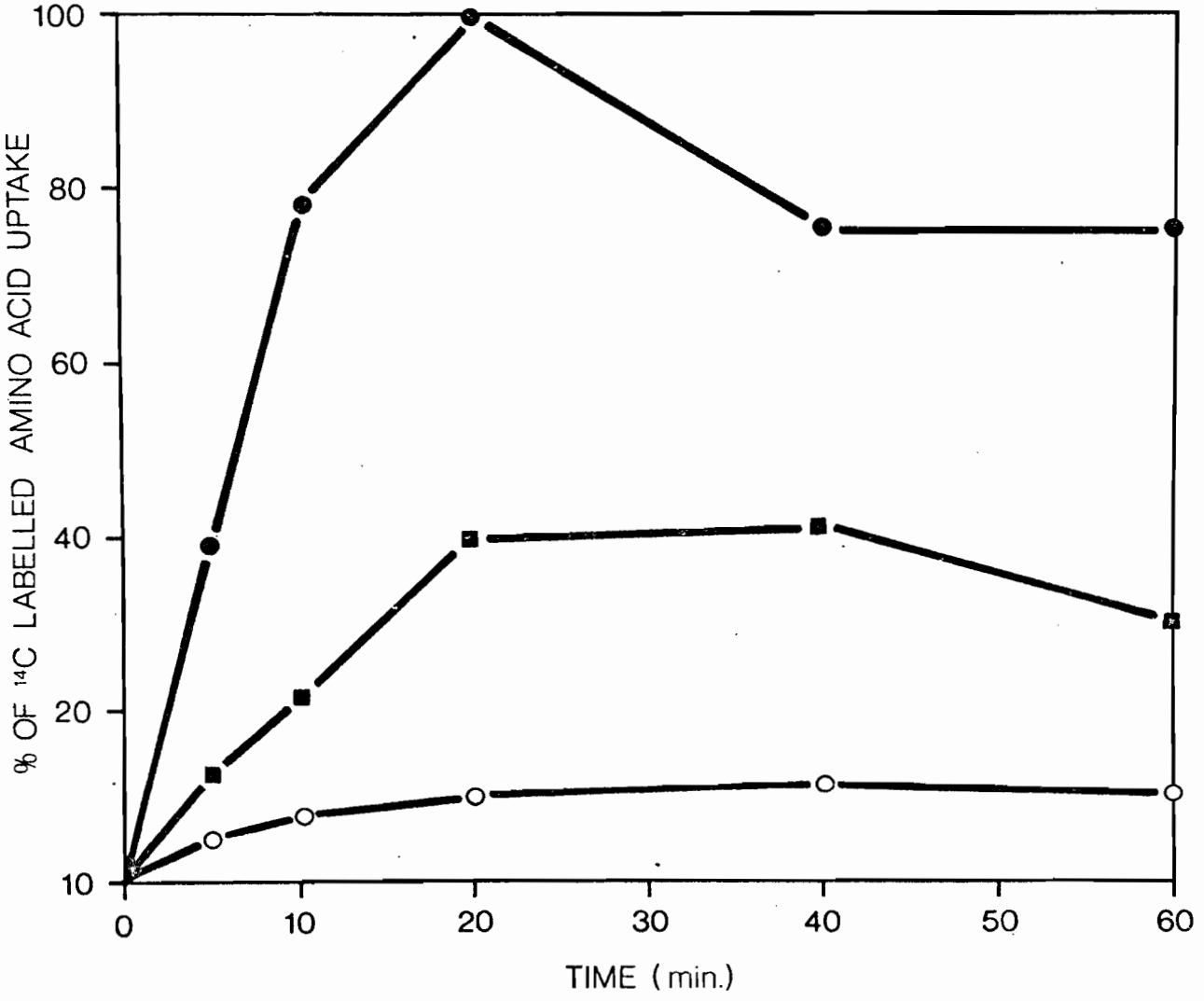

100
80
60
40
20
10
% OF ^{14}C LABELLED AMINO ACID UPTAKE
0
10
20
30
40
50
60
TIME (min.)

Fig. 27. Effect of BHA on S. aureus Wood 46 parent strain lysine uptake; ● : control; ■ : 0,28 mM ; ○ : 0,56 mM.

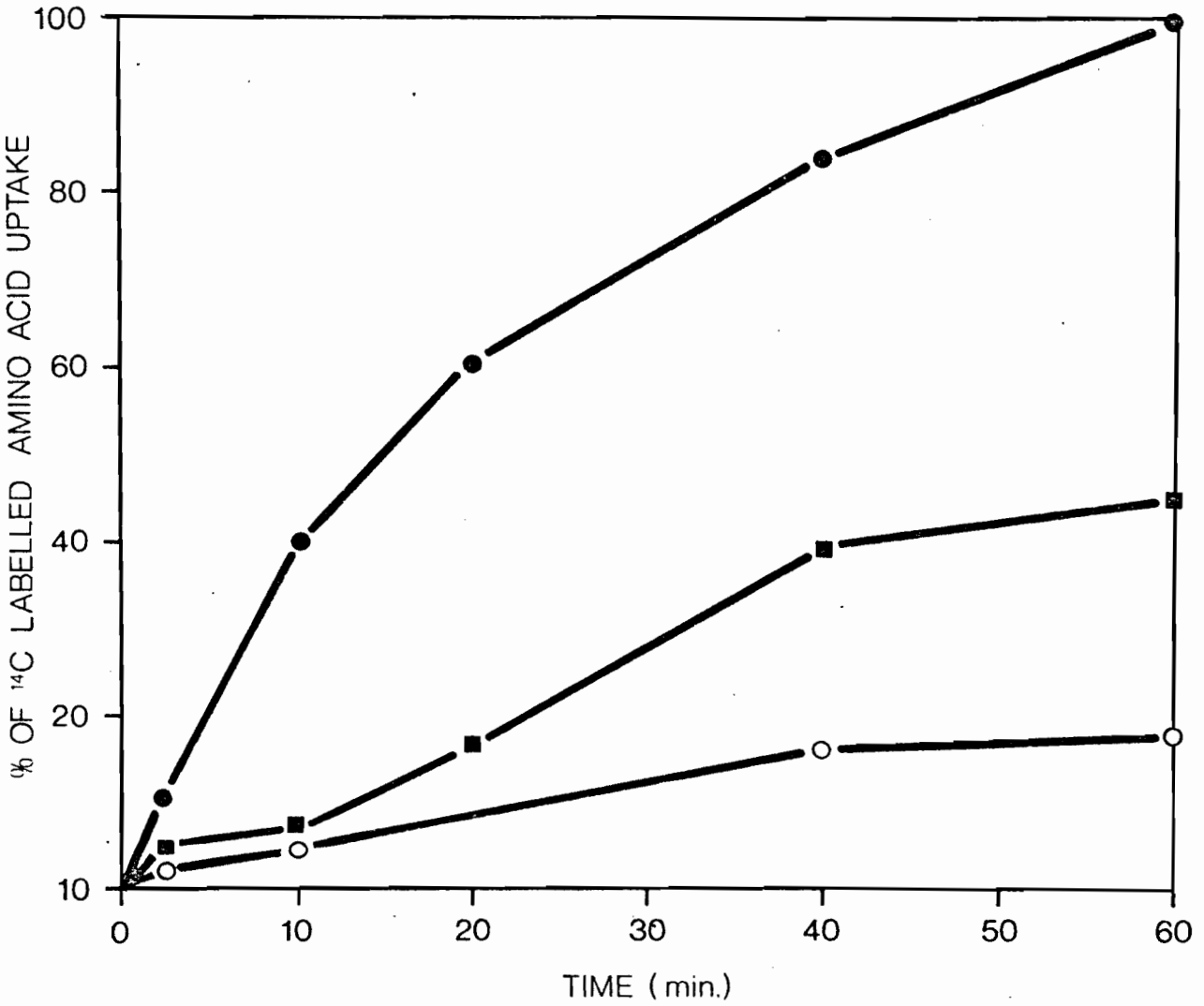

100
80
60
40
20
10
% OF ¹⁴C LABELLED AMINO ACID UPTAKE
0
10
20
30
40
50
60
TIME (min.)

Fig. 28. Effect of BHA on S. aureus Wood 46 parent strain isoleucine uptake; ● :control; ■ : 0,28 mM ; ○ : 0,56 mM.

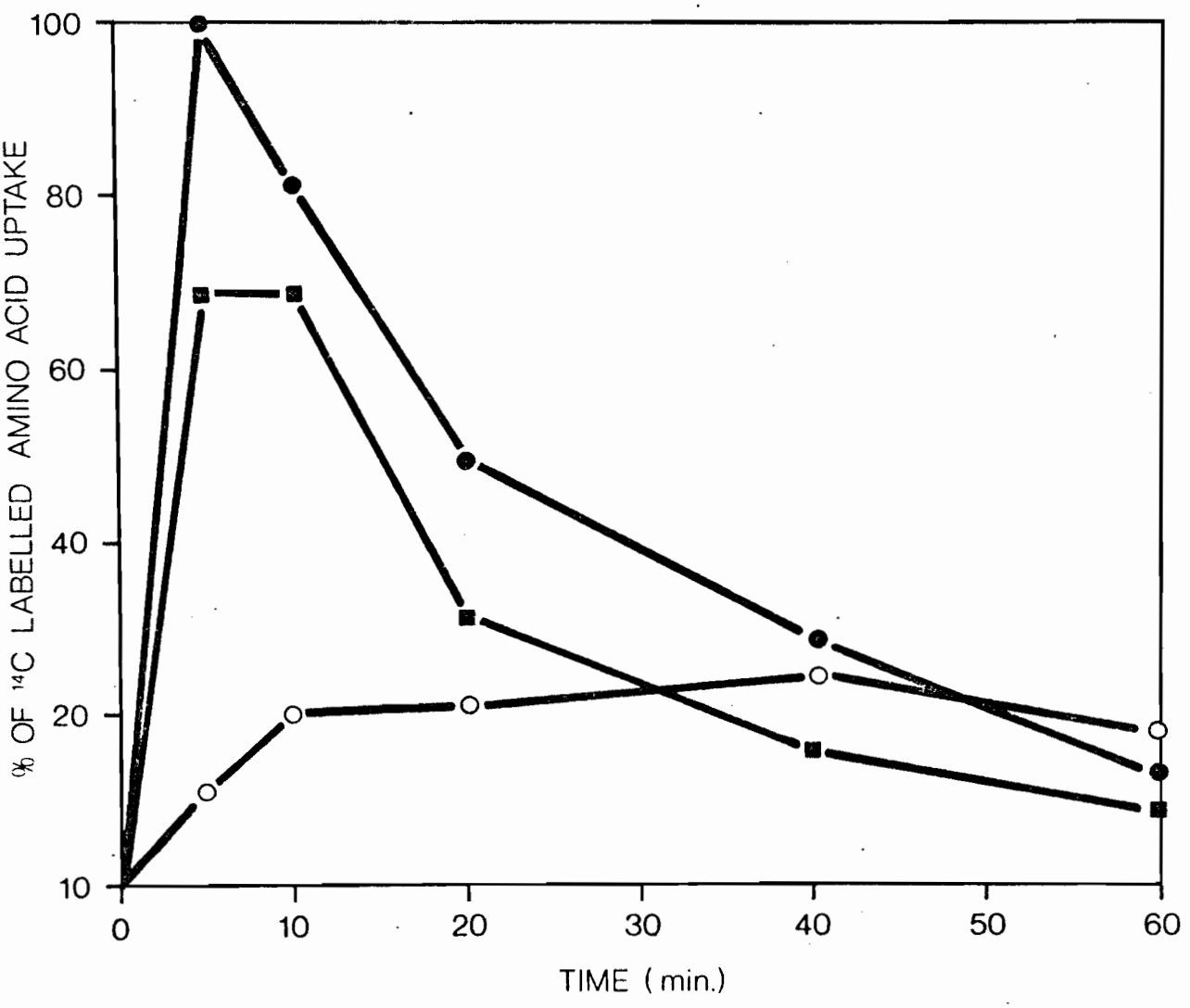
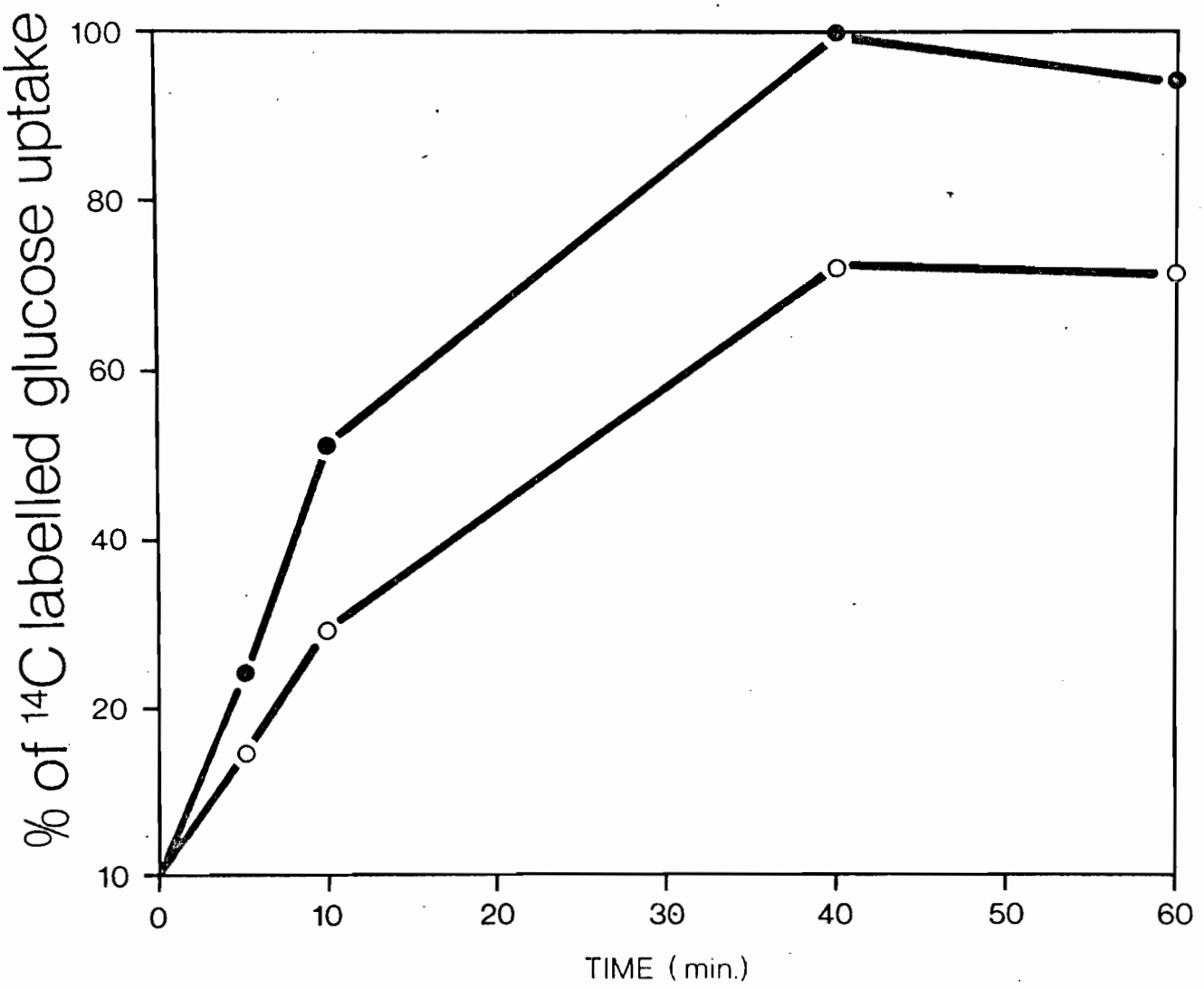

100
80
60
40
20
10
% OF ¹⁴C LABELLED AMINO ACID UPTAKE
0
10
20
30
40
50
60
TIME (min.)

Fig. 29. Effect of BHA on S. aureus Wood 46 parent strain glucose uptake; ● : control; ○ : 0,56 mM.


% of ^{14}C labelled glucose uptake
100
80
60
40
20
10
0
10
20
30
40
50
60
TIME (min.)

3.10.2 Effect of BHA on nutrient uptake in the variant strain

The effect of 0,56 mM BHA on amino acids and glucose uptake was also studied in the variant strain.

Fig. 30 shows that glucose uptake was not significantly affected since uptake in both control and treated suspensions closely paralleled one another. After 10 minutes of exposure, uptake in the treated suspension reached 85% of that of the control.

Glutamic acid initial uptake was only slightly affected as compared to uptake in the parent strain in the presence of 0,56 mM BHA since, after 10 minutes of exposure, uptake reached 69% of that of the control (Fig. 31). Isoleucine, whose uptake was very rapid, was 60% of that of the control after 2,5 minutes of exposure (Fig. 32) while that of lysine was 52% of that of the control after 10 minutes of exposure (Fig. 33). In all 3 cases the patterns of uptake closely paralleled one another and uptake was fully resistant to inhibition by 0,28 mM BHA.

3.11 Effect of BHA on ATPase activity

BHA was shown to inhibit dehydrogenase and respiratory chain activities and amino acid uptake. This action, directed towards the energy generating mechanism of the cell responsible for the production of the PMF in aerobic conditions, was shown to be relatively specific since glucose uptake was not significantly affected. In anaerobic

Fig. 30. Effect of BHA on S. aureus Wood 46 variant strain glucose uptake; ● : control; o : 0,56 mM.

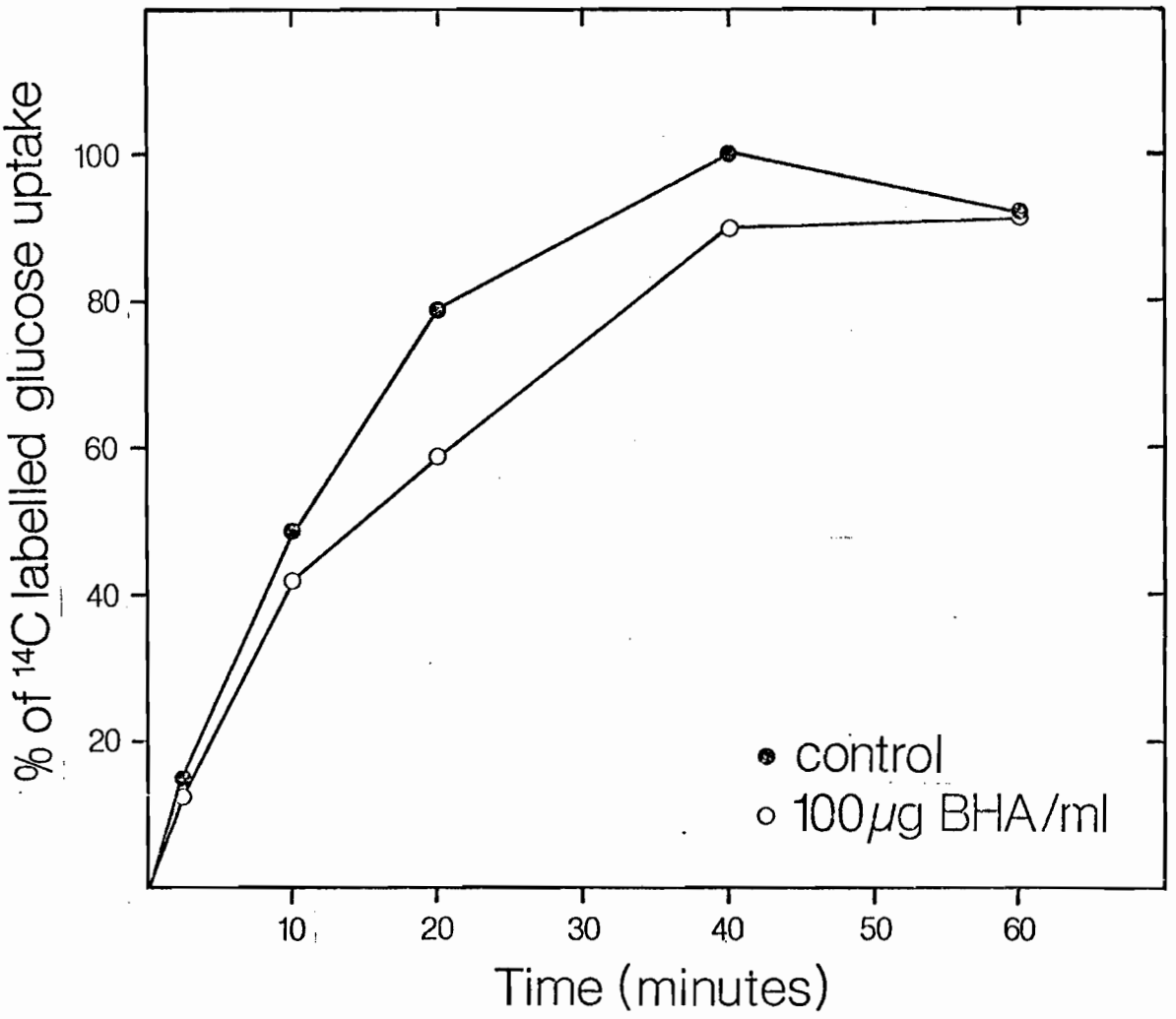

% of ^{14}C labelled glucose uptake
100
80
60
40
20
10
20
30
40
50
60
Time (minutes)
control
100μg BHA/ml

Fig. 31. Effect of BHA on S. aureus Wood 46 variant strain glutamic acid uptake (0,56 mM).

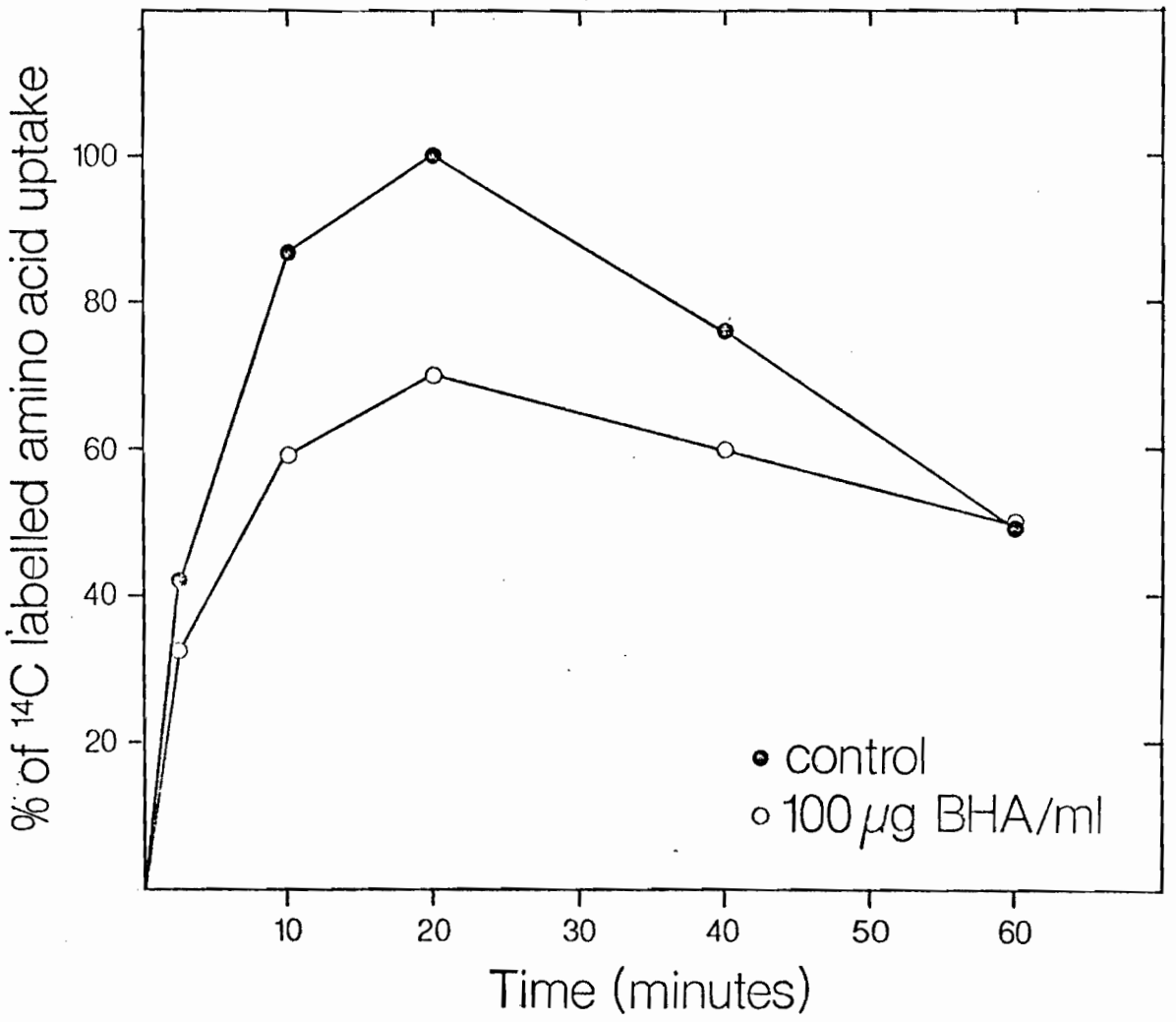

% of ^{14}C labelled amino acid uptake
100
80
60
40
20
10
20
30
40
50
60
Time (minutes)
control
100 μg BHA/ml

Fig. 32. Effect of BHA on S. aureus Wood 46 variant strain isoleucine uptake (0,56 mM).

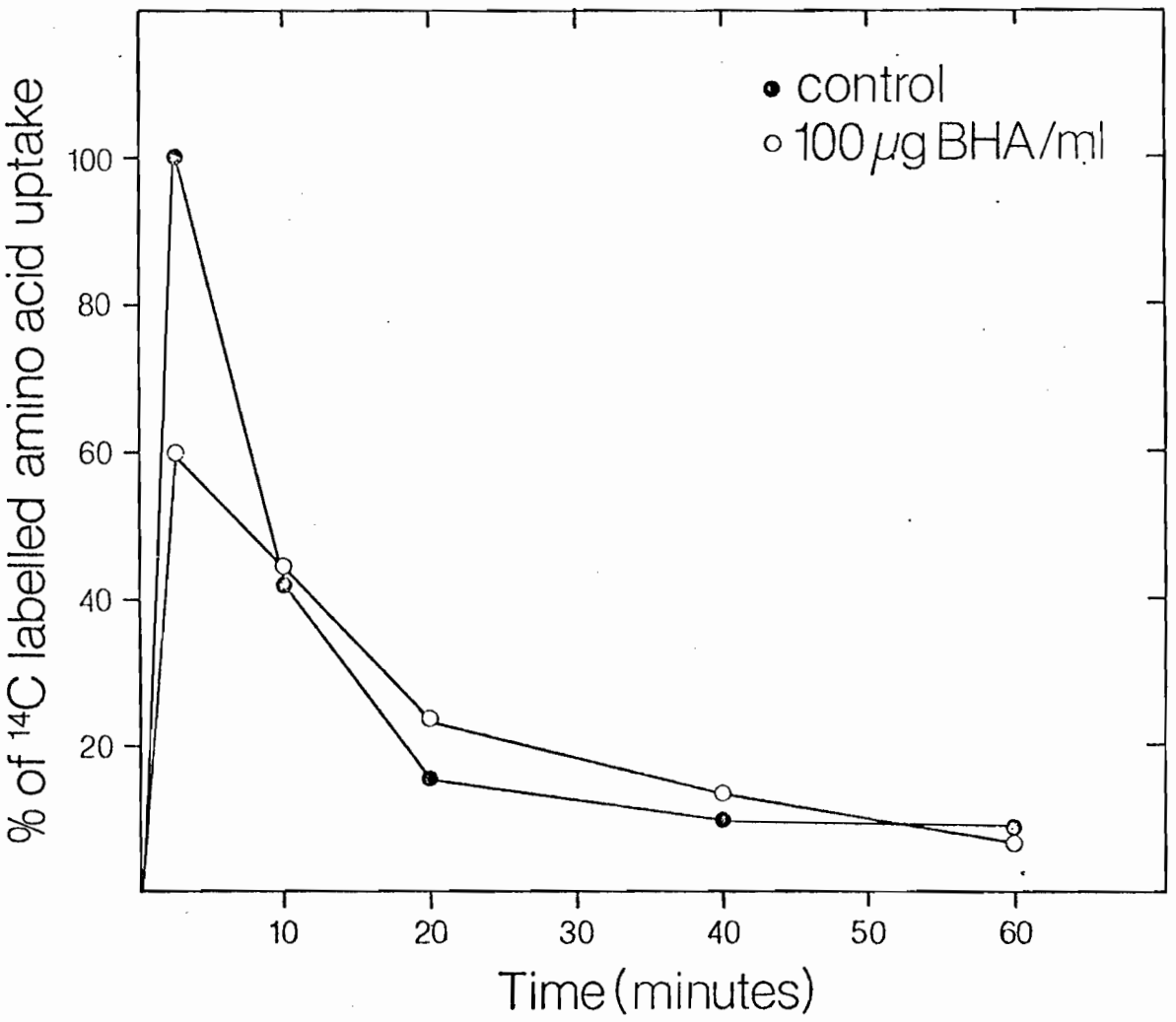
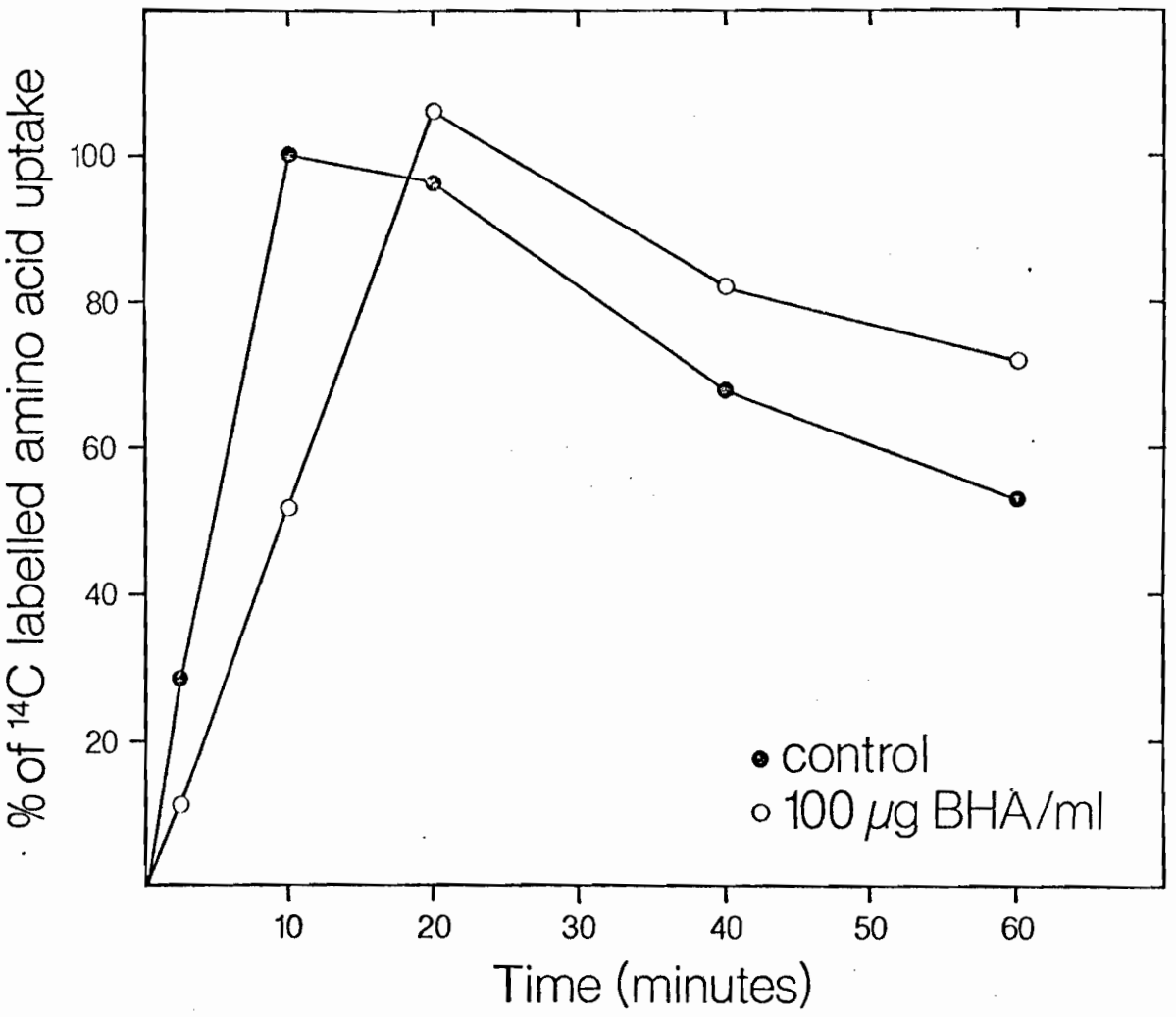

control
100 μg BHA/ml
% of ¹⁴C labelled amino acid uptake
100
80
60
40
20
10
20
30
40
50
60
Time (minutes)

Fig. 33. Effect of BHA on S. aureus Wood 46 variant strain lysine uptake (0,56 mM).


% of ^{14}C labelled amino acid uptake
100
80
60
40
20
10
20
30
40
50
60
Time (minutes)
control
100 μg BHA/ml

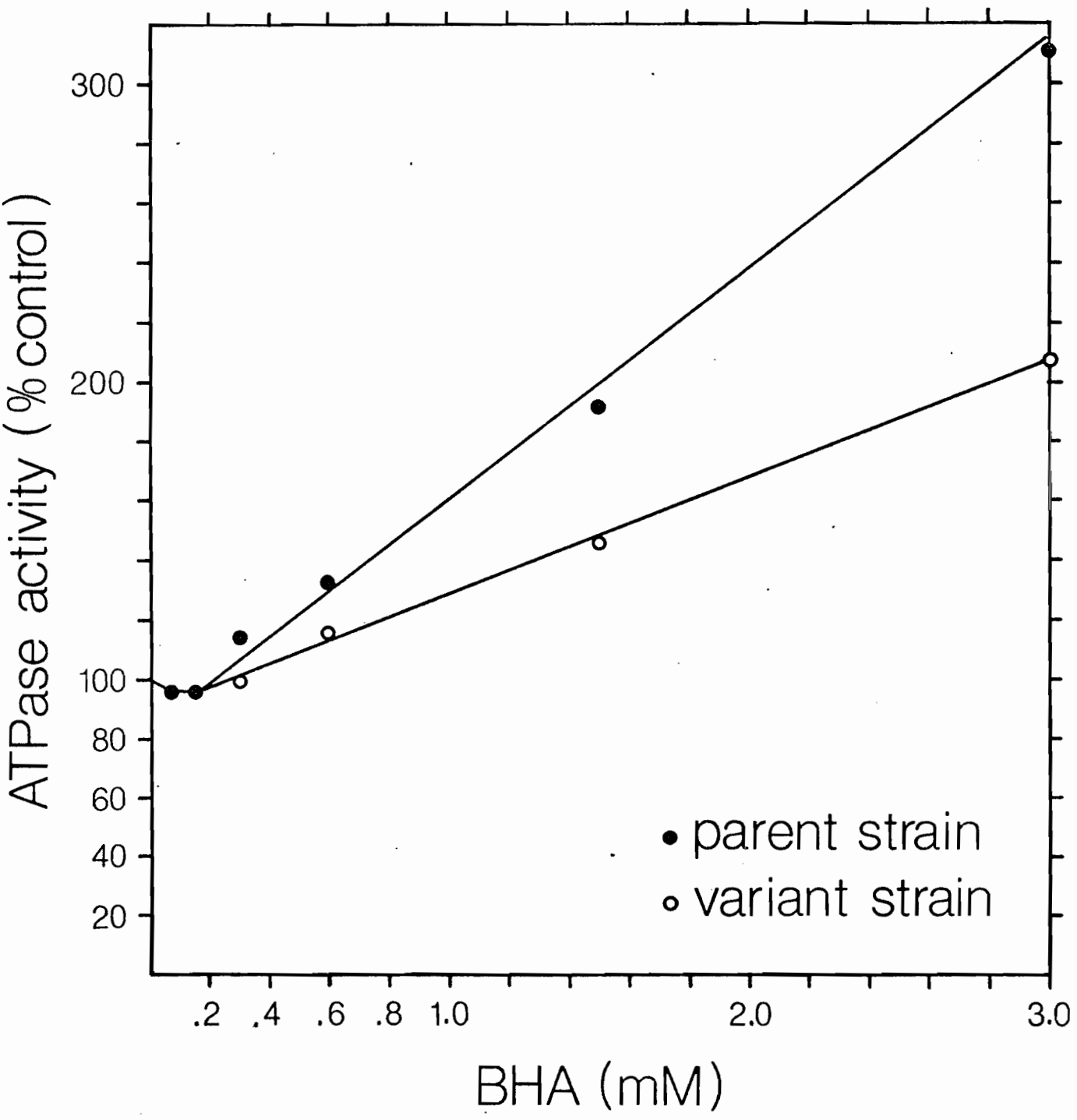
conditions and when oxygen tension becomes low enough, the membrane-bound ATPase is responsible for ATP hydrolysis resulting in an outwardly directed proton flux, thus forming the PMF (Kashket, 1981). Therefore it was of interest to determine whether this important membrane enzyme, whose activity must be carefully controlled, was affected by BHA.

Two different membrane preparations of the parent strain yielded 4,7 and 6,2 U./mg of protein while those of the variant strain yielded 6,1 and 6,6 U./mg of membrane protein.

When membrane preparations of the parent strain were incubated with BHA, there was a marked stimulation of the activity (Fig. 34). Concentrations of 0,6 mM (100 ug/ml), 1,5 mM (250 ug/ml) and 3,0 mM (500 ug/ml) of BHA resulted in 1,3, 1,9 and 3,1 fold stimulation of the activity respectively. Preparations of the variant strain were less sensitive to the process but were nonetheless stimulated 2,1 fold by 3,0 mM BHA. Lower concentrations of the antioxidant had no effect in both cases; higher concentrations of BHA were not tested because of the low water solubility of the antioxidant in this system.

Controls run without membrane and membrane preparations added after the reaction was terminated did not show this high release of P_i and BHA did not absorb at 820 nm, the wavelength used for P_i determination. These controls indicate that non-enzymatic ATP hydrolysis was not involved in these experiments and that BHA did not interfere in the assay.

Fig. 34. Effect of BHA on S. aureus Wood 46 parent and variant strains ATPase activity.


ATPase activity (% control)
300
200
100
80
60
40
20
.2
.4
.6
.8
1.0
2.0
3.0
BHA (mM)
parent strain
variant strain

Since $CaCl_2$ was shown to partially reverse growth inhibition of S. aureus caused by BHA (para. 3.2.3) and that some ATPases are activated by calcium (Salton,1974), the effect of $CaCl_2$ on ATPase activity was studied. Addition of 4,0 mM $CaCl_2$,over the range of BHA concentrations used in these experiments or in the absence of BHA, had no effect on ATPase activities of both strains.

DISCUSSION

4.1 Minimal inhibitory concentrations

The mic's of BHA against S. aureus Wood 46 grown aerobically in BHI broth, as well as in the 3 other media used in this study, were 0,28 and 0,56 mM after 6 and 24 hours respectively. This last figure is slightly lower than the value of 0,84 mM reported by other authors after 24 hours of incubation (Chang and Branen, 1975; Davidson et al, 1979; Shih and Harris, 1977). This difference may be attributed to the different media, the different strains and the different methods used by these authors to assess growth. On the otherhand, Pierson et al (1980) reported that 0,56 mM inhibited the growth of S. aureus for 48 hours.

The mic against anaerobically grown S. aureus was found to be 0,14 mM after 24 hours of incubation. To our knowledge it is the first time that the mic of BHA against anaerobically grown S. aureus has been determined. The observation that S. aureus was more sensitive to BHA under anaerobic conditions than under aerobic conditions is similar to the results of Yotis and Baman (1969) working with diethylstilbestrol (DS), a synthetic estrogen with antistaphylococcal activity. Hence 6,0 to 7,2 ug DS/ml were necessary to completely inhibit the growth of S. aureus in aerobic conditions and only 1,6 to 2,8 ug/ml were required in anaerobic conditions. This finding was also reported

for other mammalian hormones (Yotis and Fitzgerald, 1968) but the authors concluded : "The reasons for enhancement of antimicrobial activity by anaerobiosis remain obscure ". We feel that the explanation of such a phenomenon relies on the differences associated with the cell wall of S. aureus grown either aerobically or anaerobically. In an interesting study, O'Brien and Kennedy (1971) reported that the cell wall of S. aureus grown anaerobically was 5 times thicker than the wall of bacteria after aerobic growth. However, cell walls from anaerobically grown S. aureus were more sensitive to bacteriolytic enzymes like lysostaphin and lysozyme. They proposed that the wall of anaerobically grown bacteria consisted of a more loosely formed network, resulting in a greater sensitivity towards the bacteriolytic enzymes. Our results suggest that these findings could be extended to BHA which, as a result of anaerobiosis, would be more likely to reach its site of action and exert its antibacterial activity. Thus the greater sensitivity of S. aureus in anaerobic conditions is unlikely to be a characteristic inherent to the molecule of BHA itself but rather a consequence of the influence of anaerobiosis on the physiology of S. aureus. This hypothesis is strengthened by the fact that S. aureus is more sensitive to other antimicrobials like DS, mammalian hormones, lysostaphin and lysozyme, under anaerobic conditions.

4.2 Effect of BHA and other agents, added alone or in combination, on the growth of S. aureus

A final concentration of 2% (v/v) ethanol was shown to slightly delay the growth of S. aureus Wood 46 (Fig. 5). Pierson et al (1980) reported that the same concentration of ethanol had no effect on the growth of another strain of S. aureus and on the growth of S. typhimurium. Our results are however in agreement with those of Ingram (1976) who reported that addition of ethanol (4%, v/v) to growing cultures of E. coli caused a lag in the growth for approximately 30 minutes, followed by a resumption of growth at a slightly reduced rate. Fatty acid alterations occurred during this lag and continued into the growth period; these changes were essential for the function of many membrane-associated enzymes. It is likely that the slight delay we noticed in the presence of 2% ethanol occurred for the same reasons.

During the first 4 hours of exposure to 0,14 mM BHA growth was delayed after which it resumed at a slightly slower rate than that of the ethanol-containing control. S. aureus managed to grow at this particular concentration of BHA but probably needed some sort of adaptation that is likely to take place during this 4 hours lag and throughout the growth period. These preliminary experiments did not enable us to determine the exact nature of these adaptive mechanisms but it is likely that they would tend to enable the cells to grow

in the presence of the antioxidant by affecting the membrane, and/or the cell wall, and/or the energy generating mechanism and/or the macromolecular synthesis.

The effect of 0,28 mM BHA on the growth of S. aureus was much more drastic since there was a significant 2 log decrease in the viability of the culture, after which growth resumed at a rate almost similar to that of the ethanol-containing control. To our knowledge it is the first time that such a growth pattern has been reported for S. aureus in the presence of BHA. This decrease and subsequent increase in viable cells have however been reported for S. typhimurium (Pierson et al, 1980), V. parahaemolyticus (Robach et al, 1977) and E. coli (Shih and Harris, 1977) grown in the presence of BHA. Pierson et al (1980) suggested that this phenomenon was an injury-recovery process but did not prove it. We used two different media that ascertained that the decrease in viability was not caused by injured cells but rather by cell death. One of them was the optimal medium of Hürst and Hughes (1981) for counting injured and uninjured cells. This medium was made of TSA supplemented with 0,5% sodium pyruvate; the mode of action of the sodium pyruvate is to degrade the metabolic by-product hydrogen peroxide (H_2O_2), rather than supplying a required nutrient(Baird-Parker and Davenport, 1965). Since in addition microscopic examinations ascertained that clumping was not responsible for the decrease of the CFU's, our results suggest that cell death was responsible for it.

It was noted, during the bactericidal phase occurring during

the first 10 hours of contact with 0,28 mM BHA, that the colonies of S. aureus were different from those of the inoculum since they were either dwarf or normal size and with or without a zone of hemolysis associated with them. These modified colonies were not detected in the presence of lower concentrations of BHA. They were unstable since they reverted to the normal type when subcultured once on blood agar. Such dwarf-colony variants of S. aureus have been known for a long time (Swingle, 1935). They are usually obtained by treating cultures of normal cells with either chemicals (Lacey, 1969) or antibiotics (Chin and Harmon, 1971; Quie, 1969) and are characterized by having a defect in a specific metabolic pathway (Horodniceanu et al, 1974). Such colonies are known to revert readily to the original type unless they are subcultured in a medium containing a low concentration of antibiotic (Horodniceanu et al, 1974). Since the blood agar we used to subculture those dwarf-colony variants contained no BHA, nor an antibiotic, it is likely that the dwarf-colony variants manage to overcome their metabolic disorders and, as a result, to grow normally and to form standard colonies after a single subculture.

Colonies isolated during the active phase of growth in the presence of 0,28 mM BHA were also different from those of the inoculum and from those just described. They had the same size as those of the inoculum but a distinct hemolytic pattern and did not revert to the original type when subcultured once on blood agar. Such fresh isolates, taken after 24 hours in BHI broth or after a primary isolation on blood agar, were inoculated in a fresh medium containing

0,28 mM BHA and it was found that their growth curves were almost similar to that of the ethanol-containing control. Moreover, the fact that the BHA was recovered intact after 30 hours of incubation with 0,28 mM BHA suggested that S. aureus did not metabolize the antioxidant and that the variant had became resistant to that concentration of BHA.

Pseudomonas fluorescens is the only other microorganism for which a progressive adaptation to BHA has been reported (Davidson, 1979). It was the result of many subcultures at nontoxic level. The relative resistance of S. aureus Wood 46 is also an interesting phenomenon since growth in the presence of phenolic derivatives do not usually lead to resistance (Hamilton, 1971). Hugo and Franklin (1968) reported that S. aureus, grown in the presence of 3% glycerol in order to increase its cellular lipid content, was less sensitive to alkyl phenols. Resistance was directly related to the increased lipid content. This relative resistance was only the result of a phenotypic adaptation and was lost after subcultures in a glycerol-free medium. The observation according to which the cells from the cultures of the variant strain did not settle down readily to the bottom of the tube but rather yielded hazy cultures, suggested that the variant might have managed to increase its cellular lipid content since Hugo and Stretton (1966) had noticed pellicle formation in cultures of bacteria grown in the presence of 3% glycerol. We must be cautious in our conclusion however since cultures of variants

that had became sensitive to BHA showed the same behaviour. We failed however in our attempt to obtain organisms resistant to 0,28 mM BHA by growing the parent strain in the presence of 3% glycerol. It is possible that growth in the presence of glycerol brought some changes in the cellular lipid content of the parent strain but that these changes were not sufficient to account for the resistance to BHA. Preliminary experiments also indicated that both parent and variant strains were producers of an extracellular lipase. Therefore it is possible that, in the presence of glycerol and because of the presence of an extracellular lipase, the cellular lipid content could not be increased. This possibility does not exclude however that modifications in the cellular lipid composition may have occurred in the presence of BHA.

We did however increase the resistance of S. aureus to BHA by growing the cells in the presence of 4,0 mM $CaCl_2$. Since $MgCl_2$ had no effect, Ca^{2+} was probably the ion responsible for this phenomenon. Since growth was not stimulated in the presence of $CaCl_2$ in controls run without BHA, it was likely that Ca^{2+} interfered directly with the antibacterial activity of BHA and did not act merely as a growth factor. It is likely that this ion might act as a cofactor for a membrane enzyme whose activity would have been decreased in the presence of BHA or as a structure-former since Ca^{2+} can be expected to stiffen lipoprotein membrane by forming salt bridges between -COO- groups (Langmuir and Schaeffer, 1936). Ca^{2+} has also been shown to

accumulate in the cell walls while Mg^{2+} tend to accumulate in the cytoplasm (Williams and Wacker, 1967). Humphrey and Vincent (1962) also showed that all the calcium present in a calcium-depleted culture of Rhizobium trifolii was contained in the cell walls and that it could not be functionally replaced by Mg^{2+} or by other divalent cations. Therefore, Ca^{2+} may restore enzymatic activity or by stiffening the membrane and the cell wall, prevent BHA from penetrating readily the cytoplasmic membrane or the cell wall. The inability of Mg^{2+} to decrease the mic might be the result of the weaker interactions that develop between macromolecules and Mg^{2+} as compared to Ca^{2+} (Tasaki and Iwasa, 1981) or if Mg^{2+} is not the required cofactor for an enzyme that would have been inhibited by BHA.

EDTA, on the other hand, has been shown to potentiate the antibacterial activity of BHA. It was interesting to find that sub-inhibitory concentrations of EDTA and BHA in combination were highly lethal to S. aureus Wood 46. It thus appears that there is an EDTA-BHA synergism. Although EDTA is a poor antimicrobial, this chelating agent was shown to increase the antibacterial activity of several compounds like fatty acids (Sheu and Freese, 1974), lipophilic preservatives (Shibasaki and Kato, 1979) and the surfactant monolaurin (Kabara, 1980). EDTA probably acts through its chelation of metal ions which are responsible for the integrity of the cell. Thus it is likely that the chelation of cations is followed by chemical or conformational changes in the cell wall or in the membrane which, in turn, result in an increased action of BHA.

4.3 Effect of BHA on hemolytic activity

In all experiments performed, hemolytic activity began to be detectable at the end of the exponential phase. This is in agreement with the findings of Abbas-Ali and Coleman (1977) according to which alpha-toxin is a secondary metabolite. Since it is important to express enzymatic activities as specific activities, hemolytic activity was expressed as H.U./10^8 CFU and will be discussed as such.

Hemolytic activities reached 53 H.U./10^8 CFU after 11 hours in the control and 43 H.U./10^8 CFU after 12 hours in the presence of 2% (v/v) ethanol. At first sight this concentration of ethanol seems to have a slight depressing effect on the alpha-hemolytic activity. However it is noteworthy that in the control the determination was carried out on 11 hour old cultures, i.e. 3 hours after the culture had reached the stationary phase while, in the ethanol-containing control, this determination was made only 2 hours after the culture had reached the stationary phase. Therefore the growth delay could justify the difference in hemolytic activity between these 2 cultures.

In the presence of 0,14 mM BHA hemolytic activity was however drastically reduced and growth delay could not explain the low hemolytic activity found in 16 hour old cultures. Such a depressing effect of BHA on alpha-hemolytic activity has never been reported before. Ayaz et al (1980) reported that in the presence of 0,84 mM BHA enterotoxin A could not be detected in the supernatant of S. aureus but

this observation was however the result of growth inhibition, which is not the case in our experiments.

It is noteworthy that a similar response has been noticed in the presence of antimicrobials other than BHA. Hence Nordström and Lindberg (1978) showed that streptomycin and novobiocin inhibited the production of alpha- and beta-hemolysins in mutants of S. aureus resistant to these antibiotics and at the same time induced the production of protein A. Streptomycin is known to block protein synthesis (Wallace et al, 1973) by binding to the cell membrane and to nucleic acids (Anand and Davis, 1960) and novobiocin to inhibit DNA and RNA synthesis (Smith and Davis, 1967) and cause an accumulation of wall precursor nucleotides in S. aureus (Wallace et al, 1973). In another study Shibl and Al-Sowaigh (1979) reported that subinhibitory concentrations of lincomycin and clindamycin completely suppressed the production of streptolysin S by Streptococcus pyogenes. These two antibiotics are known to inhibit ribosome function (Shibl and Al-Sowaygh, 1979). Nordström and Lindberg (1978) proposed that streptomycin and novobiocin might interfere in promoter-RNA polymerase interactions and since there are promoters with weak and strong affinities for RNA polymerase, the weak interactions may be disturbed by these antibiotics. This explanation might also be true for BHA, although we don't have any evidence for its interaction with protein synthesis.

Hemolytic activity in the presence of 0,28 mM BHA reached 114 H.U./10^8 CFU after 29 hours. Such a high activity may look contra-

dictory in view of the results recorded in the presence of 0,14 mM BHA. However, we should recall that the colonies isolated during the active phase of growth in the presence of 0,28 mM BHA were different from those of the inoculum and from those isolated in the course of the treatment with 0,14 mM. Therefore it is likely that we were then dealing with a new isolate having properties different from those of the inoculum and for which 0,28 mM BHA was only a subinhibitory concentration. This observation is substantiated by the fact that the growth curve of the variant in the presence of 0,28 mM BHA was almost similar to that of the ethanol-containing control. Such a stimulation of the hemolytic activity has also been noticed in the presence of antibiotics. Hence sub-bacteriostatic concentrations of penicillin and methicillin have been shown to stimulate the production of staphylococcal hemolytic activity (Hallander et al, 1966). These authors also reported that some extra antigens were produced in cultures containing low concentrations of penicillin. They failed to characterize the activity associated with these new antigens although they proposed that it might be due to a release of beta-toxin since activity on sheep erythrocytes was detected only in the presence of low concentrations of penicillin. Lorian (1971) also reported that beta-hemolysis was produced on sheep blood agar by several strains of S. aureus and that the zone of hemolysis surrounded the inhibition zone produced with discs of cephalotin and penicillins.

The alpha-hemolytic titer of the resistant strain grown

in the presence of 0,28 mM BHA was much lower (Fig. 8). Although the exact reason for this remains unclear, it is likely that the physiological condition of the cells might be responsible for such a result since maximum activities were reported after 13 hours in this case while it was determined after 29 hours when S. aureus was grown for the first time in the presence of 0,28 mM BHA.

These preliminary results indicating that BHA was possibly affecting the hemolytic properties of S. aureus Wood 46 prompted us to study in more detail the hemolytic activity of both parent and variant strains. The first striking feature is of course the hemolytic pattern associated with isolated colonies of the variant. It is the first time to our knowledge that such a hemolytic pattern has been reported for S. aureus. However a similar hemolytic pattern has been reported by Lorian and Popoola (1972) with Streptococcus pneumoniae. This type of hemolytic activity appeared only after anaerobic incubation; the haemolysin did not hemolyze human and rabbit cells and needed air and low temperature to be activated. In our case this type of hemolysis was produced under either aerobic or anaerobic conditions and did not need low temperature for activation. The reason why a ring of red cells stands between the colony and the zone of beta-hemolysis remains unexplained. It has already been shown that under aerobic conditions peroxide production prevents erythrocyte hemolysis (Noble and Vosti, 1971). However the fact that such a hemolytic pattern was also produced under anaerobic conditions suggests that it is more probable that

some other cellular metabolite might interfere with the hemolytic activity.

This peculiar hemolytic pattern was associated with isolated colonies only. Along lines of heavy growth there was a zone of beta-hemolysis followed by a zone of partial hemolysis or of discoloration. Lines were also formed within the zones of discoloration. This observation corresponds to the description of the hemolytic pattern produced by the beta-toxin (Elek and Levy, 1950). This kind of hemolytic pattern was associated with the variant strain only.

When the parent and variant strains were grown in BHI broth without BHA the variant strain was the only one to produce an hemolytically active substance on sheep red blood cells and whose titer increased upon refrigeration. This observation along with the hemolytic pattern itself, provide evidence that a beta-hemolytic activity was associated with the variant strain but not with the parent. The results obtained with the analysis of 50 different colonies analyzed by means of the reverse CAMP test substantiated these preliminary findings.

The electrophoretic procedure of Haque (1967) gave a direct evidence that a beta-hemolysin was produced by the variant. Furthermore, since the presence of cell-associated beta-toxin was detected only in the variant strain, this toxin was thus a new metabolite, not produced by the parent strain.

The fact that the variant strain obtained by treatment with

0,28 mM BHA was shown to produce a new toxin suggests that the high titer of alpha-hemolytic activity noticed when the parent strain was grown in the presence of 0,28 mM BHA (Fig. 7) might have been the result of a cross-reaction of the beta-toxin with rabbit erythrocytes since this toxin is also slightly active on these erythrocytes (Wiseman, 1965).

The exact mechanism by which BHA induces the formation of a new hemolysin in the variant strain remains highly speculative at this point. In this regard, it is however interesting to recall the finding of Hallander et al (1966) who noticed an increase of beta-hemolytic activity in the presence of low concentrations of penicillin. According to the hypothesis of Nordström and Lindberg (1978) and if the beta-toxin is normally repressed in the parent strain, BHA might block transcription for the weak promoter controlling the beta-toxin repressor operon and, as a result, the beta-toxin would be synthesized. Although attractive, this hypothesis is unlikely since it would mean that BHA must be present in order to get the beta-toxin synthesized. However, BHA might affect the genetic material of the parent strain in such a way that production of beta-hemolysin can occur even in the absence of BHA. If so, this new property would be genotypic in character. This possibility was further strengthened when we showed that BHA was possibly mutagenic for S. aureus Wood 46. However, since it was demonstrated that the relative resistance to the antioxidant was not stable and lost upon subculturing, it might suggest that this relative resistance was

thus a phenotypic adaptation. This would be in accordance with the findings of Hugo and Franklin (1968) who showed that the resistance of S. aureus to alkyl phenols was lost when the bacteria were subcultured in a medium not favouring the growth of lipid-enriched cells.

It was shown that, concomitantly to the synthesis of a new toxin, the variant had lost the property of producing the staphylokinase. This finding corroborated those of Winkler et al (1965) who reported that most strains of S. aureus that are beta-toxin producers lack the ability to produce the staphylokinase and they postulated that lysogenisation is responsible for this phenomenon. In our experiments both parent and variant strains had the same typing pattern. However a higher concentration of phages was required to type the variant strain. Such a result substantiated preliminary findings according to which the cell wall of the variant might be different from that of the parent strain, possibly hindering the adsorption of the phages onto the surface of the cells. Thus, these results suggest that fibrinolytic properties of staphylococci are not always phage-dependent but imply instead the occurrence of structural loci on the bacterial genome as proposed by Winkler et al (1965). As mentioned above, these modifications were shown to result from a possible mutagenic action of BHA. The mutagenicity assay we performed did not completely rule out the possibility that spontaneous mutants may have arisen in the different cultures during the incubation period, although controls did not give rise to such spontaneous mutants. The mutagenicity assay also revealed that both concentrations of BHA used to perform the experiments

gave similar results. These were probably threshold concentrations since only 16 of the 22 tubes yielded modified stable colonies. These results stress the importance of the ratio (BHA concentration)/CFU in this mutagenicity assay. They are also interesting in view of the fact that BHA was shown to increase the frequency of recombinants in Saccharomyces D-3 (Fabrizio, 1974), to increase aflatoxin B_1 mutagenicity (Shelef and Chin, 1980) and to be carcinogenic in F 3444 rats (Ito et al, 1982).

It is noteworthy that no variants were detected in the presence of 0,56 mM BHA and 4,0 mM Ca^{2+}. One of the reasons may be that this concentration of BHA was too high to allow the required cellular multiplication and nuclear segregation of potential mutagenized cells. Also, since the bacterial genome is attached to the cytoplasmic membrane (Rogers, 1970) and Ca^{2+} is known to act as a structure former at both cell wall and membrane levels, it is possible that, in the presence of Ca^{2+}, the molecule of BHA could not reach the bacterial genome and therefore is prevented from exerting its possible mutagenic activity.

The mutagenicity assay was facilitated by taking advantage of the fact that the variant strain was less sensitive to bacitracin than the parent strain. The differential sensitivity to bacitracin is an interesting character since this antibiotic is known to inhibit a specific reaction in the peptidoglycan synthesis. However, this enzymatic reaction is membrane-bound (Siewert and Strominger, 1967) and it is likely that changes in membrane structure modify the accessibility of

bacitracin to such a membrane-bound enzyme. (Altenbern, 1975). This property, along with the resistance to pH and to osmotic shock suggest that the variant strain is a membrane mutant of S. aureus Wood 46 parent strain. The greater sensitivity of the parent strain to pH suggests that some enzymes might be inactivated or that the membrane was more permeable to hydrogen ions while sensitivity to osmotic shock confirmed the greater resistance of the cytoplasmic membrane of the variant strain to an environmental stress.

Mutants displaying the same hemolytic pattern to that of the variant were isolated after treatment with EMS. However these mutants were sensitive to 0,56 mM BHA. Since we did not isolate similar mutants with plasmid-curing agents, it is likely that the peculiar hemolytic pattern associated with isolated colonies of the mutants were coded for by the bacterial genome itself and not by a plasmid.

The fact that BHA purchased from 3 different companies was capable of inducing the formation of mutants does not completely eliminate the possibility that a contaminant with a mutagenic activity might be responsible for such results, although GLC analysis did not reveal any , but render this possibility very unlikely.

4.4 Effect of BHA on the cytoplasmic membrane

BHA being a highly hydrophobic molecule, the cytoplasmic membrane is likely to be the main site of adsorption since the cell wall of S. aureus contains as little as 1 or 2% lipid material (Salton, 1964).

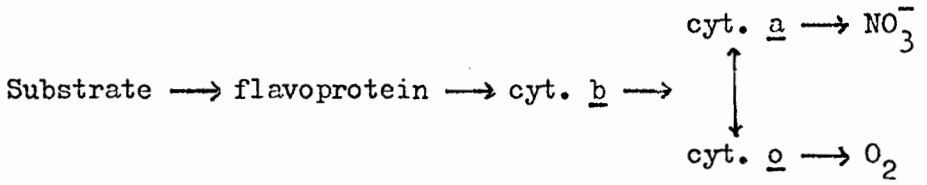
Moreover, the low water solubility of the drug probably precludes any significant diffusion into the cytoplasm. Bacterial variants such as L-forms, spheroplasts and protoplasts have been used with success to monitor the antibacterial activity of compounds acting at the membrane level (Russel et al, 1973). Lysis of bacterial protoplasts by BHA occurred at concentrations lower than those causing leakage of nucleotides from whole cells. It is an indication that the cell wall might play an important role in the relative resistance of whole cells to lysis by low concentrations of BHA. It was interesting to note that whatever the concentration, the extent of lysis of protoplasts was the same over a period of 24 hours and it suggests that lysis was complete with all 3 concentrations tested. However the rate of lysis was dependent on the concentration used. The fact that the protoplasts from the variant strain were less susceptible to lysis by BHA than those of the parent strain ascertained that they were not only more resistant to a physical stress, i.e. osmotic shock, but to a chemical stress as well. Since the amount of BHA taken up by both the resistant and parent strains was not significantly different, it is likely that the adsorption of this chemical does not induce a non-specific physico-chemical disturbance of the osmotic properties of the membrane as it has been suggested for polymixins (Few and Schulman, 1953) and other membrane-active substances (Hamilton, 1968). Hence mutations towards resistance to these membrane active substances always involves a mechanism preventing the uptake of the chemical by the membrane (Hamilton, 1968). Thus the binding of BHA to the membrane

of S. aureus Wood 46 most probably alters some specific functions of the membrane. The linearity of the adsorption isotherm shows that the number of sites for adsorption remained constant. One explanation of this constant number would be that adsorption of BHA causes the disruption of the membrane and the creation of new binding sites. This type of isotherm has also been reported for the adsorption of another antioxidant, propyl gallate, onto the cells of E. coli (Boyd and Beveridge, 1981).

Antibacterial activity of BHA was greater in culture medium compared to that in phosphate buffer. Similar results were obtained by other authors (Husseini and Stretton, 1980; Rubbo et al, 1950) who were studying the antimicrobial activity of chelators on S. aureus. They showed that the lethal effect associated with these compounds appeared to be dependent on its complexing with metal ion(s) outside the cell. No such property such as chelation has ever been reported for BHA. It is more likely that this antioxidant is more effective against cells undergoing active multiplication. Thus BHA should interfere either on the energy metabolism, on the synthesis of macromolecules or produce membrane damage on actively growing cells only. This observation, in addition to the fact that BHA induced leakage of cytoplasmic constituents and lysis of protoplasts, is also suggestive that it would be a potent inhibitor of some membrane functions involving the inactivation of some enzymatic activities, permeation or the inhibition of the electron transport system which are all functions carried out by the membrane in S. aureus (White and Freeman, 1967; Niven and Hamilton, 1974).

4.5 Effect of BHA on membrane functions of S. aureus

Reduction of cytochromes in the presence of electron donors enabled us to determine the composition of the electron transport chain of S. aureus Wood 46. Our results indicate that complete electron transport chain is functional in this organism and resembles the mammalian system in being sensitive to the inhibitors of the electron transfer reactions. These data corroborate those of Taber and Morrison (1964) who suggested the following scheme for the respiratory chain of S. aureus:



Although various theories have been proposed (Stuckey, 1972) on the mechanism of hydrogen or electron donation by antioxidants, none has been definitely proved. It is well established that bacterial respiratory chains consist of a series of cytochromes which are electron transferring molecules containing colored active groups called hemes. The hemes are comprised of porphyrin and iron. The iron atom is in the ferric (Fe^{3+}) form when the cytochromes are in the oxidized state. In the present studies when S. aureus cytochrome system (cell-free extracts) served as the electron acceptor, it appears that each cytochrome in its oxidized (Fe^{3+}) form accepted electron from BHA and

became reduced to the ferrous (Fe^{2+}) form while antioxidant BHA was completely oxidized. The reduced form (i.e. ferrous form) in turn donated electrons to the next carrier which is then reduced and so on. Kurechi et al (1980) investigated the hydrogen or electron donating ability of several antioxidants including BHA and reported that ferric ion (Fe^{3+}) is reduced to ferrous ion (Fe^{2+}) by many antioxidants when used alone or in combination. Since BHA is a very lipophilic molecule and lipids are involved in the respiratory process in S. aureus (Goldenbaum and White, 1974), our results suggest that the inhibition we observed of the electron transfer reactions resulted from such an interaction. Hence BHA, like organic solvents (Goldenbaum and White, 1974), would disrupt the structural integrity of the respiratory chain carriers and, as a result, interrupt electron flow. The presence of lipids is not essential however for the reduction of cytochromes. Thus the contradictory effect of BHA in reducing cytochromes without stimulating respiration can be explained.

It is noteworthy that proton translocation experiments did not reveal any uncoupling effect of BHA and therefore ascertained that BHA was an inhibitor of the respiratory activity.

Although Wedding et al (1967) showed that various substituted phenols could act as uncouplers of oxidative phosphorylation, Hugo and Street (1952) showed that phenol and phenoxyethanol (0,1 to 0,2%) caused 10 to 15% inhibition of oxygen consumption in E. coli when lactate, pyruvate, acetate or succinate was the substrate. We cannot exclude that these compounds might have the same effect as BHA on the respiratory chain of E. coli.

Various dehydrogenase activities were inhibited by BHA, those associated with succinate and malate being very sensitive to a concentration of BHA as low as 0,07 mM . This observation is in accordance with the fact that oxygen consumption in the presence of succinate was more sensitive to inhibition by BHA than that associated with lactate and glucose. It is possible that the localization of these different enzymatic systems in the membrane is responsible for the difference in sensitivity to BHA; i.e. those more profoundly embedded in the membrane would be less susceptible and vice-versa. This hypothesis is strengthened by the work of Bach and Lambert (1937) who studied the effects of antiseptics and solvents on certain dehydrogenase activities of S. aureus. They showed that activities associated with glucose and lactate were only partially inhibited by benzene, toluene or phenol while those associated with succinate, fumarate and glutamate were completely destroyed.

At the beginning of the DISCUSSION we reported an interesting analogy between DS and BHA, both of them being more effective under anaerobic conditions. It is of interest to note that DS, like BHA, also inhibited oxygen consumption and dehydrogenase activities in S. aureus (Yotis and Baman, 1969). This analogy is much more striking if we consider that DS has also been shown to inhibit amino acid uptake in S. aureus (Fitzgerald and Yotis, 1971). Since we noticed that the initial rate of amino acid uptake was inhibited by BHA, the antioxidant thus inhibits the transport process. It is most probably a direct consequence of the inhibition of the respiratory activity which is respon-

sible for the production of the PMF used to drive amino acid uptake. Inhibition of uptake of these nutrients is likely to result in inhibition of macromolecular synthesis and of cellular constituents and, as a result, inhibition of growth and of multiplication.

The decrease of radioactivity we noticed with all 3 amino acids after a certain time is probably the result of oxidative degradation.

The relative resistance of glucose uptake to inhibition by BHA, as compared to that of amino acids, can be explained by the fact that the former is a group translocation process whereas the latter is directly dependent on the chemiosmotic property of the membrane. Therefore it is likely that membrane permeability for glucose is not significantly affected by 0,28 and 0,56 mM BHA.

It is of interest to recall that the uptake of glucose by the phosphotransferase system requires the participation of soluble and membrane-bound enzymes. Hence enzyme 1 and the histidine-containing protein are soluble and initiate phosphoryl transfer from phosphoenolpyruvate produced via the Embden-Meyerhof pathway. Enzymes 2 and 3, which are sugar specific, are respectively integral and peripheral membrane proteins (Harold, 1978). The relative resistance of glucose uptake to inhibition by BHA suggest that these different components of the phosphotransferase system of S. aureus were not significantly affected by the antioxidant. Therefore it is possible that other soluble enzymes and other membrane enzymes, whose activities do not depend on the chemiosmotic properties of the membrane, are not significantly affected by BHA either. The same observation goes for the portion

of the Embden-Meyerhof pathway leading to the production of phosphoenolpyruvate. Hence it suggests that BHA, like DS (Yotis and Fitzgerald, 1968), retards the growth of S. aureus without penetrating the bacterial cytoplasm in significant amount.

Esters of para-hydroxybenzoic acid, which are also phenolic derivatives, have been shown to inhibit amino acid uptake in S. aureus while slightly stimulating that of glucose (Eklund, 1980). No such stimulation of glucose uptake was noticed in our experiments.

Our results provide direct evidence for a stimulatory effect of BHA on ATPase activity. Stimulation was proportional to BHA concentration. Such a stimulation of ATPase activity has been reported with steroid hormones on liver mitochondria (Blecher and White, 1960) and with DS on S. aureus ATPase (Kuback and Yotis, 1981). Since the bacterial ATPase are known to be comprised of several polypeptide subunits arranged in a cluster and that lipids have been shown to be associated with them(Klemme et al, 1971), it is likely that the enzyme is capable of interacting with the lipid region of the membrane. This hypothesis is substantiated by the fact that S. aureus ATPase has been shown to be tightly integrated within the membrane (Kuback and Yotis, 1981). Since the adsorption pattern of BHA onto the cells of S. aureus suggested that BHA, in binding to the cells, causes disruption of the membrane and the creation of new sites, it is possible that BHA interacts with membrane lipids or ATPase-associated lipids in such a way that more active sites of the enzyme become available as the concentration of BHA is increased. However, a direct interaction of the antioxidant with the

enzyme can not be ruled out.

It is known that the ATPase is a reversible enzyme capable of producing ATP when S. aureus is grown under aerobic conditions (Kashket, 1981). However our results do not allow us to speculate on the effect of BHA on the ATP synthetase activity although the inhibition of the respiratory activity we noticed suggests that the ATP synthetase activity would be depressed. Since these experiments were carried out in cell-free systems and with concentrations of BHA up to 3,0 mM , the phenomenon we noticed is very difficult to relate to the situation occurring in whole cells exposed to BHA. Nonetheless, ATPase activity is known to occur under conditions of low oxygen tension and of oxygen depletion, and to be responsible for the production of the PMF which can be used to drive secondary processes. It is therefore possible to speculate that the stimulation of ATPase activity could lead to ATP depletion and from there, to growth inhibition. Nevertheless, the interaction of BHA with such an important enzyme is probably detrimental for S. aureus.

CONCLUSION

BHA was shown to be possibly mutagenic for S. aureus Wood 46, inducing the formation of membrane mutants different from the parent strain with respect to the synthesis of beta-hemolysin, the production of staphylokinase and the hemolytic pattern (Degré and Saheb, 1982). These properties were stable and probably linked to the bacterial genome. In addition, the mutants were shown to be less sensitive than the parent strain to the antibacterial activity of BHA. However this property was not stable and might probably have resulted from an adaptation to BHA rather than a chromosomal mutation. The appearance of the liquid cultures of the variant, which was different from that of the parent strain, suggested that the former may have an increased cellular lipid content.

Adsorption of BHA onto the cells of S. aureus was rapid, both parent and variant strains adsorbing approximately the same amount of the antioxidant. At this stage, an explanation for the relative resistance of the variant to the antibacterial activity of the antioxidant is still speculative, but we feel that it might result from modifications in the cellular lipids, especially in the cell wall as indicated by the fluffy appearance of the culture of the variants, and/or from modifications occurring at the membrane level since protoplasts of the variant strain were less susceptible to lysis by BHA.

The former hypothesis is reinforced by the fact that the phage typing of the variant was possible only at a phage concentration 10 times higher than that of the parent strain. Therefore, in the variant, the cell wall would serve as an adsorbing barrier, preventing the antioxidant from reaching the membrane, which is likely to be the main site of adsorption due to the highly lipophilic character of the molecule of BHA. Moreover this property probably precludes any significant diffusion of BHA into the cytoplasm of S. aureus, the latter being hydrophilic in character. Along with these observations, the fact that BHA has been shown to induce leakage of nucleotides and lysis of protoplasts suggest that BHA is mainly acting at the membrane level (Degré and Sylvestre, 1983). This finding is substantiated by the fact that the antibacterial activity of BHA was enhanced in the presence of EDTA which is likely to increase cell permeability to the antioxidant and by the fact that Ca^{2+} ions, possibly by stiffening the membrane and the cell wall, decreased its antibacterial activity. Moreover the analogy that was made between DS and BHA also provides indirect evidence that BHA is a membrane active agent since the former is known to act in a similar way and to be a membrane active agent.

At high concentration (1,12 mM) BHA has been shown to cause cell death, which was related to leakage of intracellular material. At lower concentrations (0,56 mM and less), growth inhibition is likely to result from the inhibition of respiratory chain and dehydrogenase activities. It is the first time that a phenolic derivative has been shown

to reduce the cytochromes and at the same time inhibit oxygen consumption (Degré et al, 1983). As a result of inhibition of oxygen consumption, amino acid uptake, whose transport across the cytoplasmic membrane is driven by the PMF generated by the respiratory chain, is significantly decreased. Therefore macromolecular synthesis and total cellular synthesis are probably affected to the point that inhibition of growth and inhibition of cellular division occur.

The fact that glucose uptake, a group translocation process, was not affected, suggest that BHA has some specificity of action since not all membrane-associated functions were affected to the same extent.

Although S. aureus Wood 46 respiratory chain activity was affected to the point that oxygen consumption was significantly inhibited, it does not mean however that BHA was active only under aerobic conditions since S. aureus was shown to be even more sensitive to BHA under anaerobic conditions. The latter is not a characteristic inherent to the molecule of BHA but associated with the effect of anaerobiosis on S. aureus. The stimulation of ATPase activity, an enzyme usually active under anaerobic conditions, could possibly lead to ATP depletion which, in turn, would result in growth inhibition. The exact significance of this finding is still a matter of speculation but such a stimulation of the ATPase, an enzyme whose activity must be carefully controlled, is probably detrimental for S. aureus.

The fact that a subinhibitory concentration (0,14 mM BHA) was shown to significantly inhibit alpha-hemolytic activity of S. aureus

Wood 46 parent strain is an interesting finding with regard to its potential use as a food preservative since hemolysins are considered as factors of pathogenicity. Potentiation of the antimicrobial activity of BHA by EDTA is also interesting in this regard since this combination could be used to increase the effectiveness of BHA against Gram negative bacteria. However the possible mutagenicity of BHA as well as the possible adaptation of S. aureus to increasing concentrations of the antioxidant and therefore the selection of a resistant population, are 2 important criteria that should not be overlooked if the government is to decide to use phenolic antioxidants as food preservatives.

APPENDIX 1

Columbia Agar

Ingredient	g/l
Peptone	23
Starch	1
NaCl	5
Agar	10
Sheep blood (5%)	-

APPENDIX 2

Special medium of Collings and Lascelles (1963)

Ingredient	g/l
Tryptone	10
Lab Lemco (Oxoid)	5
Yeast extract	1
Na_2HPO_4	5
Glucose	20

Final pH: 6,5

APPENDIX 3

Glycerol medium (Hugo and Stretton, 1966)

Ingredient	g/l
Peptone (Oxoid No. 1)	10
Meat extract (Lemco, Oxoid)	5
Sodium chloride	5
Glycerol (3%)	-

APPENDIX 4

pH medium (Kent and Lennarz, 1972)

Ingredient	g/l
Bacto peptone	10
Yeast extract	10
Sodium chloride	5
Na_2HPO_4	0,4

Final pH: 5,2 and 7,0

APPENDIX 5

Hypertonic buffer

Ingredient	M
Tris-hydrochloride (pH 7,5)	0,05
$MgSO_4$	0,015
Sodium chloride	3,45

Hypotonic buffer

Tris-hydrochloride	0,05
$MgSO_4$	0,015

APPENDIX 6

ATPase reaction mixture

Ingredient	M
KCl	0,100
$MgCl_2$	0,004
ATP	0,0025
Tris-acetate buffer (pH 6,5)	0,050

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