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CHEMICAL AND BIOCHEMICAL IMPLICATIONS
OF NITRITE DURING CURING

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The products of the preservation of meat through the use of a brine containing nitrite and/or nitrate comprise a range of attractive, acceptable and relatively stable commodities with an excellent public health record. During the process of curing, much of the nitrite is lost, the amount depending upon such parameters as the pH of the meat, the temperature of processing and the initial nitrite level. A small proportion of the nitrite nitrogen is finally located in combination with muscle myoglobin as the characteristic red pigment of such products, but the fate of much of the nitrite is generally unknown.

Using nitrite labelled to 95% with isotopic nitrogen, it has been possible to study the gaseous products obtained in model cures at temperatures (30 - 37°C) relevant to uncooked cured products using muscle minces held at pH 6.0 with phosphate buffer, with the inclusion of a broad spectrum antibiotic to limit bacterial development. Fig I shows the distribution of mass numbers in the examination within a mass spectrometer of the gaseous products of such incubations under an atmosphere of a reduced pressure of argon. The peak at m/e 30 using $\text{Na}^{14}\text{NO}_2$ was replaced by a similar one at m/e 31 with $\text{Na}^{15}\text{NO}_2$ as would be anticipated for NO. The use of $\text{Na}^{15}\text{NO}_2$ permitted the differentiation of CO_2 and N_2O , which are normally unresolved at m/e 44. Even under anaerobic conditions, oxygen was detected at m/e 32. Small peaks occurring consistently at m/e 26 and 27 were considered to result from the fragmentation of ethylene or ethane. The presence of the former was confirmed by gas chromatography and by high resolution mass spectrometry. The production of ethylene was not apparently associated with contaminating bacteria since their suppression with increasing concentrations of nitrite was accompanied by an increasing output of the gas (Fig 2).

The reactions of nitrite which may be involved with its role in curing are principally concerned with its action as an oxidising and nitrosating agent and in its uptake by biochemical systems active within the muscle.

Nitrite as an oxidising agent

The oxidation by nitrite of oxyhaemoglobin has long been recognized in relation to the occurrence of methaemoglobinaemia of young infants in whom the achlorhydria of the stomach has permitted the reduction of nitrate in drinking water by the extension of the intestinal flora or who have been fed a nitrate rich product such as spinach maintained unhygienically without refrigeration. Foetal haemoglobin is particularly sensitive to the oxidising action of nitrite but the increasing resistance is gradual in childhood until the age of puberty when its transition accelerates¹. Early studies on the stoichiometric relationship between the reactants indicated the oxidation of one mole of haemoglobin by two moles of nitrite², but later results suggested a 1:1 molar ratio. More

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dimethylamine with 2.9 mM (200 ppm) nitrite proceeds to an extent of less than 1% of the theoretical within 3 hours at 37°. Under similar conditions, the nitrosation of diphenylamine, an amine not considered to be of physiological importance, proceeds virtually to completion. Thus the outcome of the contact with nitrite of the components of a biological system such as meat is greatly dependent upon the nature of any secondary amines present, which are generally unknown in foods except in specific instances such as marine fish.

If the reaction of nitrite with a secondary amine is reversible whilst that with a primary amine is irreversible, the incubation at acid pH of an N-nitrosamine with a receptor of nitrous acid such as urea could result in the denitrosation of the former. However, no evidence has been obtained of the breakdown of N-nitrosamines in this way. Furthermore, the incubation of a secondary amine and excess of a primary amine with a low level of nitrite sufficient for only partial nitrosation does result in the formation of some N-nitrosamine.

The N-nitroso derivatives of secondary amines are stable at neutral and alkaline pH values and sufficiently stable in acid for the volatile compounds of this type to be separable from their parent amines by distillation at acid pH¹⁰. Chemically they show but little reactivity. Physiologically, the lower members of the dialkyl and heterocyclic types are generally hepatotoxic when administered at moderate dosage¹¹; some species of animals are particularly sensitive and mink, for instance, have been employed in confirmation of chemical and physical observations. At lower levels of administration, many of the 80 or more N-nitrosamines studied have proved to be carcinogenic in experimental animals^{11, 12, 13}. Some of the simple N-nitrosamines have been tested and found to be effective at very low doses on a body weight basis in up to eleven different species of animal including monkeys¹⁴. Other more complex non-volatile N-nitrosamines such as N-nitrosodiethanolamine are far less toxic but retain their carcinogenic action, although they have not generally been tested so extensively and down to the low dosage levels employed for the volatile members of this class.

The possible components of a biological system such as meat which could potentially be nitrosated include proline, hydroxyproline, spermine, spermidine, tryptophan and histidine. The preparation of a N-nitroso derivative of arginine has been reported by Mirvish¹⁵, as well as that of the naturally occurring amide, citrulline. N-nitrososarcosine is unstable and decarboxylates readily to N-nitrosodimethylamine. Tryptophan has been reported to yield N-nitroso-indoleacrylic acid on nitrosation¹⁶. Creatinine can react with nitrous acid¹⁷ to yield a C-nitroso derivative amongst other products.

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N-nitrosamides are also very potent carcinogens but they are far less stable, particularly at an alkaline pH, than the N-nitrosamines. They also react readily with groups such as the sulphhydryl likely to be encountered in a biological system. Secondary amides are, however, very readily nitrosated, the rate of reaction being directly proportional to the nitrite and hydrogen ion concentrations.

The reaction between nitrite and a tertiary amine to yield an aldehyde together with the N-nitroso derivative of the secondary amine resulting from the loss of one substituent group, is favoured by high temperatures and nitrite concentrations but not by low pH values, being most effective in the pH region 3-16¹⁸. Benzyl groups are most readily cleaved from the tertiary amine to form the N-nitrosamine, followed by alkyl and then cyclic substituent groups.

Over the past decade, many methods have been devised and advocated for the selective separation and detection at low levels of N-nitrosamines and, to a lesser extent, the N-nitrosamides. In attempting the separation of any such compounds as a group, it has often been considered necessary to reduce the N-nitroso group to the corresponding asymmetrically substituted hydrazine, which then permits selective separation by virtue of the basic properties acquired. However, the excellent yields obtained with such reducing agents as lithium aluminium hydride in model systems were usually not reproduced with complex extracts of a biological system often including appreciable lipid. The volatile N-nitrosamines can generally be separated by steam distillation including that from an acid milieu which retains amines which may provoke confusion in subsequent procedures. In the presence of residual nitrite, a preliminary distillation from an alkaline environment is necessary prior to that from acid to avoid irrelevant nitrosation during the process. Such procedures have been extended to fractionation using suitable high efficiency columns such as the spinning band type. In solution in aqueous methanol, ethanol or acetonitrile, almost all volatile N-nitrosamines can be separated as a narrow band boiling between the volatile solvent or its azeotrope with water and water itself¹⁹. As a result, enhancements of concentration of 30-40 fold can be readily achieved although some losses are generally inevitable.

Solvent extraction of N-nitrosamines contained in condensates from steam distillations has also been used with great success, the immiscible solvent of choice being methylene chloride. In concentrating such extracts, care must be taken to dry them prior to distillation to avoid losses incurred through the formation of azeotropes.

The separation of non-volatile N-nitrosamines presents much greater difficulties but advantage has been taken of the adsorption of all such compounds tested on activated carbon. After washing the adsorbent to remove residual nitrite, etc, most volatile and non-volatile N-nitrosamines can be desorbed in part at least, in refluxing methanol²⁰. Overall recoveries from simple aqueous solution range from 94% for N-nitrosodiethylamine to 72% for nitrosoproline and to 0% for N-nitrosamines such as N-nitroso-N-methylaniline containing phenyl groups. The presence of lipid affects the recoveries adversely, however.

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Methods employed for the detection of nitrosamines and nitrosamides have included spectrophotometry, thin layer and gas chromatography, direct and differential polarography and ultraviolet photolysis to inorganic nitrite. None are unequivocal and all are subject to interference leading to loss of sensitivity in application. Recently, considerable advances have been made so far as volatile nitrosamines are concerned in their unequivocal detection by high resolution mass spectrometry coupled with gas chromatography, as a result of which, levels of 50 parts per thousand million or even less of added authentic nitrosamine can be characterized after concentration by vacuum steam distillation and solvent extraction²¹. No such similar universal procedure exists as yet for non-volatile nitrosamines but the recent development of a method²² involving hydrolysis with hydrobromic acid in glacial acetic acid has reduced very considerably the detection limits in thin layer chromatography, etc. As a result of the mild hydrolysis, which must be carried out with the virtual absence of water, the parent amine is released along with nitrosyl bromide, which can be detected as nitrite in solution. No nitrogenous compound other than a nitrosamine or a nitrosamide has been found to respond to this procedure which distinguishes between such compounds and the closely related N-nitramines and N-nitramides²³. Alkyl nitrites react as nitrite in glacial acetic acid both with and without hydrobromic acid.

Biochemical implications of nitrite

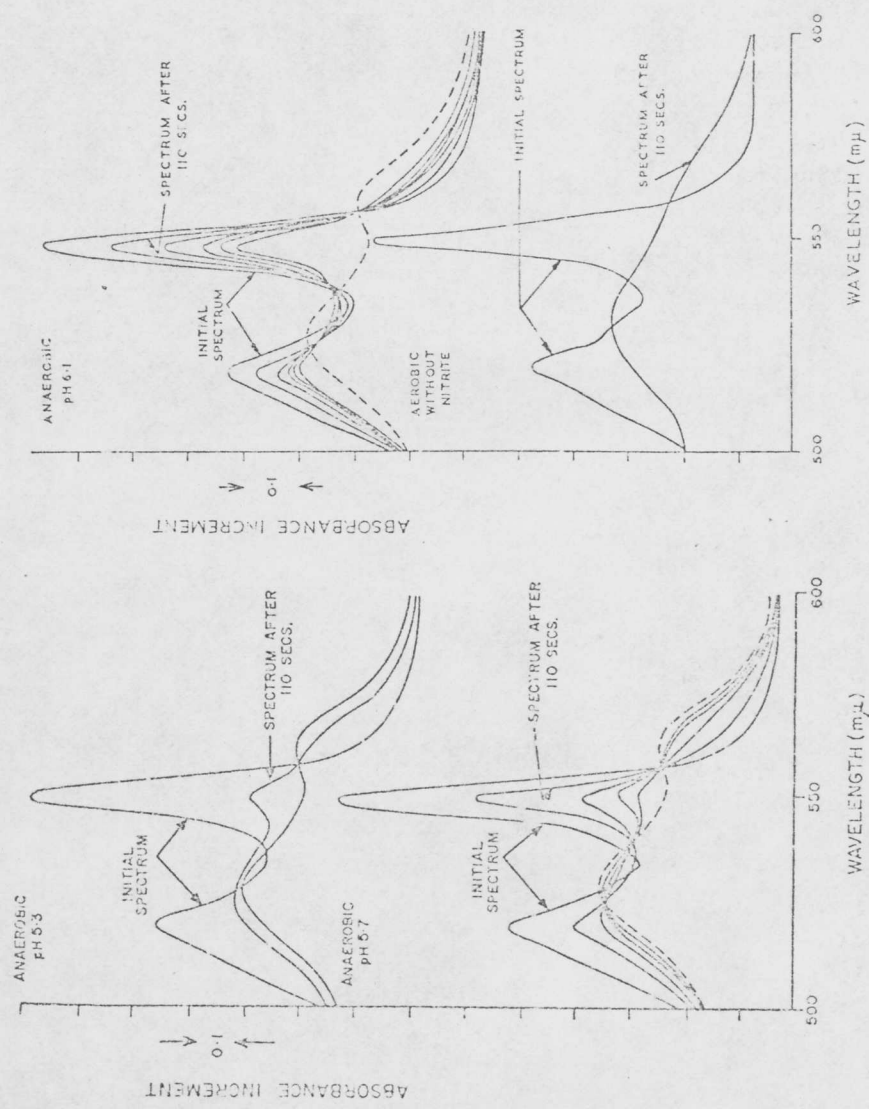
Skeletal muscle continues to respire for long periods after the death of the animal even in the presence of broad spectrum antibiotics to limit bacterial development. Under aerobic conditions, muscle respiration is inhibited by nitrite, a concentration of about 50 mM (3500 ppm) being required to effect a reduction in oxygen uptake of about 50% at pH 6.0.

At a representative pH of 6.0 the incubation of nitrite with skeletal muscle under anaerobic conditions simulating those in the interior of a block of meat results in the evolution of a nitric oxide gas fraction²⁴. The output of this fraction was not stimulated by the deliberate enhancement under aerobic and anaerobic conditions of the resident bacterial populations of the muscle minces by pre-incubation. It was stimulated, however, by reduced methylene blue which can act as an electron intermediate in a sequence of oxidation-reduction interactions.

Within the biological cell, the enzymes concerned with respiration are mainly located within the mitochondrion. After homogenization of the tissue, these sub-cellular organelles can be separated by centrifugal fractionation. In use, their high activity permits the spectrophotometric observation of molecular changes associated with the respiratory chain, without unduly high background absorption due to inert material.

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FIG. 3



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The re-reduction of nitrosylferricytochrome c by, for instance, the NADH dehydrogenase (EC 1.6.99.3) action of muscle mitochondria under anaerobic conditions results in the formation of uncomplexed ferrocytochrome c, which does not form a nitrosyl derivative at relevant pH values. In the presence of metmyoglobin, the haem pigment resulting from nitrite oxidation of the oxy-form, the nitrosyl-group or nitric oxide itself resulting from the reduction of nitrosylferricytochrome c is transferred to produce nitrosylmetmyoglobin, which is subsequently reduced to nitrosylmyoglobin by continued anaerobic mitochondrial action²⁶. The differentiation of nitrosylmetmyoglobin and nitrosylmyoglobin, which have very similar spectral properties, as intermediate and final product has been made on the basis of the lability in air of the former. Spectral observations at various time intervals indicated an increase of nitrosylmetmyoglobin production followed by a fall as the ensuing reduction to nitrosylmyoglobin proceeded. Ascorbate was able to mimic the action of mitochondrial enzymes in reducing nitrosylferricytochrome c with transfer of the nitrosyl-group to metmyoglobin and subsequent reduction to nitrosylmyoglobin. The reduction by muscle respiratory enzymes of nitrosylmetmyoglobin to nitrosylmyoglobin is achieved much more readily than that of uncomplexed metmyoglobin to reduced myoglobin. This effect is due to the ready autoxidation of reduced myoglobin back to the met-form. In an atmosphere of carbon monoxide, the ferrous form of myoglobin is stabilized by complex formation as soon as reduction occurs and no difference is then observed between the reduction of met- and nitrosylmetmyoglobin. So far as the latter is concerned, the ferrous form is more stable than the ferric and no tendency for oxidation has been observed.

Thus, the cured meat pigment, nitrosylmyoglobin, can be formed under conditions permitting enzyme action through the intermediate nitrosylferricytochrome c when using extra-mitochondrial cytochrome c. Muscle mitochondrial swelling studies have demonstrated that the membrane is permeable to nitrite and good evidence has been obtained of the conversion of endogenous mitochondrial ferrocytochrome c to nitrosylferricytochrome c and of ferrocytochrome a₃ to its nitrosyl-complex. Thus, there is every reason to suggest that the sequence of reactions elucidated using model systems is applicable to the intact muscle mitochondrion in its environment and therefore to whole skeletal muscle.

Acknowledgements

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FIG. 1

INTENSITY

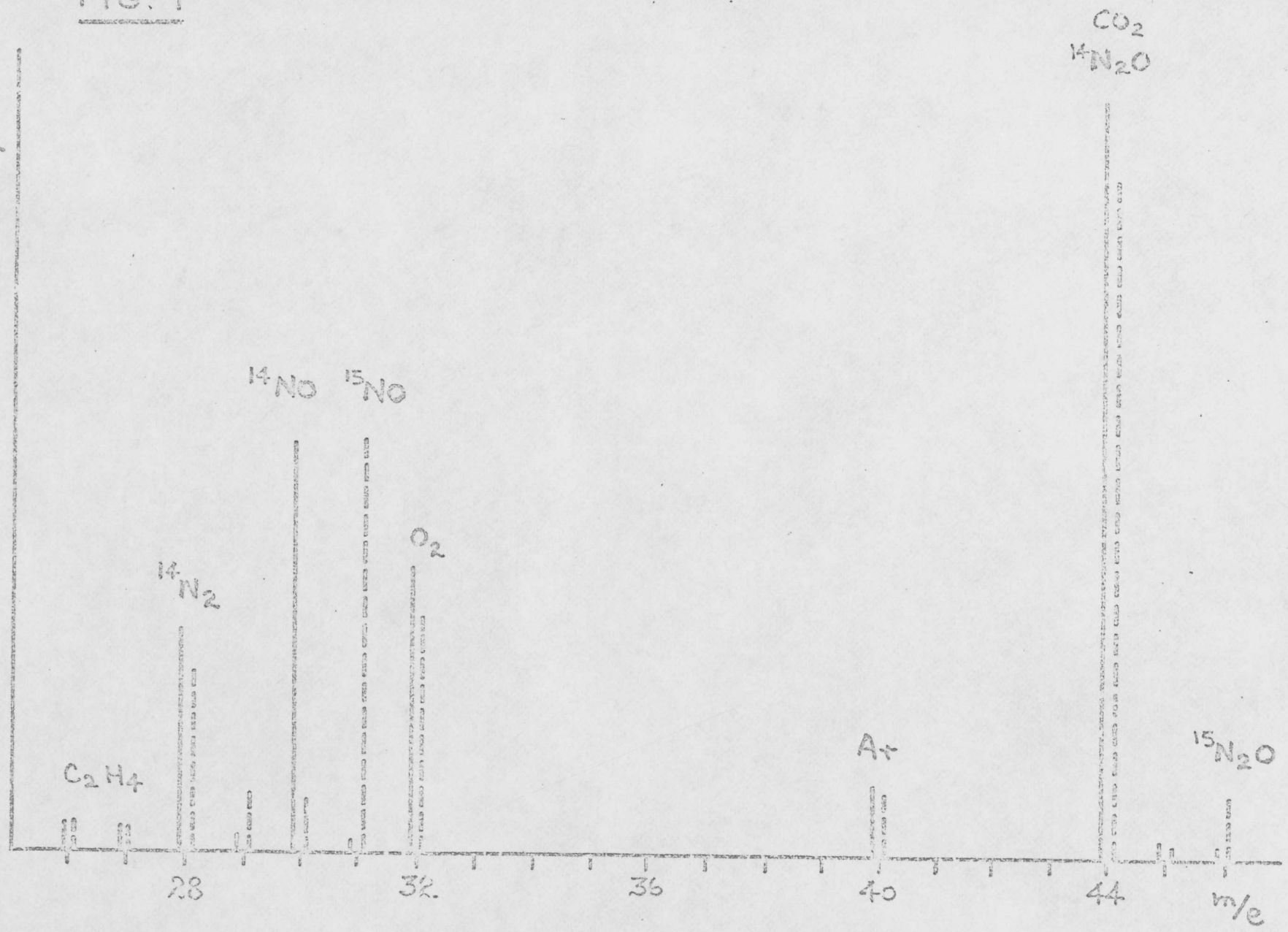
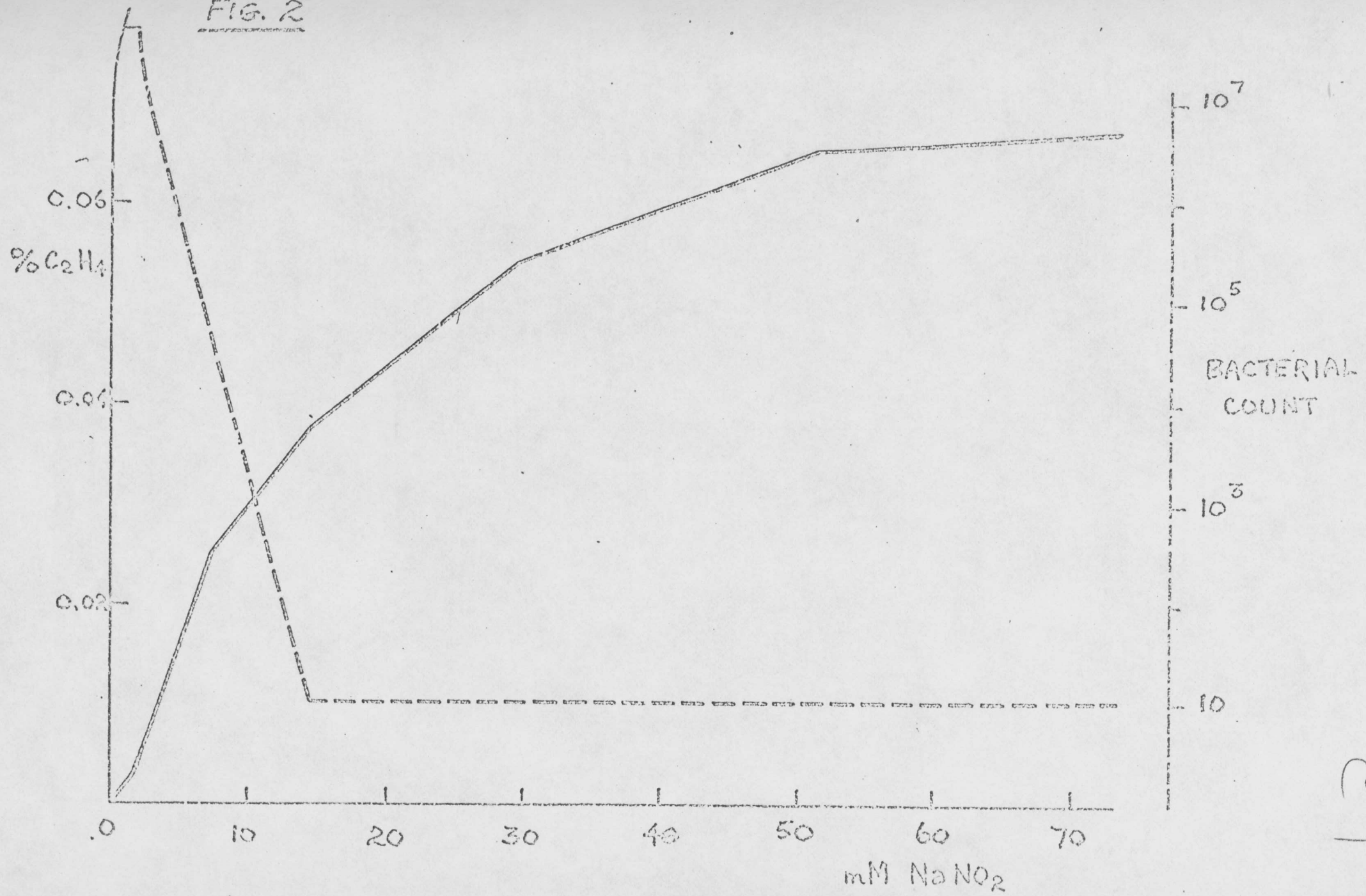


FIG. 2



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