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 (3° - 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 $Na^{14}NO_2$ was replaced by a similar one at $^{m}/_{e}$ 31 with $Na^{15}NO_2$ as would be anticipated for NO. The use of $Na^{15}NO_2$ 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 accelerates[1]. Early studies on the stoichiometric relationship between the reactants indicated the oxidation of one mole of haemoglobin by two moles of nitrite[2], but later results suggested a 1:1 molar ratio. More

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recent studies have reduced still further the nitrite level required to 0.5 - 0.7 moles per mole haemoglobin. The velocity of the conversion of oxy- to methaemoglobin has been directly related to the nitrite concentration; as well as methaemoglobin, some nitrosylhaemoglobin and even nitrosylmethaemoglobin can be formed.

A similar oxidation by nitrite or oxymyoglobin to metmyoglobin occurs readily in solution and can be observed in situ in meat. Nitrosylmyoglobin or nitrosylmetmyoglobin are not, however, detected in the absence of a reducing system. As distinct from haemoglobin, the reaction between deoxygenated myoglobin and nitrite is difficult to study owing to the intrinsic lability of the muscle oxygen storage haem pigment to autoxidation under anaerobic conditions. Attempts to determine the molar ratio of the nitrite utilized to oxymyoglobin oxidised have proved to be abortive. At the low molar levels of nitrite commensurate with those obtained for oxymyoglobin in solution, the uptake of nitrite appeared to occupy a wide range of values but nearly always below that of one mole of nitrite consumed per mole of haem pigment oxidised.

Other haem pigments which do not combine reversibly with oxygen participate in the respiratory chain as intermediates in the transfer of electrons from substrates to oxygen in the living animal. Most of such compounds in skeletal muscle are located within the mitochondrion, to which reference will be made in a further section. An important haem protein in this chain is cytochrome c which exists in ferrous and ferric forms. During the course of extraction of this compound, oxidation almost invariably occurs so that re-reduction is necessary in order to study the oxidising action of nitrite. This can be accomplished chemically by drastic agents such as dithionite or borohydride but the ferrocytochrome c produced is readily susceptible to aerial oxidation in an unphysiological manner. Initial experiments were therefore carried out on ferrocytochrome c produced by the anaerobic action of muscle mitochondria on the ferric form of the haem protein, initiated by a substrate such as pyruvate in a Thunberg tube type spectrophotometer cell. The subsequent addition from the side-arm of nitrite resulted in the gradual oxidation of ferrocytochrome c, the rate of reaction increasing markedly with fall of pH over the range 6.5 - 5.3 at least. The haem product of oxidation provided evidence of the formation of the nitrosyl- derivative of ferricytochrome c by an additional peak in the visible spectrum at 563 mm. Subsequently, however, use has been made of ferrocytochrome c produced from the ferric form through the action of hydrogen and palladium, the product being stable in air. In the absence of muscle tissue or its sub-cellular components, ferrocytochrome c is subject to oxidation by nitrite at pH 6.0 typical of many meat products. The only haem product detected so far is uncomplexed ferricytochrome c. The rate of oxidation of ferrocytochrome c by nitrite is proportional to the nitrite concentration up to at least a level of 10 mm (690 ppm) but is somewhat decreased in the absence of air.

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Of the other cytochromes of the muscle respiratory chain, ferrocytochrome b is also susceptible to oxidation by nitrite, although its action has not been studied so extensively. The terminal cytochrome a_3 of the chain, which combines directly with molecular oxygen during the course of respiration, reacts with nitrite under reducing conditions to form a nitrsoyl- complex of its ferrous form[3]. This complex is less unstable in air than uncomplexed ferrocytochrome a_3, but oxidation does proceed, probably as a result of dissociation of the nitrosyl moiety. The formation of this complex probably accounts for the competitive inhibition by nitrite of aerobic cytochrome oxidase action, to which reference will be made later.

Various other diverse cellular components have been reported to be oxidised by nitrite although mainly at low pH values not normally encountered in meat products. For instance, at pH values up to 4.0, nitrite has been reported to oxidise reduced nicotinamideadenine dinucleotide (NADH), its concurrent products of reduction being determined ad NO, N_2O and N_2 within the mass spectrometer. In equimolocular proportions, however, no reaction between nitrite and NADH was observed at pH 6.0. Sulphydryl groups are stated to reduce nitrite at acid pH; reduced glutathione (GSH) for instance, at pH 3.0 yields hydroxylamine and ammonia[5]. No gas fraction was, however, produced at 37°C from nitrite and GSH at pH 6.0. The role of muscle sulphydryl groups in the reduction of nitrite and the formation of nitrosylmyoglobin under conditions relevant to heated cured meat products has been studied extensively by Möhler and co-workers in Munich[6].

The reduction of nitrite by ascorbate at pH 3-5 to NO, N_2O was virtually eliminated at pH 6, according to Evans and McAuliffe[4], using an ascorbate:nitrite molar ratio of 100:1. Nevertheless, the anaerobic incubation of 8.6 mM sodium ascorbate with 62 mM nitrite at pH 6.0 was found manometrically to produce an appreciable NO fraction.

Nitrite as a nitrosating agent

The differentiation of primary, secondary and tertiary amines on the basis of their actions with nitrous acid has been accepted by many generations of students of organic chemistry. However, even under favourable conditions, the nitrosation of some secondary amines is very slow and the lack of reaction of tertiary amines and nitrous acid is considered to be "the most persistent myth in organic chemistry"[7].

Unlike the Van Slyke deamination of primary amines, the nitrosation of secondary amines is considered to be a reversible reaction. For any one amine, the rate of forward reaction is proportional to the square of the nitrite concentration[8], the optimum pH range of 2-3[9] being a compromise between the requirements of the amine in an uncharged state and that of the nitrosating species as NO^+. Towards neutrality, the rate of nitrosation falls off rapidly and approaches zero asymptatically. However, the main determining factor is the basicity of the amine involved. At the optimal pH, the nitrosation of a basic amine such as

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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 pH[10]. Chemically they show but little reactivity. Physiologically, the lower members of the dialkyl and heterocyclic types are generally hepatotoxic when administered at moderate dosage[11]; 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 monkeys[14]. 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 Mirvish[15], 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-[illegible]-indoleacrylic acid on nitrosation[16]. Creatinine can react with nitrous acid[17] 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 sulphydryl 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 3-16[18]. 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 asymetrically 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 itself[19]. 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 methanol[20]. 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 extraction[21]. No such similar universal procedure exists as yet for non-volatile nitrosamines but the recent development of a method[22] 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-nitramides[23]. 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 fraction[24]. The output of this fraction was not stimulated by the deliverate 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 highbackground absorption due to inert material.

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Under aerobic conditions, nitrite inhibits competitively the action of mitochondrial cytochrome oxidase (EC 1.9.3.1) in oxidising ferro- to ferricytochrome c. The inhibition probably results from the reversible formation of a complex with the oxidase, nitrosylferrocytochrome a_3. Kinetic studies indicated a dissociation constant for the enzyme-nitrite complex of 7.0 mM as compared with a value of 40 μM for the complexing of cytochrome a_3 with oxygen, so that the physiological pathway is followed preferentially to the diversion introduced by nitrite. Nitrite has also a weak uncoupling action of the phosphorylation accompanying electron transfer in respiration but its action is not comparable with that of an uncoupling agent such as 2,4-dinitrophenol[25]. Thus, as the nitrite concentration is increased over the range of approximately 0-10 mM (0-690 ppm) the overall rate of aerobic oxidation of ferrocytochrome c in the presence of mitochondria is decreased as a result of the inhibition by nitrite of cytochrome oxidase action. Above a nitrite concentration of 20 mM or thereabouts, the oxidation of the cytochrome is resuscitated at a pH dependent rate as a result of the direct action of nitrite upon ferrocytochrome c, to which reference has been made in the section dealing with its action as an oxidising agent. A similar effect is obtained under the nominally anaerobic reducing conditions which are probably representative of the curing conditions in a block of meat, except that the original ferrocytochrome c oxidation observed at low oxygen tensions in the absence of nitrite is inhibited maximally by nitrite around a concentration of 3 mM and close to the 200 ppm level, which often constitutes the legal limit within cured meat products.

Under anaerobic conditions, however, nitrite can act as an inefficient electron acceptor to the terminal portion of the mammalian respiratory chain with the oxidation of ferro- to ferricytochrome c[25]. As a result, nitrite is concurrently reduced to nitric oxide, part of which at least is located in combination with the oxidised form of the cytochrome. The anaerobic oxidation of ferrocytochrome c by nitrite is markedly less rapid than that brought about through the admission of air in the absence of nitrite as is illustrated by Fig 3, in which spectral observations were made every 110 seconds. The final spectrum in the presence of nitrite will be seen to have an additional peak at 563 nm in comparison with that resulting from the admission of air. The identity of nitrosylferricytochrome c as a product was confirmed by difference spectra in the Soret region of incubations with nitrite with reference to those in its absence. The resultant peak at 417 nm can be ascribed to either nitrosylferricytochrome c or ferrocytochrome c with reference to ferricytochrome c, but the latter possibility is ruled out by the spectrum in the visible region. Furthermore, the peak at 417 nm is relatively stable in air which would not be true of ferrocytochrome c in the presence of muscle mitochondria.

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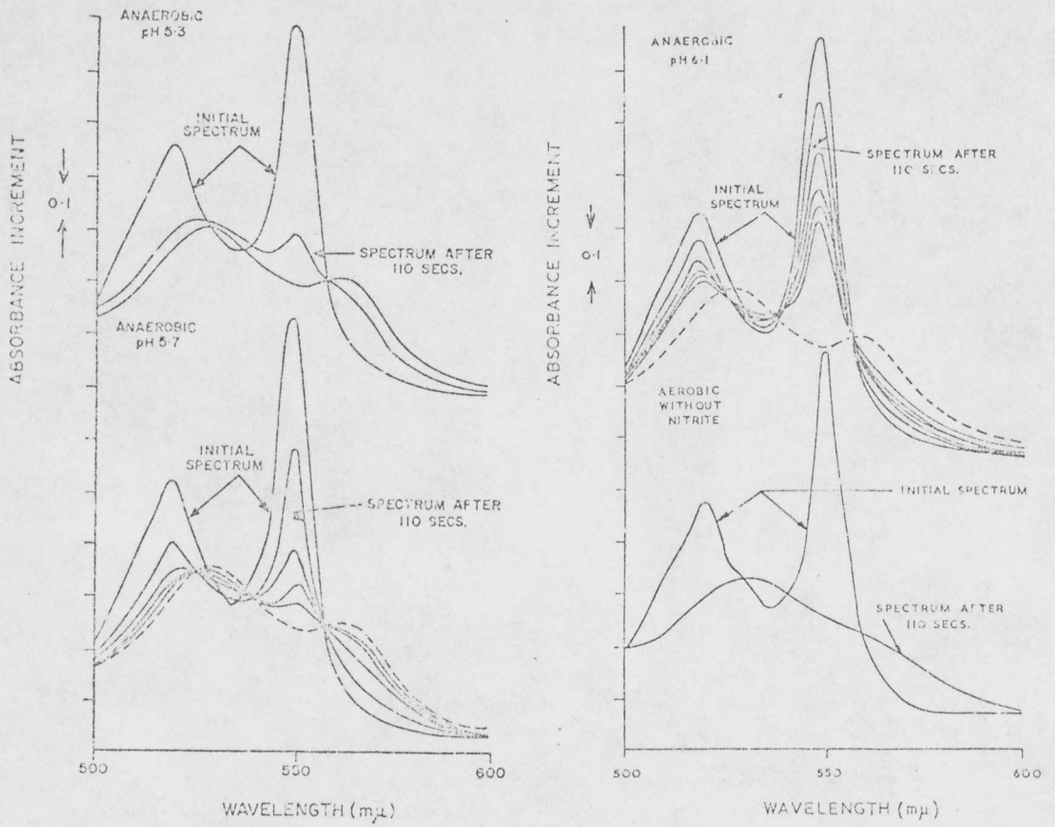
FIG. 3


ANAEROBIC
pH 5·3
INITIAL
SPECTRUM
SPECTRUM AFTER
110 SECS.
ANAEROBIC
pH 5·7
INITIAL
SPECTRUM
SPECTRUM AFTER
110 SECS.
ABSORBANCE INCREMENT
0·1
500
550
600
WAVELENGTH (mμ)
ANAEROBIC
pH 6·1
SPECTRUM AFTER
110 SECS.
INITIAL
SPECTRUM
AEROBIC
WITHOUT
NITRITE
INITIAL SPECTRUM
SPECTRUM AFTER
110 SECS.
ABSORBANCE INCREMENT
0·1
500
550
600
WAVELENGTH (mμ)

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 nitrosyl-metmyoglobin, which is subsequently reduced to nitrosylmyoglobin by continued anaerobic mitochondrial action[26]. 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 nitrosyl-metmyoglobin production followed by a fall as the ensuring 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 a_3 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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References

1 K. W. Keshane & W. K. Metcalf, Physics in Medicine & Biology 5:27:1960.

2 O. Bodansky, Pharmacol. Rev. 3:144:1951.

3 J. P. Colpa-Boonstra & K. Minnaert, Biochim. Biophys. Acta 33:527:1959

4 H. J. Evans & C. McAuliffe, McCallum-Pratt Inst. Contrib., Johns Hopkins Univ. 125:189:1956.

5 M. Lemoigne, P. Monguillon & R. Desveaux, Compt. Rend. 206:947:1938.

6 K. Möhler, Z. Lebensmittel-Unters. Forsch. 142:169:1970.

7 P. A. S. Smith & R. N. Loeppky, J. Amer. Chem. Soc. 89:1147:1967.

8 S. S. Mirvish, J. Natl. Cancer Inst. 44:638:1970.

9 J. Sander, Hoppe-Seyl Z. physiol. Chem. 348:852:1967.

10 G. Eisenbrandt, A. von Hodenberg & R. Preussmann, Z. Anal. Chem. 251:22:1970

11 H. Druckrey, R. Preussmann, S. Ivankovic & D. Schmahl, Z. Krebsforschung 69:103:1967.

12 C. M. Goodall, W. Lijinsky, L. Tomatis & C. E. M. Wenyou, Toxicol. & Applied Pharmacol. 17:426:1970.

13 J. H. Weisburger, E. K. Weisberger, N. Mantel, Z. Hadidian & F. Friedrickson, Naturwiss. 53:508:1966.

14 D. Schmähl & H. Osswald, Experientia 23:497:1967.

15 S. S. Mirvish, J. Natl. Cancer Inst. in press

16 N. J. Demjanov & N. I. Putochin, Dokl. Akad. Nauk. U.R.S.S. 2:390:1935.

17 I. Greenweld & I. Levy, J. Org. Chem. 13:554:1948.

18. G. E. Hein, J. Chem. Education 40:181:1963.

19 C.L.Walters, Laboratory Practice, in press.

20 C. L. Walters, E. M. Johnson & N. Ray, Analyst 95:485:1970.

21 G. M. Telling, T. A. Bryce & J. Althorpe, J. Agric. Food Chem, in press.

22 G. Eisenbrandt & R. Preussmann, Arzneimittel Forsch. 20:1513:1970

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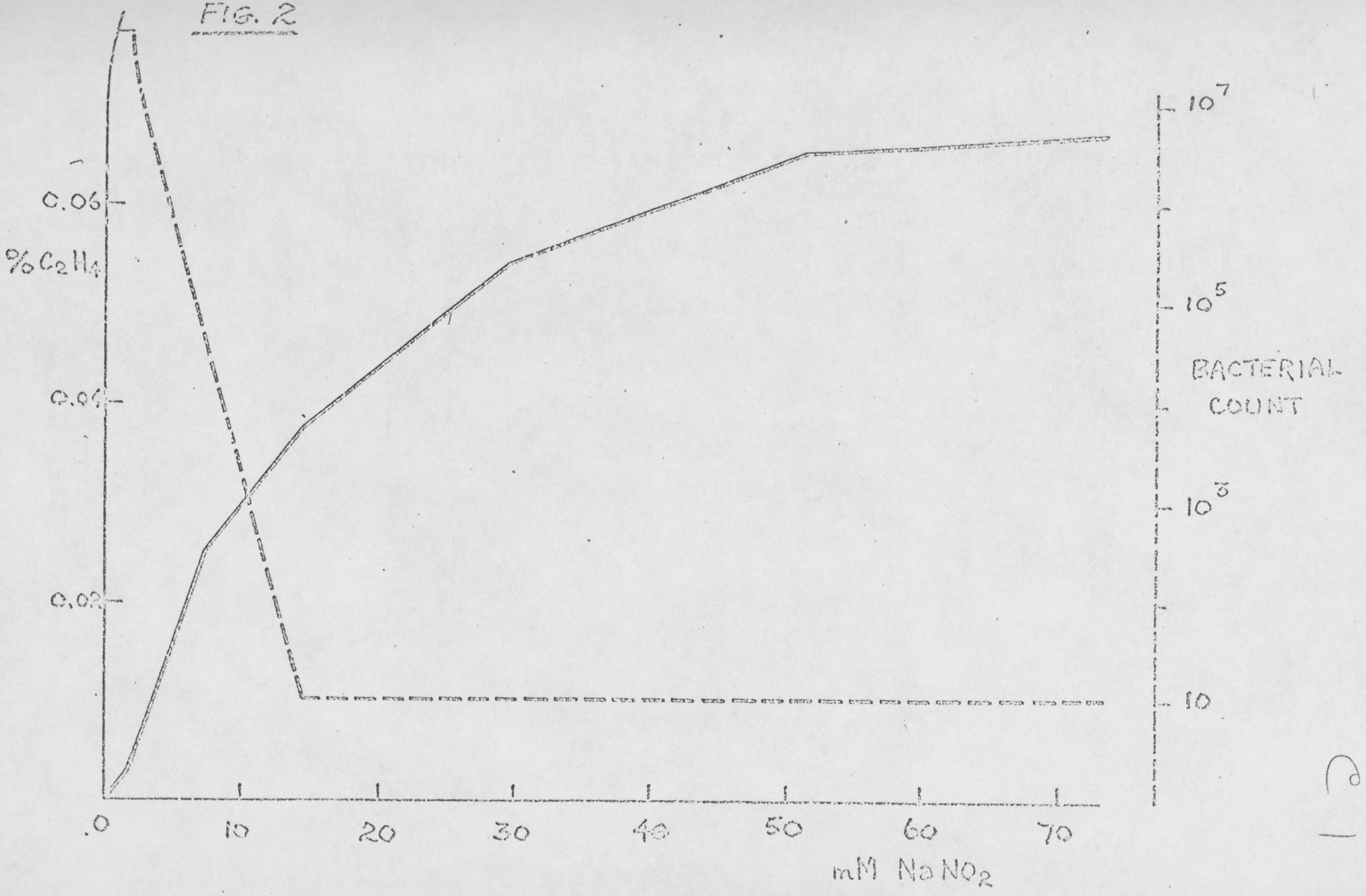
FIG. 1

INTENSITY
C_2H_4
$^{14}N_2$
^{14}NO
^{15}NO
O_2
A+
CO_2
$^{14}N_2O$
$^{15}N_2O$
28
32
36
40
44
m/e

FIG. 2


0.06
% C_2H_4
0.04
0.02
0
10
20
30
40
50
60
70
mM $NaNO_2$
10^7
10^5
10^3
10
BACTERIAL COUNT

23 E. M. Johnson & C. L. Walters, Analytical Letters, in press

24 C. L. Walters & A. McM. Taylor, Biochim. Biophys. Acta. 86:448:1964.

25 C. L. Walters & A. McM. Taylor, Biochim. Biophys. Acta. 96:522:1965.

26 C. L. Walters, R. J. Casselden & A. McM. Taylor, Biochim. Biophys. Acta. 143:310:1967.

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