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DEGRADATION AND BIOLOGICAL ACTIVITY OF DAZOMET

A Thesis
Submitted in partial fulfilment of the requirements
for the award of the Degree of
DOCTOR OF PHILOSOPHY

by

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March 1982

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To: Sai Baba
My parents
My wife

ORIGINALITY

All the work presented in this thesis has been carried out by the author except where acknowledged and has not previously been presented for a degree at this University or any other institution.

ABBREVIATIONS

ca	about
CR	correlation coefficient
D	all other products not quantified
Dazomet	3,5-dimethyltetrahydro-2H-1,3,5-thiadiazine-2-thione
DMTC	N,N-dimethyldithiocarbamate
DMTD	N,N'-dimethylthiuram disulphide
DMTU	N,N'-dimethylthiourea
HPLC	high performance liquid chromatography
K'	capacity factor
MDC	N-methyldithiocarbamate
MIT	N-methylisothiocyanate
Na-MDC	sodium salt of MDC
SD	standard deviation
TMTD	tetramethylthiuram disulphide

Other abbreviations were defined as used in the text

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1. INTRODUCTION

1.1 General Introduction and Applications of Dazomet

Dazomet belongs to a group of compounds classed as 3,5 disubstituted derivatives of 1,3,5 thiadiazine-2-thiones, also referred to as 'carbothialdines' and dithiocarbamate fungicides. They were first synthesised by Delepine in 1897, and later by Bodendorf in 1930. The structure of these compounds (Ainley et al, 1944) is:

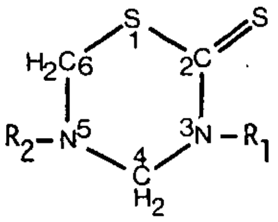


FIGURE 1.1 Structure of 3,5 disubstituted tetrahydro-2H-1,3,5-thiadiazine-2-thiones

Dazomet: $R_1=R_2=CH_3$ (3,5 dimethyltetrahydro-2H-1,3,5-thiadiazine-2-thione)

Other trade names: Basamid, Mylone, DMTT, Crag Mylone.

Other chemical and test names: dimethylformacarbothialdine, N521, crag fungicide 974.

They are also referred to as 'synthetic mustard-oil splitting substances' on account of their ability to form isothiocyanates in vivo. The choice of substituents at R_1, which is responsible for structure and quality of mustard oil to be split and R_2, which determines aqueous solubility offers a choice of a very wide range of potentially active substances (Haenel, 1967).

Dazomet was first recognised for its insecticidal activity by Davies and Sexton in 1948. They found that 3,5-dimethyl, 3-phenyl-5-hydroxyethyl, 3-phenyl-5-propyl, 3-pheyl-5-methyl, and 3-p-tolyl-5-methyl derivatives of thiadiazine-2-thiones were highly toxic to grasshoppers. Since then dazomet has found a wide spectrum of usage as a nematicide, algicide, fungicide, anti-dermatophyte, bactericide and herbicide (Martin and Worthing, 1976).

The activity of dazomet is thought to result from its volatile degradation products, rather than the intact molecule. Methylisothiocyanate (MIT) is the main product implicated in its toxicity. The other products of interest include formaldehyde, methyldithiocarbamate (MDC) and sulphur. The activity of MDC is thought to be due to further production of MIT. Any discussion on the many uses of dazomet must therefore also include a consideration of the activity of the main degradation products.

MIT and other fungicides that decompose to MIT control root rot of peas (Pisum sativum L.) caused by Aphanomyces euteiches in both greenhouse and field trials. Sodium N-methyldithiocarbamate (metham), dazomet and formulations of dazomet used for plant bed fumigation, all controlled root rot effectively in greenhouses and field at 50-200 ppm (Papavizas and Lewis, 1971a).

Papavizas and Lewis (1971b) also obtained effective control of damping off (caused by Aphanomyces cochlioides) of sugar beet in greenhouses. Dazomet when added to the soil at 50 and 100 ppm, reduced the disease by approximately 50%. The effectiveness of dazomet, metham and MIT at all concentrations was found to increase when applied under a plastic cover.

MIT and the volatile materials released from the decomposition of dazomet and metham in soil reduced the germination ability of endoconidia and chlamydaspores of Thielaviopis basicola which causes root rot of plants of economic importance (Papavizas and Lewis, 1972).

3,5 disubstituted derivatives of thiadiazine-2-thiones have been studied for their antidermatophytic activity. The 3,5-dibenzyl derivative which is available as Afungin and Fungiplex is used for the treatment of dermatomycoses. The 3-benzyl, 5-carboxymethyl derivative named Defungit has been found effective therapeutically (Kristian and Bernat, 1968; and Augustin and Bernat, 1971). They also synthesised several other 3,5 disubstituted derivatives and examined them for their antibacterial, antifungal, antiprotozoal antihelmintic and cytotoxic activity. The antidermatophytic activity of some 3,5-disubstituted derivatives was investigated against several organisms by Manohar, Narashima Murthy et al (1975). They showed that a few of the compounds were comparable to or better than griseofulvin in this respect.

The antitubercular effect of several of these derivatives has been tested against Mycobacterium tuberculosis (Odlevora, Augustin and Nemec, 1971). These studies showed that compounds with a benzylfurylmethyl substituent at position 3 and benzyl or carboxymethyl at position 5 were the most effective. The antitubercular activity was found to be in the range of 1-5 $\mu g.ml^{-1}$ and compared favourably with the activity of well known antitubercular agents under the same conditions.

Tzirkov and Dirimanov (1978) tested dazomet upon pure cultures of soil microorganisms. They found that it was highly toxic and inhibited the growth of Aspergillus flavus, Aspergillus niger and Aspergillus terreus and strains of Azobacter chroococum.

Potato cyst-nematodes (Heterodera rostochiensis and H.pallida) are the most important cyst-nematodes found in British soils. Dazomet, metham and MIT have been shown to give good control of these organisms (Whitehead, 1975).

Plumb, Jenkyn and Broom (1978) investigated the effect of pesticides on perennial ryegrass sward. They found that dazomet did not have consistent effect on the control of root browning. It did, however, increase dry matter yields in nitrogen deficient soils.

MIT and formaldehyde which are implicated in the activity of dazomet are used individually as vapour fumigants. They are general sterilising agents that kill fungi, bacteria and nematodes in the soil, and have been commonly used for treating bulk soil for plotting and bed use. A covering is normally held above the soil surface to allow circulation of the vapours. (Martin and Worthing, 1976).

The other possible decomposition product sulphur has been long in use as a fungicide and acaricide. It has been used in the field for application as a dust to foliage (Johnson, Krog and Poland, 1963).

Other compounds in the class of dithiocarbamates have been in use mainly as foliage fungicides. The different dithiocarbamates and their uses have been described by Martin and Worthing (1976). Some of the common dithiocarbamate fungicides, their structure, uses and types of formulations are listed in Table 1.1.

TABLE 1.1: Main Uses, Persistence and Typical Formulations of Some Commonly Used Dithiocarbamates

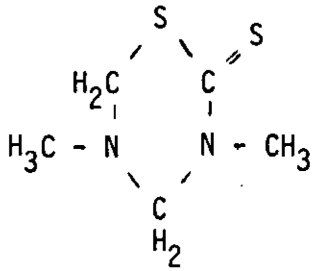
COMMON NAME:CHEMICAL NAME STRUCTURE	MAIN USES	PERSISTENCE	TYPICAL FORMULATIONS
Dazomet: tetrahydro-3,5-dimethyl-2H-1,3,5-thiadiazine-2-thione 	Soil fungicide and nematicide	Brief	98% w/w Prill
Mancozeb: complex of zinc ion and maneb	Non-systemic fungicide; potato blight	Long	80% w/w w.p; also with zineb
Maneb: manganese ethylenebisthithiocarbamate $[\text{-SCS.NH.CH}_2\text{CH}_2\text{CS.S Mn-}]_X$	Non-systemic fungicide	Long	80% w/w w.p; also with zinc oxide, zineb or other metals
Metham-sodium: sodium methyldithiocarbamate $CH_3NH.CS.SNa$	Soil fungicide and nematicide	Brief	33% w/v solution
Nabam: disodium ethylenebisdithiocarbamate $CH_2NH.CS.SNa$ $CN_2NH.CS.SNa$	Non-systemic fungicide	-	22% w/v solution, used with zinc sulphate

TABLE 1.1 Continued

Propineb: zinc propylenebisthiocarbamate $[-SCS.NH.CH_2CH(CH_3)NH.CS.SZn-]_X$	Non-systemic fungicide; downy mildews and potato blight	Moderate	70% w/w w.p.
Thiram: tetramethylthiuram disulphide $(CH_3)_2N.CS.S$ $\quad\quad\quad\quad\quad\ \ \vert$ $(CH_3)_2N.CS.S$	Non-systemic fungicide; foliage and seed use	Moderate	80% w/w w.p.
Zineb: zinc ethylenebisthiocarbamate $[-SCS.NH.CH_2CH_2NH.CS.SZn-]_X$	Non-systemic fungicide; foliage use	Moderate	70% w/w w.p.

1.2 Methods of Analysis

The various methods described for the analysis of dithiocarbamates may be divided generally as follows:

1.2.1 Direct spectrophotometric methods: ultraviolet; visible spectra-based on compleximetric reactions; infrared; nuclear magnetic resonance and mass spectra.

1.2.2 Determinations based on decomposition products: Analysis of e.g. carbon disulphide, amine or isothiocyanate.

1.2.3 Methods based on titration of dithiocarbamates with acids, bases, or heavy metals.

1.2.4 Methods based on chromatographic techniques: paper, thin layer, column and high performance liquid chromatography (HPLC).

1.2.5 Other methods: e.g. polarographic, oxidation, reduction, and fluorimetric reactions.

1.2.1 Direct spectrophotometric methods

Direct spectrophotometry of dithiocarbamates can often be used to advantage as long as there is no interference absorption from other components of the material to be analysed.

The ultraviolet absorption spectra of various dithiocarbamic acids and salts together with thiuram disulphides have been summarised by Raizman and Thompson (1972). Dithiocarbamic acids and salts in general show two fairly strong ultraviolet absorption bands in the 250 and 290 nm regions. The thiuram disulphides also show these two bands but at much lower intensities, in addition to a characteristic intense absorption in the 220 nm region.

The dithiocarbamate esters or dithiourethanes (which includes the thiadiazine-2-thione type of compounds) show the same absorption bands as the thiurams but the longer wavelength bands are much stronger (Raizman and Thompson, 1972).

Many of the heavy-metal salts of dithiocarbamates absorb in the visible regions. Cupric dimethyldithiocarbamate, for example, has an additional absorption band at 425 nm (Keppel and Munsey, 1957). The ultraviolet spectra of several salts of diethyldithiocarbamic acid in chloroform have been measured by Chitton (1953) and several methods have been described for the analysis of dithiocarbamates and thiurams involving reaction with copper or other metal salts (Morrison and Shephard, 1946; Kres, 1951; Kerssen and Riepman, 1959.

The reactions of several 3,5-disubstituted tetrahydro-1,3,5-thiadiazine-2-thiones with copper salts has been studied. Grahl and Dedek (1967) found that the 3,5-dibenzyl derivative turns yellow when shaken with an aqueous solution of copper sulphate. However, the absorption of the resulting complex at 440 nm is very unstable. Better results were obtained with chloroform soluble copper salts such as copper stearate.

Other direct spectroscopic methods such as infrared, nuclear magnetic resonance, and mass spectra have not been used as extensively as ultraviolet spectroscopy in the analysis of dithiocarbamates. Infrared spectra of dithiocarbamates have been investigated by several workers (Chatt, Duncanson et al, 1956; Fisher and Uhlich, 1960; Nakamoto, Fujita et al, 1963). The application of infrared technique for determining ferbam, zineb and tetramethylthiuram disulphide in a mixture of other types of pesticides was described by Fisher and Uhlich (1960). Nuclear magnetic resonance spectra of carbamate pesticides including dazomet (Keith and Alford, 1970) and mass spectra of several dithiocarbamate esters (Duffield,Djerassi et al, 1967) have been reported.

1.2.2 Determinations based on decomposition products

The methods for direct estimation of dithiocarbamates described previously (Section 1.2.1) are very useful for pure samples. However, interference in the analysis occurs when trace amounts of other dithiocarbamates, or metal ions which lead to the formation of metal complexes are present as impurities. Hence determinations based on decomposition products are most widely used.

Acid hydrolysis leading to the formation of the parent amine and the evolution of carbon disulphide is the key reaction used in the analysis of both dithiocarbamate salts and thiuram disulphides. Either carbon disulphide or the corresponding amine is determined. Usually carbon disulphide is selected and determined iodimetrically, colorimetrically or by gas chromatography. The reactions which lead to the formation of these compounds have been summarised by Thorn and Ludwig (1962).

The most common technique, and that most suitable for macro determinations, is the so-called xanthate method in which the carbon disulphide is collected in alcoholic potassium hydroxide and determined iodimetrically.

Raizman and Thompson (1972) described a generalised procedure for most commercial dithiocarbamate salts (including manganese and zinc ethylenebisdithiocarbamates - maneb and zineb) based on various methods recommended in the literature. The apparatus used consists of a digestion flask and a reflux condenser which is then connected in series to one or two hydrogen sulphide scavenge-traps followed by a methanolic potassium hydroxide scrubber for collecting carbon disulphide. Air is aspirated through the system to pass the gaseous products through the scrubber train. The amount of carbon disulphide produced is determined by titrating with standardised iodine solution using phenolphthalein as indicator.

The evolved carbon disulphide can be measured spectrophotometrically. The most commonly used method is based on the absorption of the carbon disulphide in ethanolic solutions of copper acetate containing triethanolamine and diethylamine (Idelson, 1960). This solution is known as Viles reagent (Viles, 1951). The wavelength used for measuring the resultant dithiocarbamate was not generally agreed upon until Cullen (1964) investigated this matter. He found that both 1:1 and 1:2 copper/dithiocarbamate complexes were formed in the medium. The former has a maxima at 380 nm, and the latter at 435 nm. Due to high base line at 380 nm he suggested use of 435 nm by limiting the amount of copper ion to promote a 1:2 complex formation.

Munnecke, Domsch and Eckert (1962) studied the fungitoxicity of air passed through columns of soil treated with mylone and other dithiocarbamates (captan, nabam, thiram, terraclor and zineb). The gases released when some of the dithiocarbamates degrade included hydrogen sulphide and carbon disulphide. Carbon disulphide was trapped in Viles reagent and determined spectrophotometrically.

The carbon disulphide and hydrogen sulphide evolved after acid digestion may be determined by gas chromatography (Bighi, 1964; Bighi and Saglietto, 1966).

After acid decomposition of a dithiocarbamate salt, besides the liberation of carbon disulphide, a corresponding amine is also produced, which remains in the aqueous phase as a nonvolatile amine salt. This mixture may be made alkaline and the volatile amine distilled and determined by acid consumption (Bighi and Penzo, 1963). The amine may be assessed colorimetrically by reacting with carbon disulphide and copper (11) ion to form the copper dithiocarbamate (Henermann, 1957). The amine salt could be directly determined by X-ray diffraction methods (Brock and Louth, 1955), or by paper chromatography (Zijp, 1956).

Methylisothiocyanate is another volatile product formed when dithiocarbamates degrade. Munnecke, Domsch and Eckert (1962), Munnecke and Martin (1964) and Munnecke, Martin and Moore (1967) investigated the release of methylisothiocyanate from several dithiocarbamate fungicides. Methylisothiocyanate was trapped in ethanol and determined by measuring the ultraviolet absorption at 243 nm.

1.2.3 Methods based on titration of dithiocarbamates with acids, bases or heavy metals

Two moles of acid are consumed when a dithiocarbamate is decomposed with acid resulting in the amine salt:

$$R_2NCSS\,Na + HX \rightarrow R_2NCSSH + NaX$$
$$R_2NCSSH \rightarrow R_2NH + CS_2$$
$$R_2NH + HX \rightarrow R_2NH.HX$$

The dithiocarbamate can be decomposed with dilute sulphuric acid, and the excess acid can be back-titrated with base, using phenolpthalein indicator (Shankaranarayana and Patel, 1961). Hulanicki and Shiskova (1965) affected the decomposition of water soluble dithiocarbamates with perchloric acid. Back-titration was followed potentiometrically.

Chemical methods for determining dithiocarbamate esters are not highly developed. A procedure is recommended, however, for the analysis of the cyclic dithiocarbamate ester dazomet (Stansbury, 1964). It is based on the same method described above for dithiocarbamate salts, but the decomposition products formed in a measured excess of hydrochloric acid includes methylamine hydrochloride, carbon disulphide, and formaldehyde. The amount of hydrochloric acid consumed, which is determined by titrating the excess with standard sodium hydroxide in presence of methyl red-boromocresol green indicator, is a measure of the dazomet originally present.

Titration of dithiocarbamic salts and water soluble dithiocarbamates with heavy-metal ions forms basis of very useful titrimetric methods. Bicovsky and Bicovska (1959) described a method in which soluble alkali-metal dithiocarbamates, and xanthates, are converted to the corresponding chloroform soluble nickel chelate. The chloroform soluble material is then titrated with mercury (11) ions, using diphenyl carbazone as indicator.

Carbon disulphide can be determined by converting it to dithiocarbamic acid which is then titrated with mercury (11) ions in pyridine using copper (11)-EDTA complex as an indicator (Sedivec and Flek, 1959).

Silver ions form an insoluble complex with thiuram disulphides, and this has been used as a basis for quantitative determination. Li and Li (1962) described a procedure in which tetramethylthiuram disulphide was reacted in dioxane solution with excess silver nitrate solution. The resultant precipitate was filtered and the excess silver ions in the filterate back-titrated with thiocyanate solution.

1.2.4 Methods based on chromatographic techniques

Various chromatographic techniques have been described for separating and identifying dithiocarbamates.

The most commonly used techniques have been paper chromatography. Several derivatives of dithiocarbamic acid salts, esters and thiuram disulphides have been separated. However, no single method is generally applicable for the analysis of all dithiocarbamates.

The α-amino acid salts of dithiocarbamic acids have been separated using ammonium hydroxide-propanol solvent systems (Jensovsky, 1955; Zahradnik and Kobrle, 1959). The compounds were detected using ammoniacal silver nitrate. McKinley and Magarvey (1960) studied the separation of some dithiocarbamate salts derived from dimethylamine (e.g. ferbam, thiram and ziram) and ethylenediamine (e.g. maneb, nabam and zineb). Satisfactory separation of dimethylamine derivatives was achieved on a fibreglass paper impregnated with formamide using chloroform, petroleum ether, and a mixture of n-hexane and chloroform as mobile phase. However, the ethylenebisdiamine dithiocarbamates remained relatively immobile. But good separation of nabam and its products of oxidation can be obtained by using butanol, ethanol and water as developing solvent (Ludwig and Thorn, 1960). Iodine-azide reagent was used for detecting the compounds.

A method applicable for the separation of simple dithiocarbamates and thiuram disulphides was described by Lu and P'o (1963). They used various organic solvents and water as mobile phases and ace~~no~~tonitrile-butadiene copolymer in benzene-acetone solution as stationary phase; acidified sulphate was used for detecting the compounds. Separation of disulfiram (tetraethylthiuram disulphide) using aqueous methanol was studied by Gockeritz (1966). However, he found that the compound decomposed on paper.

Gockeritz (1966) also studied the paper chromatography of 3,5-disubstituted thiadiazines. He used heptane and mixtures containing other organic solvents as mobile phases to separate Afungin (3,5-dibenzyl), Ujothium (3-benzyl, 5-carboxymethyl) and other pharmaceutical preparations containing thiadiazines. The dithiourea and ethanolamine impurities in the preparations were also separated. The compounds were detected mainly with iodine-azide reagent.

A quantitative photometric procedure applicable in principle to all thiadiazine from which involatile amines or amino acids could be split was described by Schmandke (1965). The chromatograms were developed in ligroine-methanol-water and treated with acetic acid, ninhydrin and acidified copper nitrate. The resultant red complex was determined colorimetrically. Acid decomposition of dithiocarbamates followed by subsequent determination of the amine salts by paper chromatography has also been used to identify dithiocarbamate rubber accelerators and antioxidants (Zijp, 1956).

Thin layer chromatography using silica gel has been used to separate manganese and zinc ethylenebisdithiocarbamates (maneb, zineb) and their degradation products (Fishbein and Fawkes, 1965). The compounds were detected by either dithizone or potassium ferricyanide ferric chloride.

Column chromatography on alumina was used by Bellamy et al (1947), for separating zinc dimethyldithiocarbamate and tetramethylthiuram disulphide from other chemical impurities in rubber. Similarly Parker and Berriman (1952) separated several zinc dialkyldithiocarbamate and thiuram disulphides on silica gelcelite columns. They investigated several developing solvents and reagents for separating and detecting the dithiocarbamates. Metal chelates of dialkyldithiocarbamates have been separated by column chromatography using activated alumina (Al-Mahdi and Wilson, 1951) or silica (Smith and Hayes, 1959).

High Pressure Liquid Chromatography (HPLC) has been introduced only relatively recently for the analysis of dithiocarbamates.

With the advent of low dead volume instruments, highly sensitive (although non-specific) detectors, and extremely efficient support materials, HPLC has become a very useful tool for the analysis of various chemicals of agricultural and medical importance. The advantages of HPLC were summarised by Horgan (1974):

i) analysis of compounds that are thermally unstable;
ii) analysis of compounds with low volatility;
iii) minimal clean-up of samples;
iv) non-destructive detectors;
v) easy sample scale-up for preparative purposes, and
vi) high resolution due to the participation of both the stationary and the mobile phase.

The individual components of the system as well as the various materials and the separating mechanism have been described by Done (1974) and Bristow (1976).

The ability of dithiocarbamates to form complexes with heavy metals was described previously. This property has been widely used for the analysis of metal-ions by HPLC but not of the ligand.

Separation and identification of nickel (Liske, Guiochon and Colin, 1979) and other metal ions such as cobalt, copper, zinc and lead bisalkyldithiocarbamate complexes (Lehotay, Liska et al 1979) has been studied on silica gel columns. Good separation was obtained of individual metal-dithiocarbamate complexes, but when different metals or dithiocarbamates are present, exchange reactions may occur between the metals and the dithiocarbamates to give mixed ligand complexes. A number of metal complexes of diethyl, benzylmethyl and diethoxyethyl-dithiocarbamate have also been separated on silica gel columns (Heizmann and Ballschmiter, 1977; Moriyasu and Hashimoto, 1978).

Several metals and mixtures of metals have been separated on bonded nitrile column using diethyldithiocarbamate as a ligand (Gaetani, Laureri and Mangia, 1979). The same system has been used for determining levels of platinum excreted in urine after administration of anti-tumour agent (Bannister, Sternson and Repta, 1979). However, repeated use of the column le~~a~~d to loss of data reproducibility.

Schwedt (1977, 1978, 1979) studied reverse phase chromatography of a number of metal-ion complexes of diethyl and tetramethylenedithiocarbamates. He successfully chromatographed complexes of cobalt, copper, nickel, mercury, and lead, although the latter was found to be unstable.

Direct HPLC of dithiocarbamates has not been studied as widely as the heavy metal-dithiocarbamate complexes.

Thruston et al (1972) investigated HPLC of several carbamate pesticides on both reverse phase and silica columns. They successfully separated dazomet and other carbamate pesticides on a silica column using isopropanol-hexane as mobile phase.

Several reviews have been written on the analysis of pesticides including carbamates by HPLC (Ishii, 1974; Moye, 1975; Lawrence and Turton, 1978).

1.2.5 Other methods

The polarographic behaviour of dithiocarbamate salts has been fairly extensively investigated (Fujinaga and Yamashita, 1964; Zuman et al, 1953; Pasciak, 1963). Zuman et al (1953) determined the carbon disulphide evolved from decomposition of dithiocarbamates by treating it with ethanolic solution of diethylamine to form the corresponding dithiocarbamate. Polarographic methods for the determination of several dithiocarbamate pesticides (e.g. ziram, zineb, ferbam) have been investigated by Nangniot (1960). The behaviour of some heavy metal dithiocarbamates was studied by Fujinaga and Yamashita (1964). The oxidation-reduction potential of tetraethylthiuram disulphide and diethyldithiocarbamates has been investigated by Gregg and Tyler (1950).

A nonspecific approach based on the use of oxidising agents to convert the dithiocarbamate sulphur into sulphate ion, which can then be determined gravimetrically, as barium sulphate, is often used for the determination of dithiocarbamates. Various oxidising agents have been investigated (Wojahn, 1952). As in the case of other compounds containing the $-CSS^-$ function the dithiocarbamate salts could be determined by titrating with iodine to a normal starch end point (Sankranarayana and Patel, 1961).

The ability of thiuram disulphides to be reduced back to the corresponding dithiocarbamates forms another basis of analysis. The dithiocarbamate formed could be determined by methods already described previously. An alternative method based on measuring the stoichiometry of the reaction step has also been studied. Reaction of the disulphide with cuprous iodide and the determination of the resulting cupric diethyldithiocarbamate (Domer, Fredga and Linderholm, 1949) is the method most widely used. Conductometric titration of tetramethylthiuram disulphide with standardised copper (11) sulphate solution and using hydroquinone as reducing agent can be carried out to measure the reduction step itself (Scheele and Gensch, 1953). Lowen (1961) described a method in which a thiuram disulphide is reduced with standard sodium sulphide and the end point is determined electrometrically, using platinum-calomel electrode.

Very few pesticides fluoresce sufficiently to enable their direct determination. Usually, a derivative is prepared either by hydrolysis, oxidation, chelation, or reaction with a derivatising reagent to yield a highly fluorescent product (Argauer, 1977). Nitrogen containing pesticides, such as carbamates, ureas and triazines, can be converted to amines and phenols. Both amines and phenols either fluoresce strongly themselves or readily form highly fluorescent derivatives after hydrolysis or after hydrolysis and reaction with reagents such as d~~e~~ansyl chloride, fluorescamine, NBD chloride (4-chloro-7-nitrobenzo-2,1,3-oxidiazole) and o-phthaldehyde. The application of fluorescent derivatising reagents for the preparation of fluorescent probes and fluorescent tags for amines, amino acids, phenols, and proteins has been reviewed by White and Argauer (1970).

1.3 Degradation Studies

1.3.1 dazomet

The instability of dazomet in aqueous solutions was described as early as 1893 by Mulder. He found that by the addition of heavy metal salts, the corresponding metal dithiocarbamates, e.g. $[H_2N.C(:S)S]_2$ Pb were formed. Levi and Gimignani (1929) later studied the reaction of dazomet in aqueous solutions containing silver ions and reported the formation of both dimethyl dithiocarbamate and methylene dithiocarbamate silver complexes.

The reactions of dazomet in aqueous solutions were not further reported until 1956, when van der Kerk studied the fungicidal activity of dazomet and sodium N-methyldithiocarbamate. He concluded (without furnishing chemical evidence) that dazomet is hydrolysed in aqueous solutions to form formaldehyde and an ester of N-methyl dithiocarbamate which is then further decomposed to form methylisothiocyanate. Decomposition to methylisothiocyanate was also thought to occur with sodium N-methyldithiocarbamate (Figure 1.2).

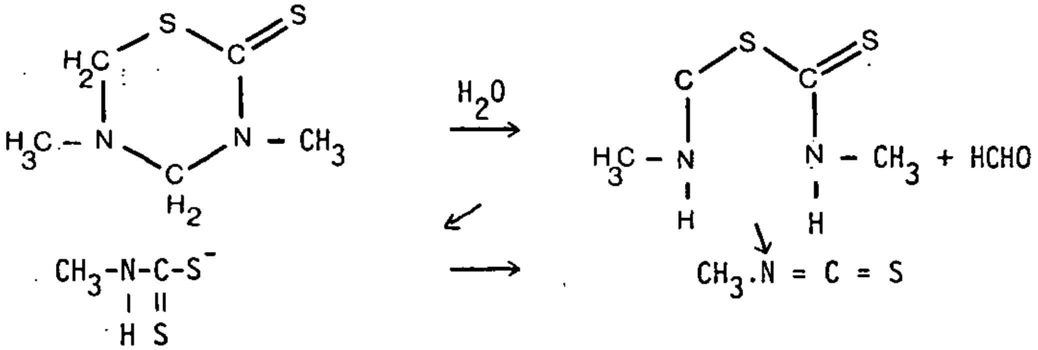


FIGURE 1.2 Degradation of dazomet and N-methyldithiocarbamate to methylisothiocyanate (van der Kerk, 1956)

Goksoyr (1964) carried out spectrophotometric studies to check van der Kerk's suggestion. He compared the ultraviolet spectra of dazomet in carbon tetrachloride and water, and suggested that in water the dazomet ring splits to give a lower absorbance ultraviolet spectra characteristic of a dithiocarbamate derivative. However, the dithiocarbamate was not positively identified.

Goksoyr also investigated the formation of dimethyl dithiocarbamate and methylene dithiocarbamate reported by Levi and Gimignani (1929). He found that dazomet formed a yellow precipitate with cupric sulphate which was not extractable with carbon tetrachloride, indicating that dimethyl dithiocarbamate was not present. Small quantity of cupric dimethyl dithiocarbamate (1:2) could however be detected only at very high concentrations of the dithiocarbamate. Attempts by Goksoyr to synthesise methylene dithiocarbamate as described by Levi and Gimignani (1929) were not successful. He thus concluded that neither dimethyl dithiocarbamate nor methylene dithiocarbamate were ordinarily formed as degradation products of dazomet.

Another mechanism for the degradation of dazomet has been suggested by Vacek and Flegr (1964) who studied the reactions of dazomet with lead (11) acetate. They proposed that methylammonium methyldithiocarbamate occurs as a primary product of dazomet hydrolysis (Figure 1.3).

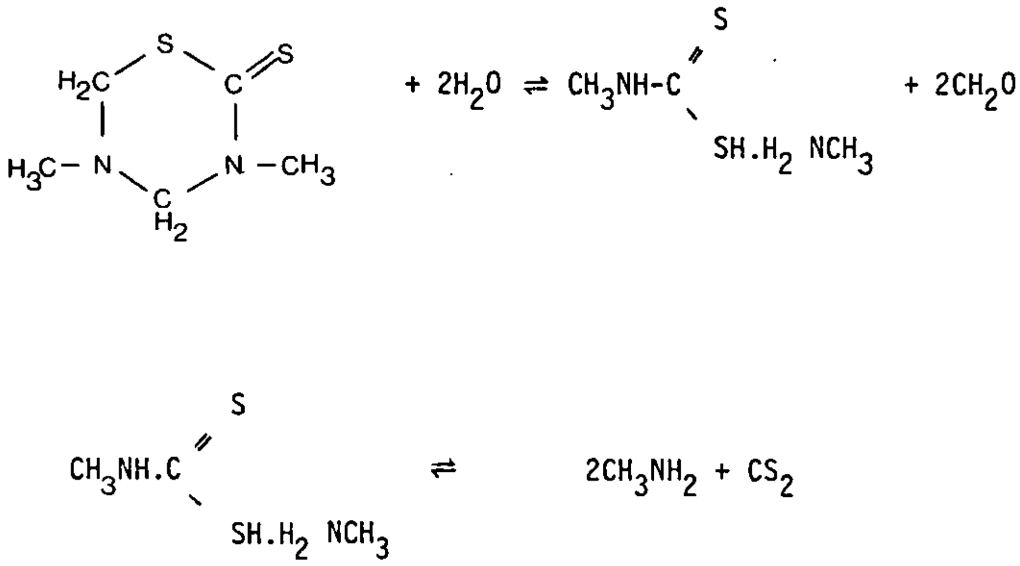

+ 2H2O ⇌ CH3NH-C(=S)SH.H2NCH3 + 2CH2O
CH3NH.C(=S)SH.H2NCH3 ⇌ 2CH3NH2 + CS2

FIGURE 1.3 Degradation of dazomet to methylammonium methyldithiocarbamate (Vacek and Flegr, 1964)

1.3.2 N-methyldithiocarbamate

It is necessary to consider the reactions of N-methyldithiocarbamate since it has been implicated as one of the degradation products of dazomet.

There are three main pathways by which N-monosubstituted dithiocarbamic acid may decompose in acid and alkali medium (Takami, et al, 1973a). This is shown in Figure 1.4.

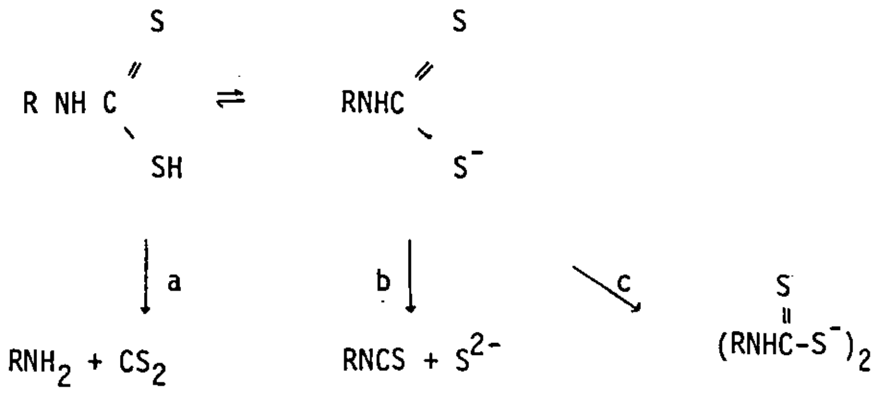


FIGURE 1.4 Acid and base decomposition pathways of N-monosubstituted dithiocarbamic acids (Takami et al, 1973a)

One route is the acid decomposition to the corresponding amine and carbon disulphide (reaction a). These reactions were studied by Zuman and Zahradnik (1957) for a number of mono and dialkyl dithiocarbamic acids in acid solution. They followed the reaction polarographically and found it to be first order. The calculated reaction-rate constant decreased with increasing pH values. Zahradnik and Zuman (1958) proposed the following forms in which dithiocarbamic acids may exist (Figure 1.5).

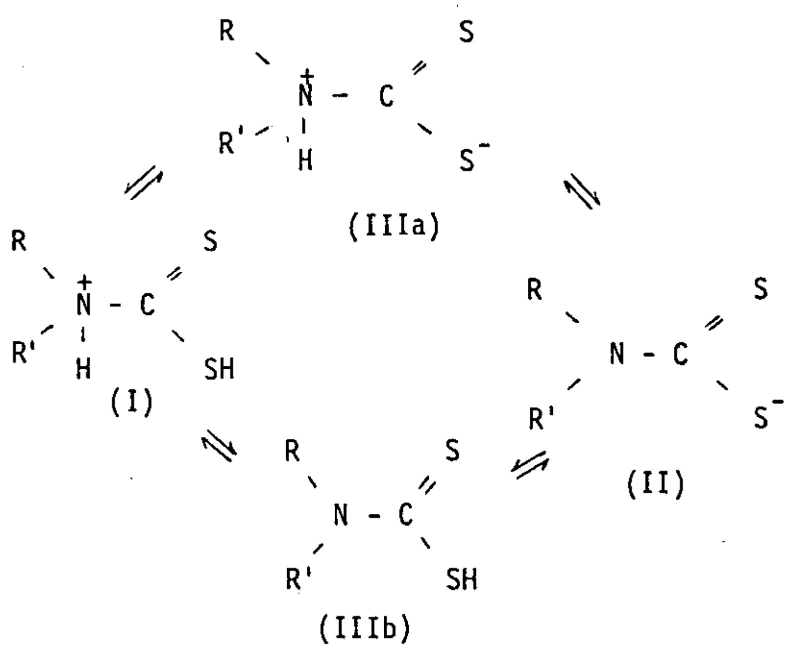


FIGURE 1.5 Forms of dithiocarbamic acids in acidic and basic conditions (Zahradnik and Zuman, 1958).

Form I could exist only in acid solution, and form II under alkaline conditions. The acid decomposition was explained in terms of proton addition according to the following equilibrium reactions (Figure 1.6):

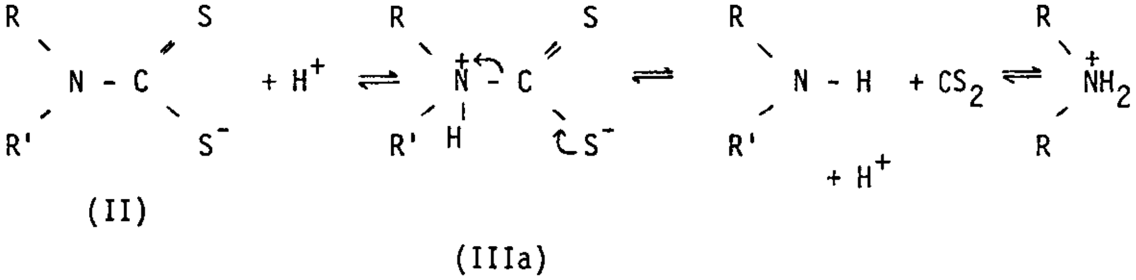


FIGURE 1.6 Mechanism for acid decomposition of dithiocarbamic acid by proton addition (Zuman and Zahradnik, 1957).

The form IIIa was considered to be the reactive form of dithiocarbamic acid in acid solution which splits into carbon disulphide and amine.

Miller et al (1962), however, ruled out the possibility of IIIa due to spectral evidence. Form IIIb was supported based on findings of solvent effects in aqueous methanol and dioxane systems (Joris et al 1970a). They found that a decrease in the dielectric constants of the solvents enhanced the decomposition of the dithiocarbamate having smaller N substituents such as methyldithiocarbamic acid and retarded those having bulkier ones. Thus they proposed a transition form (Figure 1.7) in which the N-C bond was solvated. This was a critical factor in the stability of the dithiocarbamate. A fast proton pre-equilibrium between the acid form and the anion form occurs predominantly in acid solutions before splitting of the molecule to carbon disulphide and amine.

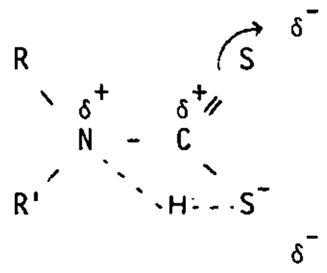
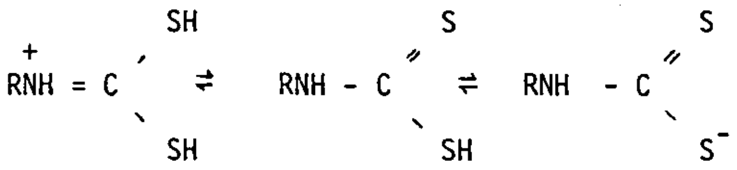


FIGURE 1.7 Fast proton pre-equilibration between the acid and anion form of dithiocarbamic acid (Joris et al, 1970a)

The role of a further protonated form IV (Figure 1.8) of N-monosubstituted dithiocarbamic acids in strongly acid solutions was considered by Takami et al (1973b).



(IV) (iii) (ii)

FIGURE 1.8 Protonated forms of N-monosubstituted dithiocarbamic acids in strongly acid solutions (Takami et al, 1973b)

They suggested that decomposition reaction in strongly acid solutions proceeds via (iii) in the equilibrium IV⇌(iii), followed by rate-determining decomposition of (iii), but not via IV. This mechanism was in accordance with the findings of Joris et al (1970b). However, Takami et al (1973b) proposed a transition state (Figure 1.9) which was in conflict with that of Joris et at (1970a). The lone pair of nitrogen and π-electrons of thiocarbonyl

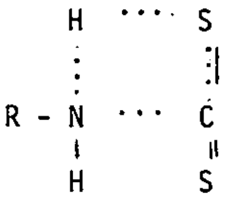


FIGURE 1.9 Transition state for the decomposition of N-monosubstituted dithiocarbamic acids in acid solutions (Takami, et al 1973b)

group lie on the same plane unlike the state proposed by Joris et al (1970a) in which the lone pair and SH are required to situate on the same plane (Figure 1.7). The C-N bond cleavage occurs concertedly with proton transfer.

In addition to the formation of carbon disulphide and methylamine, other products have also been reported to be formed when N-methyldithiocarbamate decomposes in acid solution. Turner and Corden (1963) and Joris et al (1970b) reported the formation of hydrogen sulphide, N,N'-dimethylthiourea, N,N'-dimethylthiuram disulphide and methylisothiocyanate, but the mechanisms have not been unequivocally established.

The other reactions of dithiocarbamic acids involves the formation of isothiocyanate (reaction b; Figure 1.4) and thiuram disulphides (reaction c; Figure 1.4) in alkaline solutions.

Halls (1969) studied the decomposition of N-methyldithiocarbamates and found that it was unstable at pH > 7. The observed decomposition products were methylisothiocyanate, hydrogen sulphide and traces of sulphur.

Two different mechanisms have been proposed for the decomposition of monoalkyl dithiocarbamates. The first mechanism consists of base catalysed decomposition, according to the following mechanism (Figure 1.10) proposed by Wronski (1959) and Hodgkins et al (1961).

(1) $RHNCSS^- + B \rightleftharpoons RNCSS^{2-} + BH^+$

(2) $RNCSS^{2-} \rightarrow RNCS + S^{2-}$

FIGURE 1.10 Mechanism for base decomposition of N-methyldithiocarbamic acid (Wronski, 1959; Hodgkins et al 1961).

However, Takami et al (1970c) suggested a different mechanism (Figure 1.11) in which the conjugate base (iia) is unreactive and the isothiocyanate and sulphide ion are formed directly from (ii) through the following transition state (Figure 1.12):

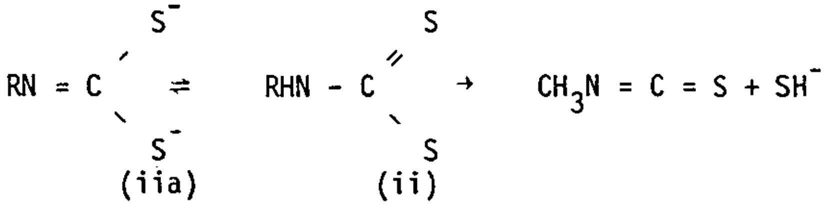


FIGURE 1.11 Mechanism for base catalysed decomposition of N-methyldithiocarbamate involving a conjugate base form (Takami, et al, 1970c)

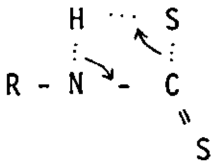
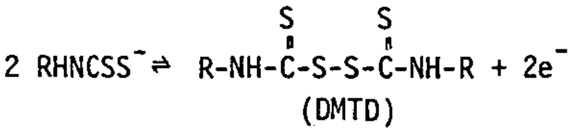


FIGURE 1.12 Transition state for the decomposition of N-monosubstituted dithiocarbamic acid in basic solutions (Takami, et al (1973c)

The other mechanism for base decomposition involves oxidation. Turner and Corden (1963) observed that at pH 9.5 vapam (sodium N-methyldithiocarbamate) decomposes to methylisothiocyanate and elemental sulphur. The rate of decomposition was dependent on oxygen content of the solution. Joris, et al (1970b) suggested the following decomposition mechanism (Figure 1.13) involving the formation of N,N'-dimethylthiuram disulphide (DMTD). But the mechanism has not been finally established.

(1) 

$$2\ RHNCSS^- \rightleftharpoons R\text{-}NH\text{-}\overset{\overset{S}{\|}}{C}\text{-}S\text{-}S\text{-}\overset{\overset{S}{\|}}{C}\text{-}NH\text{-}R + 2e^-$$

(DMTD)

(2) $DMTD + \frac{1}{2}\ O_2 \rightleftharpoons 2\ RNCS + H_2O + 2S$

FIGURE 1.13 Oxidation mechanism for the decomposition of vapam (sodium N-methyldithiocarbamate) at pH 9.5)

Studies on the degradation of dazomet show that the compound is unstable in aqueous conditions. However, very little work has been done to quantify the degradation of dazomet. The volatile products released from secondary breakdown reactions have been studied, and some of the products quantified, but these do not give any clear understanding of the primary degradation process which is essential for inferring its biological activity.

1.4 Biological Action of Dithiocarbamates

A considerable amount of literature has been published on the biological effects of the dithiocarbamates since the first patent for their use as fungicides by Tisdale and Williams (1934). Most of the 'fundamental' studies on fungitoxicity have been undertaken on the intact fungus or its spores. These studies are useful especially for certain inferences on structural relationships and mode of action. Another area of investigation has been the effect of dithiocarbamates on specific enzyme systems. Numerous workers have investigated the effects of dithiocarbamates on such processes as respiration and nitrification as well as many isolated enzyme systems. However, no clear answer appears as to the mode of biocidal action in intact cells. Several reviews (Janssen and Kaars Sijpestijn, 1961; Thorn and Ludwig, 1962; Albert, 1979; Lukens, 1971) have been written, essentially cataloguing these observations.

Any consideration of the biological action of dithiocarbamates has to take into account the activities of their decomposition products. It is possible to consider these effects in three general areas.

1.4.1 Interaction with thiols

The cysteyl branches on proteins serve as functional groups for enzymic activity and determine in part, the structural form and characteristics of the proteins. Fungicides may combine with thiols on coenzyme or apoenzyme and prevent the functioning of the system in a reaction that may be nonspecific (Owens and Blaak, 1960).

McCallan, Miller and Weed (1954) examined the effects of several fungicides on respiration of conidia of five fungi. They found that ferbam (ferric dimethyldithiocarbamate), lime sulphur ($caS.S_x$) and phenol were strong inhibitors of respiration of certain fungi, but had little or no effect on respiration of others.

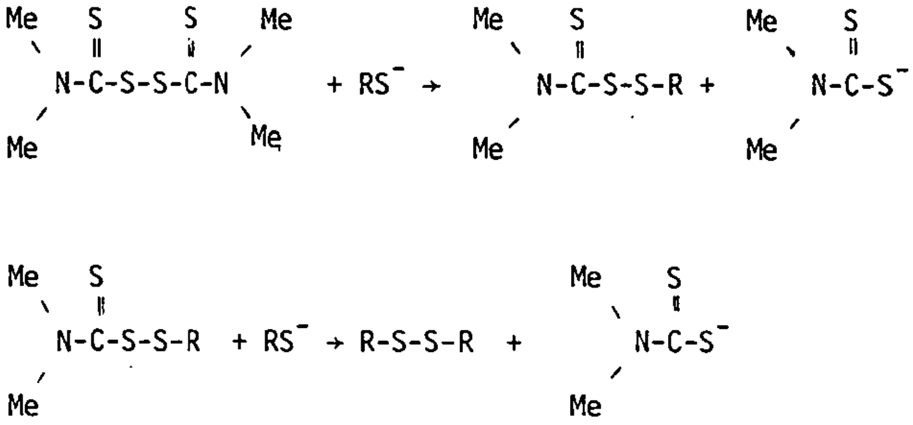
Owens (1960) distinguished metabolic inhibition in the tricarboxylic acid cycle of Neuraspora sitophila by sulphur containing fungicides. He found that sulphur, sodium sulphide, thiram (tetramethylthiuram disulphide) and ferbam, all stopped the conversion of acetate to citrate and slightly inhibited succinate oxidase. Ziram (zinc dimethyldithiocarbamate), nabam (disodium ethylenebisdithiocarbamate), and maneb (manganese ethylenebisdithiocarbamate) all inhibited aconitase. Methylisothiocyanate had no effect on acetate-to-citrate metabolism, though it inhibited the dehydrogenases of malate and succinate.

Inhibition of protein synthesis in vitro by isothiocyanates has been reported by Leblova-Svobodova (1965). He showed that the inhibition was not due to competition of isothiocyanates for amino acids in the system. Lukens, 1971, suggested that the isothiocyanates act by combining with thiol groups on the enzymes of the system.

Leakage of membrane constituents has also been reported in the presence of dithiocarbamates. Wedding and Kendrick (1959) showed that sodium-N-methyldithiocarbamate caused leakage of cellular constituents from Rhizoctonia Solani. They suggested that the dithiocarbamate acts by attacking thiols of the membrane.

Methylisothiocyanate, the decomposition product of N-methyldithiocarbamate reacts with thiols to form methyldithiocarbamic acid ester (Thorn and Ludwig, 1962). However, there was no evidence of membrane damage as reflected by loss of selective ion permeability, and other thiol reactants (such as captan and dichlone) have been shown to be similarly inactive on the fungus membrane (Kottke and Sisler, 1962; Miller and McCallan, 1957).

The dithiocarbamates are unique among fungicides in that their fungitoxicity increases with the ability of the compounds to ionize (Klopping and van der Kerk, 1951). The free dimethyldithiocarbamate, however, does not react with thiols (Owens and Rubinstein, 1964), but thiram, the disulphide of dimethyldithiocarbamate, does react with thiols without requiring oxygen. Hence, Owens and Rubinstein (1964) suggested that the mechanism may involve the formation of a free radical. The reaction can also proceed via a polar exchange mechanism (Campaigne, Tsurugi and Meyer, 1961). The final product is the disulphide of the thiol and the dithiocarbamate ion of thiram (Figure 1.14).



$$(Me)_2N-C(=S)-S-S-C(=S)-N(Me)_2 + RS^- \rightarrow (Me)_2N-C(=S)-S-S-R + (Me)_2N-C(=S)-S^-$$

$$(Me)_2N-C(=S)-S-S-R + RS^- \rightarrow R-S-S-R + (Me)_2N-C(=S)-S^-$$

FIGURE 1.14 The reaction of thiram with thiols (Campaigne, Tsurugi and Meyer, 1961)

The inhibitory activity of dithiocarbamates has been shown to be antagonised by imidazole derivatives. Kaars Sijpesteijn and van der Kerk (1952; 1954) showed that L-histidine and a number of imidazole derivatives displayed antagonistic activity towards tetramethylthiuram disulphide and dimethyldithiocarbamate.

1.4.2 Oxidation-reduction reactions

Miller, McCallan and Weed (1953) proposed that sulphur may exert its fungitoxicity by removal of protons from the protoplast. Fungi which are sensitive to sulphur, reduce the element quantitatively to hydrogen sulphide. These protons may be removed from the dehydrogenases of the system thus impairing the energy production mechanism of the cell. Sulphur may also compete in a similar manner with oxygen for protons from terminal oxidases (Horsfall, 1956).

Tweedy (1964) also suggested that the reduction of sulphur to hydrogen sulphide by fungi may involve the cytochrome system and may uncouple phosphorylation.

The various aspects of biological effects of sulphur and its mode of action have been reviewed by Horsfall (1956) and Martin (1964), and it is not certain whether sulphur or a compound of sulphur permeates fungus cell and causes toxicity.

1.4.3 Metal chelation

Heavy metals are required by the cells to catalyse certain metabolic processes. It may serve this function as a chelate with a biological component.

Metal ions may act as catalysts by binding as cofactors to the sites on enzymes. Fungitoxic chelators may compete with natural cofactors to bind through metal to sites on the enzymes (Rich, 1960).

Leibermeister (1950) investigated the effects of sodium dimethyldithiocarbamate on several strains of *Staphylococci* and on *Escherichia coli* and found greater inhibition in serum agar than in other media. Addition of copper sulphate to serum-free media increased inhibitory effect of the dithiocarbamate. He considered that the antibacterial action of this and other copper-binding compounds was not due to removal of copper from the medium, but was primarily due to formation of toxic copper complexes in the medium.

Owens (1953) studied various enzymes obtained from both plant and animal sources, e.g. pancreatic amylase, malt amylase, polyphenol oxidase and catalase, chosen to represent amino, sulphydryl-, copper- and iron-dependent enzymes respectively. He found that sodium diethyldithiocarbamate, iron and zinc dimethyldithiocarbamate, and tetramethyldithiocarbamate all inhibited to a varying degree all the enzymes tested. Owens (1953) presented evidence to indicate that inhibition resulted from complex formation with metals of the metal-containing enzymes or by interference in electron shifts between sulphydryl or amino groups of the enzyme and substrate molecules. Other enzymes e.g. cholinesterase, alkaline phosphatase, alcohol dehydrogenase and succinic dehydrogenase, which depend on metal ions as cofactors have also been shown to be inhibited. These have been reviewed extensively by Thorn and Ludwig (1962).

Horsfall (1956) studied the fungitoxicity of several metal complexes. He found that there was a parallel relationship between fungitoxicity and stability constants of metal complexes and suggested that the toxic reactions may occur as a result of interaction with essential enzymes or due to catalysis of toxic reactions in the cell.

Different chelate toxicants have different permeability to cells. This results in a peculiar pattern of growth and/or germination inhibition with certain fungi by a number of dithiocarbamates and their oxidation products. This was originally described by Dimond et al (1941) for spore germination tests (Figure 1.15).

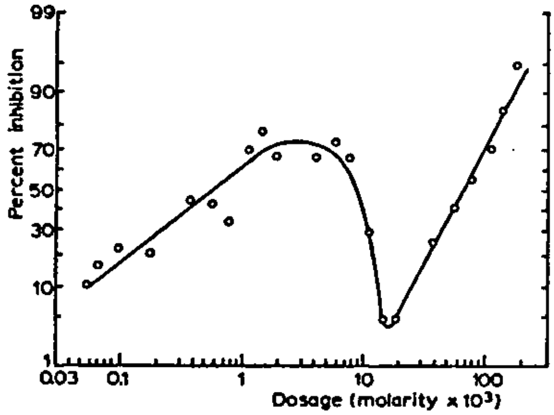


FIGURE 1.15 Bimodal dose response curve for TMTD (tetramethylthiuram disulphide (from Dimond et al, 1941).

They suggested that the two positive slopes represented two distinct toxicants while the negative slope represented a change to the second toxicant at the expense of the first as the dose of the chelator was increased.

This phenomenon was later shown to occur during studies of the growth inhibition of Aspergillus niger and Penicillium italicum by sodium dimethyldithiocarbamate and tetramethylthiuram disulphide in solid media (Kaars Sijpesteijn and van der Kerk, 1953; 1954).

They also showed that the first zone of inhibition could be reversed by certain α-keto acids. Goksoyr (1955) suggested that with dimethyldithiocarbamate (DDC) the first toxicant was 1:1 copper:DDC complex (Figure 1.16a) formed in the medium. With increasing concentration of DDC a 1:2 complex (Figure 1.16b) was formed resulting in decreased potency and thus a negative slope.

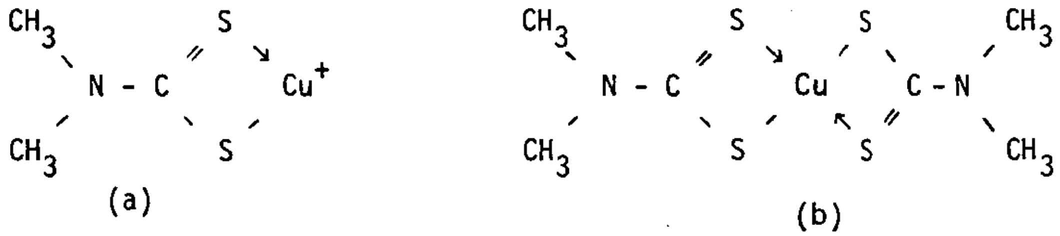


FIGURE 1.16 1:1 (a) and 1:2 (b) complexes of copper dimethyldithiocarbamate (Goksoyr, 1955)

With the chelation of all the metal, the second toxicant was considered to be DDC itself or its 1:1 complex with other metals.

Horsfall (1956) suggested, however, that the first zone of inhibition may be due to the chelation of required metals in the cell.

Later Kaars Sijpesteijn and Janssen (1958) studied the solubility properties of the copper complexes and suggested that the 1:2 complex penetrates the cell bettter than the 1:1 complex, but the latter is the actual toxic moiety which combines with an enzyme. The 1:2 complex must be transformed into 1:1 complex within the cell to show any toxicity.

1.5 Toxicity Studies and Applications of Dithiocarbamates in Clinical Medicine

Lysenko et al (1957) studied several compounds for their ability to protect against ionizing radiation in mice. They found good effect with sodium diethyldithiocarbamate. Cohen et al (1957) and Du Bois et al (1961) suggested that the dithiocarbamates shield from radiation damage by increasing electron flow towards radiosensitive site in the cellular oxidation-reduction chain.

Sodium diethyldithiocarbamate has also been found useful against exposure to vapours of nickel carbonyl (Sunderman and Sunderman, 1958; West and Sunderman, 1958).

Since the initial discovery by Hald et al (1948) that Antabuse (tetraethylthiuram disulphide) rendered humans sensitive to ethyl alcohol, the use of Antabuse for the treatment of alcoholism and its effect on enzymes involved in alcohol metabolism has been well documented (reviewed by Thorn and Ludwig, 1962; Lukens, 1971).

Studies on the toxicological properties of the dithiocarbamates and the related thiuram disulphides show them to be low in toxicity. However structural differences are important in the overall toxicity of these compounds.

Tetramethylthiuram disulphide has been found to be approximately ten times more toxic to rabbits than the tetraethyl derivative

(Antabuse) (Hanzlik and Irvine, 1921). Studies on mice (Kirchheim, 1951), chicks, goslings and turkey poults (Waibel, 1957) show similar results. Waibel found that turkey poults were able to tolerate tetramethylthiuram disulphide at much higher concentration in their diet (200ppm) compared to chicks and goslings (40 and 150 ppm respectively).

Toxic reactions involving allergic sensitisation of skin have also been observed. Griepentrog (1960) showed that tetramethyl, dipyrrolidyl and polyethylene thiuram disulphides - all produced allergic skin response.

The dithiocarbamic acid salts have been found to be generally more toxic than the corresponding disulphides. Toxicological studies for a number of dithiocarbamic acid salts have been reported (Dicke, Allen and Richter, 1947). These authors and others (Rarattini and Leonardi, 1955; Koroblev, 1960) have also shown that as with the disulphides, the dimethyl derivatives are more toxic than the diethyl derivatives. Some difference in toxicity of different metal salts has also been observed. Hodge et al (1952) and Hodge (1956) showed that ferric salt of diethyldithiocarbamate has much lower acute toxicity than the zinc salt, though the overall toxicity of either salt was not high.

1.6 Aim of the Project

The aim of this project was to study the degradation of dazomet under different conditions and correlate this with its biological activity.

2. MATERIALS AND METHODS

2.1 Chemicals

Dazomet

Dazomet was supplied by B.A.S.S. (UK) Ltd, Agrochemicals Division, Hadleigh, Essex as a solid formulation containing 98-99% w/w of dazomet. It was purified by recrystallising 3 times from acetone (Goksoyr, 1964).

Dithiocarbamic acid salts

The sodium salts of N-methyl-, NN-dimethyl, and NN-diethyldithiocarbamate were supplied by J D Campbell and Sons Ltd, Warrington, Sigma London Chemical Co Ltd, Poole and Aldrich Chemical Co Ltd, Gillingham, respectively. Ammonium NN-tetramethylenedithiocarbamate was supplied by Sigma London Chemical Co Ltd, Poole. Sodium N-methyl and N-ethyldithiocarbamate and ammonium dithiocarbamate were synthesised from corresponding amine and carbon disulphide according to the method described by Klopping and van der Kerk (1951).

Thiuram disulphides

Thiram (NN, N'N'-tetramethylthiuram disulphide)was supplied by Robinson Bros Ltd, West Bromwich, and disulfiram (NN, N'N'-tetraethylthiuram disulphide) was supplied by Sigma London Chemical Co Ltd, Poole. NN'-dimethylthiuram disulphide was prepared from N-methyldithiocarbamate according to the method described by Takami et al (1973).

The above chemicals were all stored in a vacuum desiccator over phosphorous pentoxide.

Metal salts

Analytical-reagent grade nickel sulphate, cobalt nitrate, copper nitrate, mercury (11) nitrate and lead nitrate were used.

Other chemicals and reagents

N-methylisothiocyanate and fluorescamine were supplied by Sigma London Chemical Co Ltd, Poole. Methylamine hydrochloride and caffeine were supplied by Aldrich Chemical Co Ltd, Gillingham. Methanol (HPLC grade) was supplied by Fisons Scientific Apparatus, Loughborough.

All other chemicals, unless otherwise stated, were of AR grade and obtained from either BDH Chemicals Ltd, Poole; Aldrich Chemical Co Ltd, Gillingham; or Sigma London Chemical Co Ltd, Poole.

2.2 Buffer Solutions

Sodium phosphate buffer

0.05M NaH_2PO_4 and Na_2HPO_4 were prepared and the two solutions mixed to obtain the required pH.

Borate buffer

0.02M boric acid adjusted to pH 8.7 with 0.2M sodium hydroxide.

2.3 Sterilisation Procedure for Media and Equipment

All the media were sterilised by autoclaving (20 mins at 121°C unless otherwise stated) or by membrane filtration using 0.45 μm cellulose acetate filters (Oxoid). D-glucose was sterilised by autoclaving at 115-116°C for 10 mins.

Most of the equipment was sterilised either by autoclaving or in an oven at 160°C for 1½ hours.

2.4 Culture Media

Minimal salts media for bacteria (enriched)

	per litre
KH_2PO_4	9.25g
K_2HPO_4	5.57g
Na_2SO_4 (anhydrous)	0.20g
NH_4Cl	3.00g
$MgCl_2.6H_2O$	0.20g
Casamino acids - vitamin free (Difco)	0.5 g
D-glucose	3.0 g

	per litre
Vitamins:	
Thiamine hydrochloride	1.0 mg
Nicotinic acid	1.0 mg
Trace elements:	
$FeSO_4.7H_2O$	25.0 mg
$ZnSO_4.7H_2O$	25.0 mg
$MnSO_4.3H_2O$	25.0 mg
H_2SO_4 (0.1N)	0.5 ml

Casamino acids and D-glucose (autoclaved separately as solution concentrates), vitamins and trace elements (both sterilised by membrane filtration as solution concentrates) were added to the media immediately before use.

Minimal salts media for yeast (vitamin enriched)

As minimal salts media for bacteria - except for an additional requirement of vitamins (modified according to Goksoyr, 1955):

	per litre
Aneurine hydrochloride	1.0 mg
Calcium pantothenate	2.0 mg
Pyridoxine	2.0 mg
Inositol	2.0 mg
Biotin	0.2 mg

Minimal salts agar

Minimal salts agar slopes for either yeast or bacteria were prepared by adding 15g.l^{-1} agar No 3 (Oxoid) to the respective media described above. Casamino acids, D-glucose, vitamins and trace elements were added separately, as sterile solutions after autoclaving and cooling to 50°C to avoid thermal degradation.

Nutrient broth

	per litre
Bacteriological peptone (Oxoid)	10.0g
Lab Lemco (Oxoid)	10.0g
NaCl	5.0g

The pH of the broth was adjusted as required before sterilising by autoclaving.

Nutrient agar

Nutrient agar was prepared by adding 15g.l^{-1} agar No 3 (Oxoid) to the nutrient broth media before autoclaving.

Nutrient media for filamentous fungi

	per litre
Maltose	38.0g
Yeast extract (Oxoid)	2.5g
Mycological peptone (Oxoid)	8.0g
Malt extract (Oxoid)	2.0g

The pH was adjusted to 5.0 before autoclaving.

Agar slopes for filamentous fungi

The agar slopes were prepared by adding 15g.l^{-1} agar No 3 (Oxoid) to the nutrient media for filamentous fungi before autoclaving.

2.5 Test Organisms

Bacillus licheniformis NCTC 1097; *Saccharomyces cerevisiae* CM1 19391; *Pseudomonas aeruginosa* NCTC 9433; *Pseudomonas aeruginosa* NCTC 6749; *Staphylococcus aureus* NC1B 8625.

Aspergillus niger ATCC 16404; *Aspergillus flavus* CMI 15959 and *Penicillium italicum* CMI 143617.

2.6 Degradation Studies on Dazomet

2.6.1 Stability of dazomet in distilled water

1 ml of freshly prepared stock solution (25 mg.ml^{-1}) of dazomet in acetone was pipetted into a 25 ml volumetric flask and the volume made up with distilled water to give a final concentration of 1000 ug.ml^{-1} (6.173 mM). The solutions were then trasferred to narrow neck reaction bottles sealed with rubber bungs and incubated at required temperatures. Samples were withdrawn at various time.

intervals and diluted (1:100 using distilled water) before recording their ultraviolet spectra using Unicam SP800 spectrophotometer (1 cm path).

2.6.2 Separation of products using thin layer chromatography (TLC)

i) *Preparation of TLC plates*

30g silica gel G (Merck) was shaken vigorously with 60 ml water and the resulting slurry spread with a single motion (0.6 μm thickness) on 20 x 20 cm glass plates using a Shandon (UNOPLAN) spreader. Calcium oxalate impregnated plates (Srivastava and Dua, 1976) were prepared by using 15g calcium oxalate in the slurry before spreading plates (0.6 mm thick). The spread plates were allowed to air dry at room temperature before transferring to a rack and activating in an oven at 110°C for 1 hour. They were allowed to cool and stored in a desiccator over self-indicating silica gel (Fisons)

ii) *Separation and detection of dazomet and its degradation products by TLC*

Two TLC separation procedures were used:

a) The method using calcium oxalate impregnated silica gel TLC plates was originally described for separating closely related aliphatic amines (Srivastava and Dua, 1976). Samples of degraded dazomet, and standards (ca, 30 μg) e.g. N-methyldithiocarbamate, N-methylisothiocyanate, NN'-dimethylthiourea, NN'-dimethylthiuram disulphide and methylamine hydrochloride were applied on a calcium oxalate plate using a micropipette (Corning Ltd, Stone, Staffs). The spots were dried in a cool current of air (using a hairdryer) and the plates developed in butanol/ethanol/ammonium hydroxide (80:20:20) solvent system. When the solvent front reached ca 2 cm from the top edge, the plates were removed. The spots were visualised by spraying with a solution of 50% carbon disulphide in methanol, heated at 40°C for 5 minutes and then sprayed with a solution of 1% silver nitrate. Brownish spots were detected immediately or after heating at 40°C for 15 minutes.

b) Plain silica gel plates were layered with samples of degraded dazomet and standards (according to procedure described above) and developed in chloroform/methanol (5:95) solvent system (Drescher and Otto, 1968). The spots were visualised as before.

2.6.3 Quantitative determination of dazomet using paper chromatography

The method is based on splitting of non-volatile amines from thiadiazine-2-thiones (Schmandke, 1965). Samples of dazomet (up to 15 µg) were quantitatively layered on Whatman No 1 chromatography paper (20 x 22 cm) with the aid of micropipettes (Corning Ltd, Stone, Staffs). The spots were dried in a cool current of air (using a hairdryer) and the chromatograms developed in ligroine (petroleum spirit 90-105^{o})/methanol/water (2:1:1). When the solvent front reached about 15 cm, the chromatograms were removed and air dried before spraying with 50% acetic acid and drying in a warm current of air using a hairdryer. This was followed by spraying with Ninhydrin solution (0.2% in acetone) and incubation at 105°C for 10 minutes. Red spots were formed which were sprayed with acidified copper nitrate (5% acidified by adding H_2SO_4), cut out, and eluted in 5 ml methanol. The optical density of the resulting solution was measured at 504 nm. A calibration graph of dazomet concentrations up to 15 μg was prepared.

Degradation studies of dazomet were carried out in universal bottles, sealed with plastic screw caps and containing 1000 $\mu g.ml^{-1}$ dazomet solution in distilled water. The bottles were incubated at 55°C. Samples were removed at various time intervals and extracted with an equal volume of chloroform. 30 μl of the chloroform extract (≡ 30 μg dazomet) was layered on chromatography paper and treated as described previously. The amount of dazomet in the various samples was determined from the calibration graph which showed a linear relationship up to 15 μg. For quantities above 15 μg, the samples were appropriately diluted with chloroform before applying to the chromatography paper.

2.6.4 Spot test for detection of formaldehyde (Moham Ram and Turner, 1968)

Reagents:

		g per 100 ml
A.	Phenyl-hydrazone HCl	7.5
B.	Ferric ammonium citrate	5.0
C.	Tripotassium phosphate	20.0

A, B and C were prepared individually and mixed in 5:5:1 parts by volume respectively and constituted the active reagent. Filter strips (Whatman, No 3; 8" x 1½") were pulled through the reagent solution and dried on a white tile under a warm current of air (using a hairdryer). The strips were cut into 1½" square pieces and could be stored for several days. To spot test for the presence of formaldehyde, two reagent impregnated filter pieces were tested simultaneously. On one square a drop of formaldehyde solution (4 $\mu g.ml^{-1}$) was placed to serve as a control, while on the other piece a drop of test sample was placed; both were immediately followed by 1 drop of 6NHCl. Presence of formaldehyde was indicated by development of red colour within 5-10 seconds.

2.6.5 Determination of formaldehyde using Nash's reagent

The procedure for determining formaldehyde was described by Nash, based on Hantzsch reaction (Nash, 1953). Formaldehyde in the medium is trapped as a semicarbazone and determined colorimetrically at 415 nm. Nash's Reagent:

Ammonium acetate	30.0 g
Acetyl acetone	0.4 ml
Distilled Water to	100.0 ml

Freshly prepared reagent is colourless and stable for several weeks if kept in a refrigerator. Over prolonged periods of storage the reagent starts to turn yellow and should be discarded.

A calibration graph of formaldehyde solutions in distilled water was prepared by mixing 5 ml test solutions (0-4 $\mu g.ml^{-1}$ formaldehyde solution) with 2 mls of Nash's reagent in bacteriological test tubes (with plastic caps) and incubating the tubes in a water bath at 55°C for 40 minutes.

The tubes were then removed and the optical density measured at 415 nm against a reagent blank (using a SP8-100 spectrophotometer; 1 cm path).

To determine the amount of formaldehyde released when 1 mole of dazomet degrades, a standard solution of formaldehyde (0.133 mM) and an equivalent amount of dazomet (predegraded at 55°C in universal bottles sealed with plastic screw caps) were each mixed with Nash's reagent, and the development of the colour followed against a reagent blank at 415 nm on an automatic chart recorder connected to SP8-100 spectrophotometer equipped with a thermostatically controlled cell holder. The total development of colour for the standard solution of formaldehyde took 40 minutes.

2.6.6 Determination of methylamine by fluorimetry

Reagent:

Fluorescamine		0.03 g
Acetone (dried over anhydrous $MgSO_4$)	to	100 ml

A procedure described by Coppola and Hana (1974) for the determination of glycine using fluorescamine reagent in dietetic beverages was used.

A calibration graph of methylamine hydrochloride solutions in distilled water (triple distilled water was used) was first prepared by mixing 2 ml test solutions (0.5 μg methylamine hydrochloride) with 2 ml borate buffer on a vortex type mixer, and while the tube was being rapidly shaken, 1 ml of fluorescamine reagent was added. Because of fast hydrolysis of fluorescamine in water, rapid mixing is essential for reproducible results. The relative fluorescence intensity was measured using a Perkin-Elmer 1000 Fluorescence Spectrophotometer (396 nm (excitation) and 480 nm (fluorescence)) by adjusting the highest standard (5 $\mu g.ml^{-1}$) to 100% and blank (0 $\mu g.ml^{-1}$) to 0%.

To study the production of methylamine during the degradation of dazomet in phosphate buffers (procedure described in 2.6.7)

0.5 ml sample of test solution was made up to 50 ml. with triple distilled water and 2 ml of this solution was treated exactly the same as the standards. 0.5 ml of phosphate buffer alone was treated similarly to serve as buffer correction blank.

2.6.7 Determination of dazomet and its degradation products by High Performance Liquid Chromatography (HPLC)

The liquid chromatograph

The basic components of a liquid chromatograph have been described previously (Section 1.2).

For most work with HPLC, a constant flow rate Waters Associates 6000 pump was used. The solvent (previously degassed under vacuum) was pumped (1.5 ml.min^{-1}) on to the column fitted with a Rheodyne 7010 valve injector with a 10 μl loop. Samples were injected using a plastic disposable syringe. The eluate from the column was passed through an ALC 202 detector at 254 nm, and the peaks recorded on a Servascribe recorder.

Preparation of the column

A stainless steel column (10 cm x 5 mm i.d) was packed with 5 μm (Hypersil-ODS (octadecylsilyl silica) (both supplied by Shandon Southern Products Ltd, Runcorn, Cheshire), according to the procedure described by the manufacturers and using an air pump (Olin Energy Systems Ltd, Sunderland). The performance of the column was evaluated using the chromatographic conditions described by Knox and Parcher (1969).

Degradation of dazomet in phosphate buffers

Dazomet solutions were prepared in phosphate buffers (1000 µg.ml^{-1}; 6.175 mM) and transferred to universal bottles sealed with plastic screw caps. To study the degradation of dazomet under nitrogen atmosphere, nitrogen gas was bubbled into the reaction bottles for about two minutes. Similarly the effect of oxygen saturation was investigated by bubbling oxygen gas through the samples. Degradation in the presence of methylisothiocyanate, N-methyldithiocarbamate (sodium salt), methylamine hydrochloride and carbon disulphide was studied by incorporating these in the reaction mixtures. The bottles were incubated at 55°C - unless otherwise stated, and at various time intervals, samples were removed. These were diluted with methanol (7 + 3) and mixed with equal volumes of methanol/buffer (7 + 3) containing 0.04% caffeine before injecting 10 µl into the column. Caffeine served as an injection standard and did not interfere in retention time with other components of the degradation mixture. The eluting solvent was either phosphate buffer (pH 7.0)/methanol (7 + 3) or phosphate buffer (pH 5.0)/ methanol (7 + 3) containing 0.1% cobalt nitrate. The former solvent system could only be used for studying dazomet and its neutral degradation products, while using the latter, the highly polar N-methyldithiocarbamate could also be studied. The lower pH was needed with cobalt nitrate solvent to prevent the precipitation of transition-metal phosphates.

Calibration graphs were prepared for dazomet, N-methyldithiocarbamate and N-methylisothiocyanate by preparing various concentrations of each substance in phosphate buffer, diluting with methanol (7 + 3), and mixing with an equal volume of buffer/methanol (7 + 3) containing caffeine as an injection standard before injecting 10 μl into the column. The concentration of caffeine was selected to give a maximum detector response at a particular sensitivity for the range of concentrations to be studied.

Degradation of dazomet in minimal salts media and nutrient broth

Dazomet was dissolved in 0.5 ml acetone and made up to 100 ml in a volumetric flask with either minimal salts media or nutrient broth, to give the required concentration. The solutions were transferred to 250 ml conical flasks, plugged with cotton wool, and incubated in an orbital shaker at 30°or 37°C. At various time intervals samples were removed and treated in the same way as described above.

Degradation of dazomet in presence of microorganisms

Cells were grown overnight in either nutrient broth or minimal salts media, centrifuged (5000 rpm for 15 min using MSE Mistral 6L) and resuspended to an optical density of 0.05 (Unicam

SP500; 650 nm; 1 cm path) in 250 ml conical flasks with cotton wool plugs. The cells were then shaken in an orbital shaker at 30°or 37°C and grown to an optical density of 0.2, after which, 0.5 ml dazomet solution (in acetone) was added to give the required final concentration. 1 ml samples were withdrawn at various time intervals, membrane filtered (Millipore; 0.45 µm) to remove the cells, and treated in the same way as already described.

2.7 Analysis of Dithiocarbamates by HPLC

Solutions of dithiocarbamates or thiuram disulphides (0.5g per litre) were prepared in methanol-water and injected into the column. The eluting solvents were different ratios of methanol and water, containing 0.1% (w/v) of metal salts (nickel sulphate, cobalt nitrate, copper nitrate or lead nitrate).

2.8 Investigation of Biological Activity of Dazomet and its Degradation Products

2.8.1 Minimum inhibitory concentrations (MIC)

MIC values for bacteria and yeast were determined by inoculating 5ml of minimal salts media in a series containing graded concentrates of the compound to be studied with 0.06 ml (2 drops from a standardised Pasteur pipette) overnight culture of cells.

The tubes were incubated at 37°C and the presence or absence of growth noted after 24 hours. For filamentous fungi, the organisms were grown on agar slopes and 0.06 ml spore suspension inoculated into 5 ml nutrient media (for filamentous fungi) series containing graded concentrations of the compounds to be studied. The presence or absence of growth was noted after incubating the tubes at 30°C for 48 hours.

2.8.2 Cytotoxicity against KB cells

The cytotoxicity of dazomet, N-methyldithiocarbamate isothiocyanate, and formaldehyde was tested against Human oral epidermoid carcinoma cells (KB cells; ATCC No CCl 17). The growth media and other materials needed for these experiments were all supplied by Flow Laboratories Ltd, Irvine, Ayrshire. KB cells were inoculated in tissue culture flasks containing 10 ml MEM medium (Minimum Essential Medium with Earle's Salts - with additions of 10% newborn calf serum, 2 mM glutamine, 1% non-essential amino acids and antibiotics) and incubated at 37°C under 5% CO_2/95% air mixture to maintain the pH of the culture solution. After 48 hours the cells were harvested (using 0.25% w/v trypsin) and diluted to 5×10^4 cells.ml^{-1} with fresh medium. 100 μl of the cell suspension was pipetted into each well of a microtitration plate (12 x 8 rows) and allowed to incubate at 37°C under CO_2/air mixture for 48 hours, after which, the media in each well was removed by suction and replaced with 200 μl fresh media.

Solutions of the test compounds were prepared in 0.5 ml Dimethyl sulphoxide (Fisons) and diluted in 9.5 ml medium to give a stock solution of 1.25 mg.ml^{-1}. A further dilution of 0.5 mg.ml^{-1} was also prepared. The stock solutions were all sterilised by membrane filtration (Millipore: 0.45 μm). 50 μl of each of the stock solutions was then pipetted in duplicate into the first well of a series of 8 wells in each row of the microtitration plates, and a range of 8 dilutions (1 in 5) were prepared.

The plates were incubated under CO_2/air at 37°C for 24 hours, washed in turn with distilled water and BSS (Earle's Balanced Salts for Suspension Cultures) and stained using Jenner-Geimsa stain (Paul, 1975). Live cells stained pink. The plates were observed under a microscope and the MIC's recorded as clear-unstained wells.

2.8.3 Growth inhibition studies

Cells of B.licheniformis were grown overnight at 37°C in minimal salts media, centrifuged (5000 rpm for 15 min using MSE Mistral 6L), and resuspended in fresh media to optical density (OD) of 0.05 (Unicam SP500; 650 nm; 1 cm path). The cells were then shaken at 37°C in an orbital incubator until OD of 0.2. Equal volumes (10 ml) of the cell suspension and minimal salts media containing various concentrations of the compounds to be studied were mixed in 150 ml conical flasks and shaken in a water bath at 37°C. Samples were removed at various time intervals and the OD measured. The samples were returned to the appropriate flasks after the measurement was completed.

To study the effect of L-cysteine on growth inhibition, the same procedure was used as described above, except that 1.2 ml of 0.05M L-cysteine was also added to the final incubation mixture.

2.8.4 Oxygen consumption determinations using oxygen electrode

The measurements were carried out using a Rank oxygen electrode (Rank Brothers, Bottisham, Cambridge). The principle of operation of the electrode was first described by Clark (1956). The cylindrical reaction vessel (2.8 cm long and 2.1 cm diameter) consisted of an outer water jacket which was connected to a water bath set at required temperature, and rested on a magnetic stirrer running at about 300 rev.min^{-1}. A small nylon covered magnetic flea gave continuous stirring of the contents. The electrode vessel was fitted with a perspex cap which fitted the vessel bore very closely, and could be raised or lowered by a rack and pinion adjustment. Any additions were made through a small hole in the cap. The height of the electrode was adjusted to exclude all air bubbles. A polarising voltage of 0.6V was applied to the electrode, and the current was passed through a sensitivity control to a Servoscribe recorder with chart speed of 1 cm.min^{-1}. The rate of utilisation of oxygen was given by the slope of the recorded line.

The electrode was standardised using distilled water saturated with air (Umbreit, Burris and Stauffer, 1972). For saturated solutions the sensitivity control was adjusted to give a full scale deflection on the chart paper. The oxygen content of the solutions was found from standard tables (Hodgman and Lange, 1958).

Cultures of *B.licheniformis* or *S.cerevisiae* were grown overnight on glucose or succinate substrate, and harvested by centrifugation (5000 rpm for 15 min using MSE Mistral 6L). The cells were resuspended in fresh media at optical density (OD) of 0.05 or 0.5 (Unicam SP500; 650 nm) for *B.licheniformis* and *S.cerevisiae* respectively and shaken in an orbital incubator until OD of 0.25 or 0.75, respectively was obtained. The cells were centrifuged again and resuspended in substrate free media. 2 ml of cell suspension was mixed with 3 ml substrate free media containing various concentrations of the compounds to be investigated. The stock solutions of the compounds were freshly prepared in media and stored in a refrigerator until needed when they were diluted with media to the required concentration immediately before any determination. This was to minimise any errors due to degradation or volatility of the samples. In a routine test the total volume in the electrode was 4 ml. The reaction was started by adding 0.1 ml substrate (1M glucose or succinate) after a steady reading (baseline) had been obtained. Oxygen consumption was usually allowed to proceed for 10-15 minutes.

In between determinations the flasks were occasionally shaken to aerate the cultures. A high endogeneous respiration was observed with yeast cultures. This was reduced significantly by bubbling air (from an air line connected to a 0.45μ millipore membrane filter) through the cultures. Corrections for endogeneous respiration were made for all determinations.

The effect of L-cysteine on oxygen consumption by the cells was investigated by adding 0.05 ml of 0.05M solution of L-cysteine (prepared in media) to the incubation mixture.

To study the effect of pre-degraded dazomet on cells, solutions of dazomet were degraded in 250 ml conical flasks according to the procedure described previously, and 3 ml samples of these solutions were treated as described above.

2.8.5 Oxygen consumption determinations using Warburg apparatus

The oxygen consumption of bacterial suspensions was determined by using the general methods outlined by Umbreit, Burris and Stauffer (1972).

The main compartment of each flask contained 1 ml cell suspension in substrate free media at optical density of 0.3. (Cells were grown according to procedure described in Section 2.8.4) and 1.5 ml of various concentrations of the compounds to be investigated, except in controls where 1.5 ml of minimal salts media alone was used. The side arm contained 0.5 ml substrate (0.2M), while the centre well contained 0.2 ml of 20% w/v KOH solution and fluted filter paper. The flasks were allowed to equilibrate oscillating in the Warburg bath for about 10 minutes, after which all the taps were closed, and the contents of the side arms were immediately tipped into the main compartment.

The determinations were performed at 37°C with a shaking rate of 100 oscillations per minute. Readings were taken at 15 minute time intervals for 90 minutes.

3. RESULTS

3.1 Degradation Studies on Dazomet

3.1.1 Stability of dazomet in distilled water

A freshly prepared solution of dazomet in distilled water shows two absorbance peaks at 250 and 277 nm (Figure 3.1). The lower wavelength peak is much less in intensity than that at the higher wavelength. The nature of the spectra is similar to the spectra of sodium N-methyldithiocarbamate (Na-MDC) in distilled water (Figure 3.2). However a corresponding solution of dazomet in carbon tetrachloride shows only a single peak at 290 nm, and this wavelength peak is of much higher intensity than in distilled water. Figures 3.3-3.7 show that when dazomet is incubated in distilled water at various temperatures, the 277 nm peak shifts to 280 nm. The 250 nm peak does not change in wavelength but increases slightly in intensity, though it is still lower in intensity than the 280 nm peak. The observed effect at 250 nm was more pronounced at higher temperatures. With further incubation at various temperatures, both peaks decreased in intensity. The relative decrease in peak heights was greater with increasing temperatures. With increasing incubation times, the characteristic spectra almost disappeared and an additional peak appeared at 228 nm.

3.1.2 Analysis of degradation products using TLC

TLC of degraded dazomet and some standards was investigated using two separation procedures. Typical chromatograms using calcium oxalate and plain silica plates are shown in plates 3.1 and 3.2, respectively. The R_f values for components of degraded dazomet and standards is given in Table 3.1. Separation of degraded dazomet (in pH 7 phosphate buffer at 55^0C for 4 hours) using calcium oxalate plates (Plate 3.1) showed four spots and comparing their R_f values with corresponding standards (Table 3.1), methylamine and N,N'-dimethylthiourea (DMTU) can be clearly identified. Pure samples of both dazomet and Na-MDC degrade on the plate. Methylamine can be identified in the spots arising from the sample of Na-MDC. Another spot separated from Na-MDC can also be observed in degraded samples of dazomet and has a similar R_f value to both N-methylisothiocyanate (MIT) and N,N'-dimethylthiuram disulphide (DMTD). One spot separated from a pure sample of dazomet has similar R_f value to a spot observed in the degraded sample of dazomet.

Separation of degraded dazomet using plain silica plates showed two spots (Plate 3.2). The R_f values for these spots are similar to those of corresponding samples of dazomet and Na-MDC standards (Table 3.1). However, Na-MDC, MIT and methylamine all have very similar R_f values.

TABLE 3.1

Analysis of the degradation components of dazomet using TLC and Paper chromatography

Standards / RF values	Calcium oxalate plate (1)	Plain silica plate (2)	Paper chromatography(3)
Methylamine hydrochloride	0.28	0.76	-
DMTU	0.69	+	-
DMTD	0.88	-	-
MIT	0.89	0.81	-
Na-MDC	0.29,0.90*	0.79	-
Dazomet	0.47,0.79*	0.69	0.91
Degraded dazomet (4)	0.29,0.69, 0.80,0.91	0.68,0.79	0.91
Degraded dazomet (5)	-	0.76	-

1. using butanol/ethanol/ammonia (80:20:20) as developing solvent
2. using methanol/chloroform (95:5) as developing solvent
3. using ligroine/methanol/water (2:1:1) as developing solvent
4. degraded in pH 7 phosphate buffer at 55°C for 4 hours
5. solution in distilled water stored for 4 months at 45°C

- not studied

* sample unstable and seen to degrade

+ not separated

3.1.3 Degradation study of dazomet by colorimetric procedure using paper chromatography

Figure 3.8 shows a calibration graph for the determination of dazomet by the colorimetric procedure using paper chromatography. The graph of OD at 504 nm versus concentration shows a linear relationship up to 15 µg. At high concentrations saturation is observed.

The amount of dazomet present in solutions of distilled water at 55°C was determined by using the linear portion of the calibration curve (Figure 3.9). The samples which were removed at various time intervals, were treated in the same way as the standards. The R_f values of the resulting spots after chromatographic separation were the same for both (Table 3.1). Figure 3.9 shows that the degradation of dazomet is rapid falling to approximately ~~66~~34% of the initial concentration within 30 minutes of incubation.

3.1.4 Determination of formaldehyde release

Formaldehyde was determined using Nash's reagent. A calibration graph of formaldehyde concentrations up to 4 µg.ml^{-1} shows a straight line passing through the origin with a slope of 0.222 OD units/µg.ml^{-1} (Figure 3.10). The correlation coefficient and standard deviation of 0.9998 and 0.010 µg.ml^{-1}, respectively indicates a very good linear response.

The amount of formaldehyde released from a 0.133 mM solution of dazomet which was previously predegraded by incubating at 55°C for 1½ hours is shown in Figure 3.11. The figure shows an increase in OD at 415 nm due to reaction of Nash's reagent with formaldehyde in the solution. The trace obtained on an automatic chart recorder, shows that the development of colour was rapid, reaching an OD of 1.865 after 40 minutes, after which, very little change or slight increase in OD was observed. An equivalent concentration of a standard solution of formaldehyde was treated similarly and is also shown in Figure 3.11. Again the reaction with Nash's reagent was rapid but the maximum OD after 40 minutes was only 1.09; which is nearly half the value compared to the corresponding solution of predegraded dazomet.

The slightly lower OD reading obtained for the predegraded sample of dazomet could be due to some undegraded dazomet still remaining in solution. An attempt was made to extract the undegraded dazomet with various organic solvents before incubating the test solution with Nash's reagent. However, the results obtained were very erratic. This may be due to either the interaction of the solvent with the reagent or due to the solubility of formaldehyde in these solvents, or due to a combination of both these effects.

The release of formaldehyde from solutions of dazomet was also investigated using a spot test technique. When a drop of standard formaldehyde solution was tested on a reagent impregnated filter paper, red colour developed within 5 seconds. Similar investigations of a fresh solution of dazomet also showed positive results for the presence of formaldehyde.

3.1.5 Determination of methylamine release

Methylamine was determined by fluorimetry using fluorescamine reagent. A calibration graph of methylamine concentration up to 5 $\mu g.ml^{-1}$ shows a straight line passing through the origin with a slope of 191.7 scale units/$\mu g.ml^{-1}$. The correlation coefficient and standard deviation of 0.9998 and $4.7 \times 10^{-3} \mu g.ml^{-1}$, respectively indicates a very good linear response (Figure 3.12).

The amount of methylamine produced when dazomet was degraded in phosphate buffers at pH 5.0, 6.0, 7.0 and 7.5 is shown in Figures 3.18-3.21, respectively. The determinations were made on aliquots of samples removed for HPLC analysis of other components of degradation mixture (3.1.6). The figures show that the maximum amount of methylamine released after 5 hours incubation was nearly the same (approximately 6 mM) at pH 6, 7 and 7.5 (Figures 3.19-3.21). However, at pH 5 (Figure 3.18) the maximum amount detected was slightly higher (6.7 mM). In each case the profiles for the formation of methylamine were very similar to the corresponding decay curves for dazomet.

3.1.6 Determination of dazomet and its degradation products by HPLC

Figure 3.13 shows a typical HPLC chromatogram using a reversed-phase octadecylsilyl silica column. Using methanol + phosphate buffer as eluting solvent, dazomet, caffeine (internal standard) and MIT were eluted in that order. One peak due to MDC was not retained and was eluted with the solvent front. The capacity factors for these components of degradation are shown in Table 3.2. Attempts were made to retain the MDC peak to obtain a better chromatographic separation and also to positively identify it. However, water alone as eluting solvent had no effect. A decrease in pH to suppress ionisation, also had no effect, but instead appeared to result in the loss of the peak. When ion-pair reagents such as tetrabutylammonium hydroxide and cetrimide were added to the eluent, again complete loss of the peak occurred.

A novel technique of adding transition metal ions such as cobalt (11) or nickel (11) in the eluting solvent, retained the MDC peak depending on solvent composition (Table 3.3). In each case the retention time of MDC was reduced by increasing the methanol content of the solvent. Cobalt (11) was selected for the study of degradation of dazomet because of its shorter retention time. Since the degradation studies were carried out in phosphate buffers, it was necessary to use pH 5 phosphate buffer in the eluting solvent in order to prevent precipitation of cobalt phosphate.

All components of the degraded solutions of dazomet were separated satisfactorily using the metal pairing technique. Thus, dazomet, MIT, DMTD and MDC were eluted in that order. The presence of cobalt (11) ions had no effect on the order of elution of the unpaired components (Table 3.2). The retention times were also not affected by varying the concentration of cobalt (11) nitrate. The capacity factors of the various components of degradation and the corresponding standards are shown in Table 3.2. It was found that pure samples of DMTD standards were unstable and decomposed readily to form MDC.

Calibration graphs were prepared for the determination of dazomet, MDC and MIT by HPLC (Figures 3.15-3.17). All give straight lines with correlation coefficient of the order 0.999, indicating a very good linear response of the detection system. The correlation coefficients, slopes and standard deviations are shown on the individual graphs. The lines all pass through the origin except for MDC (Figure 3.16) which shows a negative intercept, suggesting a small but a constant amount of decomposition.

TABLE 3.2

Analysis of dazomet and its degradation products by HPLC-expressed as capacity factors (K')

Eluting solvent / Component of degradation	Methanol-buffer[1]	Methanol-buffer + cobalt (11)[2]
Dazomet	1.07	1.38
Caffeine (internal standard)	1.71	2.19
MIT	2.60	3.13
DMTD+	(-)	3.19
MDC	0	18.88

1. Methanol-phosphate buffer (pH 6.0)(30 + 70)
2. Methanol-phosphate buffer (pH 5.0) (30 + 70) containing 0.1% cobalt nitrate

+ Readily decomposes to form additional peak corresponding to MDC

(-) Not identified

3.1.7 Determination of dithiocarbamates and thiuram disulphides by application of transition-metal pairing technique

The technique developed in the previous section (3.1.6) for the analysis of dazomet and its degradation products in presence of cobalt (11) as a pairing reagent, was extended to the analysis of various dithiocarbamates and thiuram disulphides.

After initial trials using separately prepared complexes, a range of solvents containing different proportions of methanol and 0.1% solutions of nickel (11) sulphate or cobalt (11) nitrate were examined. A series of sodium or ammonium salts of dithiocarbamates and related thiuram disulphide were individually injected and their capacity factors were determined (Table 3.3). These complexes were readily detected at 254 nm, with no background interference from the metal salts in the solvent. The peak efficiencies of the complexes were comparable to those of uncomplexed elutes.

A number of important fungicides based on ethylenebisdithiocarbamic acid $(CH_2NH\ CS_2)_2^{2-}$ including zineb, maneb and mancozeb could not be chromatographed using cobalt as reagent. Attempts using silver (1) nitrate also did not show any peaks. Other metal ions such as iron (111), mercury (11) and lead were also found to show no peaks. Results obtained using copper (11) as reagent, were erratic and reproducible peaks could not be obtained.

TABLE 3.3

Determination of dithiocarbamates and thiuram disulphides by the application of transition-metal pairing technique expressed as capacity factors (K')

Eluting solvent	methanol-water			methanol-buffer^1
	(70 + 30) + Ni (11)		(60 + 40) + Co (11)	(30 + 70) + Co (11)
Dithiocarbamate (Na salt) -				
N-methyl	0.57	1.25	0.71	11.8
N-ethyl	1.28	3.37	2.71	
N,N-dimethyl	1.57	4.12	3.85	
N,N-diethyl	8.28	32.1	*	
Thiuram disulphide -				
N,N'-tetramethyl (thiuram)	0.85	1.25	1.57	
N,N'-tetraethyl (disulfiram)	3.71	11.8	12.7	

1. 0.05M phosphate buffer adjusted to pH 5

* peak not observed

Preliminary studies showed that when a mixture of dithiocarbamates or thiuram disulphide was injected onto the column, several peaks resulted due to formation of mixed complexes and alkyl interchanges. This effect was investigated further and reported (see Appendix 6.5).

3.1.8 Degradation of dazomet in phosphate buffers

Degradation of dazomet in phosphate buffers at pH 5, 6, 7 and 7.5 is shown in Figures 3.18-3.21, respectively. The figures show that the decomposition is rapid falling to approximately ~~66~~ 34% of the initial concentration within 30 minutes of incubation. The degradation rate is then slower up to 90 minutes, followed by very little change up to 300 minutes. The differences in degradation profiles at various pH are minimal. However, kinetic analysis of the degradation data does show some differences. The rate constants and the rates of reaction were evaluated for dazomet and its main degradation products, based on a kinetic model and this has been treated separately in Chapter 4. The effect of pH was slightly more apparent on the production of MDC. Under all pH conditions, the amount of MDC produced was maximum after 60 minutes incubation and decreased thereafter. At pH 5 (Figure 3.18) the maximum concentration of MDC produced was 4.4 mM; while at pH 6 (Figure 3.19) and pH 7 (Figure 3.20), slightly higher concentrations (4.7 and 5.0 mM respectively) were formed. However, at pH 7.5 (Figure 3.21) the amount formed was much lower (3.6 mM).

With the other degradation product MIT, the rate of formation and the maximum amounts (approximately 0.5 mM) produced were nearly the same under all pH conditions. However, the maximum amount of MIT formed (after 5 hours incubation) was almost twice as much compared to degradation under nitrogen rich environments and nearly half the amount produced under oxygen rich environments (Table 3.4).

All samples of dazomet (except under nitrogen rich environments) formed yellowish precipitate after about 4 hours incubation in phosphate buffers at 55°C. A test of the samples on filter paper saturated with lead acetate solution revealed absence of hydrogen sulphide. There was no detectable odour either. A UV spectra of the dissolved precipitate in chloroform was identical to spectra of elemental sulphur (Figure 3.22). Yellow crystalline needles of elemental sulphur could be recrystallised from the dissolved precipitate and melted at 111°-112°C (literature value 112°C; Hodgman and Lange, 1958). The precipitate was also detected in solutions of dazomet exposed to air in distilled water at room temperature. The pH of the solutions also changed with longer period of exposure; such that it was 6.0 on mixing, 7.2 after 24 hours and 7.8 after 80 hours. When air was passed through the solution, the pH rose much faster reaching 8.2 in 48 hours.

The influence of some products arising from the degradation of dazomet on the formation of MDC was also studied. In the presence of methylamine (Figure 4.5) the smallest amount of MDC

TABLE 3.4

Influence of oxygen and nitrogen rich environments on the formation of MIT*

pH Condition+	5	6	7	7.5
Normal	0. 53	0.56	0.50	0.49
Oxygen	1.00	1.12	1.12	1.24
Nitrogen	0.25	0.19	0.25	0.28

+ dazomet degraded in phosphate buffers at 55°C and under oxygen and nitrogen rich environments

* MIT formed after 5 hours incubation (mM)

(maximum 3 mM) was detected, while in the presence of carbon disulphide (Figure 4.6) nearly 4 mM was detected. However, in the presence of MIT (Figure 4.4), the maximum amount formed was as much as 5.7 mM. When dazomet was incubated in the presence of Na-MDC (Figure 4.3), the production of MDC was not inhibited and a maximum of nearly 4 mM was formed. The influence of these products on the kinetics of degradation are considered separately in Chapter 4.

3.1.9. Degradation of dazomet in minimal salts media and nutrient broth

Figures 3.23-3.38 show the degradation profiles for dazomet at different concentrations and under incubation conditions required for growing bacteria (B.licheniformis and P. aeruginosa NCTC 6749) and yeast (S.cerevisiae). The figures show that under these conditions, the degradation profile of dazomet follows a characteristic exponential form and in each case either reaches zero concentration or tends towards it. The differences between the various curves are minimal, however kinetic analysis of the data does show some difference. The rate constants and half lives of dazomet were evaluated under these conditions and are treated separately in Chapter 4.

Figure 3.23 shows the degradation profile of dazomet in minimal salts media at 500 $\mu g.ml^{-1}$ (3.086 mM) and at 37°C (control). The amount of MDC detected increased with time, reaching a maximum of 1.5 mM after 150 minutes and decreases thereafter to 1 mM after 300 minutes incubation. The formation of MIT was not detected for 30 minutes, after which it increased in concentration, reaching a maximum value of 0.5 mM. In the presence of B.licheniformis under the same condition (Figure 3.24), the maximum amount of MDC was formed after 150 minutes incubation, but the amount was slightly less (0.9 mM) compared to the control (Figure 3.23). MIT was detected after 30 minutes incubation,but the maximum amount formed was slightly more (0.8 mM). However, in the presence of P.aeruginosa NCTC 6749 (Figure 3.25), the amounts of both MDC and MIT produced with time were very similar to the control (Figure 3.23).

Figure 3.26 shows the control for the degradation of dazomet in minimal salts media at 100 $\mu g.ml^{-1}$ (0.617 mM) and $37^{o}C$. The amount of MDC detected was maximum (0.39 mM) after 60 minutes incubation and decreased slightly thereafter with time reaching a value of 0.29 mM after 300 minutes. MIT was detected after 15 minutes incubation, and the maximum amount produced was proportionally higher than under similar conditions at 500 $\mu g.ml^{-1}$ (3.086 mM) dazomet (Figure 3.23), reaching a maximum value of 0.25 mM. In the presence of B.licheniformis (Figure 3.27) and P..aeruginosa NCTC 6749 (Figure 3.28), the maximum amount of MDC detected was much lower (only .1mM). Similar amounts of MIT (0.1 mM) were also detected in the presence of B.licheniformis (Figure 3.27), but the maximum amount produced (0.25 mM) in the presence of P. aeruginosa NCTC 6749 (Figure 3.28) was the same as the control (Figure 3.26).

Figure 3.29 shows the control for the degradation of dazomet at 50 $\mu g.ml^{-1}$ (0.309 mM) and 37^{o}C. Maximum amount of MDC (0.11 mM) was detected after 90 minutes incubation, and decreased thereafter reaching 0.4 mM after 300 minutes. MIT was detected after 45 minutes incubation and reached a maximum value of 0.1 mM. In the presence of B.licheniformis under similar conditions (Figure 3.30), the production of both MDC and MIT was very similar as the control (Figure 3.29).

Degradation of dazomet was also studied in minimal salts media at 30^{o}C. Figure 3.31 shows the control at 500 $\mu g.ml^{-1}$ (3.086 mM). The amount of MDC detected with time was greater, reaching a maximum value of 0.6 mM after 6 hours incubation, and changed very little thereafter, reaching a maximum concentration of 0.5 mM after 12 hours. MIT was not detected until after 4 hours and increased very slowly with time reaching a maximum value of 0.25 mM after 12 hours. In the presence of S.cerevisiae under the same conditions (Figure 3.32), the maximum amount of MDC and MIT detected was lower (0.2 mM for each).

Figure 3.33 shows the degradation of dazomet in minimal salts media at 100 $\mu g.ml^{-1}$(0.617 mM) and 30^{o}C (control). Maximum amount (0.09 mM) of MDC was detected after 6 hours incubation, the amount decreased rapidly thereafter to 0.02 mM after 12 hours. MIT was detected after 3 hours and reached a maximum value (0.09 mM) after 6 hours incubation, changing very little thereafter. In the presence of S.cerevisiae under the same conditions (Figure 3.34), the production of both MDC and MIT was very similar to the control (Figure 3.33).

Degradation of dazomet in nutrient broth at 500 μg.ml^{-1} (3.086 mM) and 30°C is shown in Figure 3.35 (control). MDC was not detected for the whole of the incubation period. However, MIT was detected after 1 hour incubation, rising to 0.7 mM after 6 hours and changing very little thereafter. In the presence of S.cerevisiae under the same conditions (Figure 3.36), the formation of MIT was very similar to the control (Figure 3.35).

Figure 3.37 shows the degradation of dazomet in nutrient broth at 100 μg.ml^{-1} (0.617 mM) and 30°C (control). Again, no MDC was detected. MIT was detected after 1 hour, reaching a maximum value of 0.19 mM after 5 hours incubation, and changing very little thereafter. In the presence of S.cerevisiae under the same conditions (Figure 3.38), the formation of MIT was very similar to that by the control (Figure 3.37).

3.2 Investigation of the Biological Activity of Dazomet and its Degradation Products

3.2.1 Minimum inhibitory concentrations (MIC's)

Table 3.5 shows the MIC values of dazomet, Na-MDC, formaldehyde and MIT obtained against both Gram-positive and Gram-negative bacteria and against fungi. The values for dazomet range from 2-5 μg.ml^{-1} against S.aureus to 40-60 μg.ml^{-1} against E.coli. For Na-MDC the values range from 5-10 to 40-60 μg.ml^{-1}, respectively against these organisms. The Gram-positive organisms and the fungi were generally more sensitive to both dazomet and Na-MDC, than the Gram-negative bacteria. The Gram-negative bacteria and fungi were more sensitive to MIT compared with the Gram-positive bacteria. For formaldehyde, the overall toxicity against Gram-positive and Gram-negative organisms is similar, but all the fungi were found to be more resistant. Growth was observed up to 90 μg.ml^{-1} formaldehyde.

With all MIC determinations using dazomet and Na-MDC, it was observed that the tubes showed turbidity at concentration ranges, both above and below the MIC values. This effect is shown in a typical example using E.coli against dazomet (Plate 3.3) and Na-MDC (Plate 3.4). Microscopic examination revealed precipitation in the tubes containing concentrations of the compounds above the MIC values, whereas at concentrations below MIC values, organisms were observed. In contrast, both formaldehyde (Plate 3.5) and MIT (Plate 3.6) showed normal turbidity cut-off point at the MIC and above.

TABLE 3.5

The minimum inhibitory concentrations of dazomet, Na-MDC, formaldehyde and MIT against bacteria and fungi

MIC (μg.ml⁻¹)+ / Organism	dazomet	Na-MDC	formal-dehyde	MIT
Gram-positive:				
B.licheniformis	5-10 (.031-.062)	5-10 (.039-.078)	10-15 (.333-.500)	15-20 (.205-.274)
S.aureus	2-5 (.012-.031)	5-10 (.039-.078)	5-10 (.167-.333)	10-15 (.137-.205)
Gram-negative:				
E.coli	40-60 (.248-.372)	40-60 (.310-.465)	15-20 (.500-.667)	5-10 (.068-.137)
P.aeruginosa NCTC 9433	20-40 (.124-.248)	10-15 (.078-.116)	10-15 (.333-.500)	5-10 (.068-.137)
Fungi:				
A.flavus	5-10 (.031-.062)	5-10 (.039-.078)	*	2-5 (.027-.068)
A.niger	15-20 (.093-.124)	15-20 (.116-.155)	*	5-10 (.068-.137)
P. italicum	5-10 (.031-.062)	5-10 (.039-.078)	*	2-5 (.027-.068)
S. cerevisiae	10-15 (.062-.093)	5-10 (.039-.078)	*	5-10 (.068-.137)

* growth was observed up to 90 μg.ml^{-1} (3.0 mM)

+ figures in brackets are concentrations in mM

The UV spectra of Na-MDC was determined in minimal salts media containing a full supplement of metal ions (Figure 3.69). The figure shows that after addition of small amounts of Na-MDC, a peak appears at 324 nm. The increase in intensity of the peak is very little up to 0.16 mM Na-MDC. However, with further increase in concentration to 0.4 mM, the height of peak increases by nearly four times. When the concentration is increased to 0.8 mM very little change is observed and the increase in peak intensity is of the order of less than half. At double this concentration (0.16 mM), the peak height also increases by nearly two times. Further increase in concentration results in much less increase in the peak height, and at 3.97 mM however, a reduction is observed.

In the presence of Mg (11) ions (0.98 mM) alone in the media (Figure 3.70), the peak at 324 nm changes very little or increases slightly up to 1.99 mM concentration of Na-MDC. At a little higher concentration (2.33 mM), however, the increase in peak intensity is of the order of nearly two times. With further increase in concentration of Na-MDC, to 2.78 mM, the intensity of the peak decreases slightly. An increase is again observed at 3.18 mM Na-MDC. At slightly higher concentration (3.57 mM) very little change is observed, but the increase is greater at 3.97 mM.

3.2.2 Cytotoxicity against Human KB cells

The toxic concentrations of dazomet, Na-MDC, MIT and formaldehyde against Human KB cells (oral epidermoid carcinoma cells) are shown in Table 3.6. Formaldehyde was found to be most toxic (0.8 - 4 $\mu g.ml^{-1}$) and Na-MDC, the least toxic (20-40 $\mu g.ml^{-1}$). Both dazomet and MIT were marginally less toxic than formaldehyde (4-10 and 2-8 $\mu g.ml^{-1}$, respectively). The effect of sub-toxic concentrations of dazomet (1.6 $\mu g.ml^{-1}$), Na-MDC (8 $\mu g.ml^{-1}$), formaldehyde (0.4 $\mu g.ml^{-1}$) and MIT (0.8 $\mu g.ml^{-1}$) on KB cells is shown in Plates 3.8-3.11, respectively. Dazomet, Na-MDC and MIT all have the effect of changing the shape of the cells from normal squamous (see control - Plate 3.7) to rounded forms, besides reducing their number. Formaldehyde, however, had a slight stimulatory effect on the cells (Plate 3.10) and the normal squamous shape of the cells was maintained.

TABLE 3.6

Cytotoxicity of dazomet, Na-MDC, formaldehyde and MIT against Human KB cells

Compound	Toxic concentration ($\mu g.ml^{-1}$)$^+$
Dazomet	4-10 (.025-.062)
Na-MDC	20-40 (.155-.310)
Formaldehyde	0.8-4 (.027-.133)
MIT	2-8 (.027-.109)

+ Figures in brackets are concentrations in mM

3.2.3 Effect of dazomet, Na-MDC, MIT and formaldehyde on oxygen consumption by B.licheniformis and S.cerevisiae

The effect of dazomet and Na-MDC on oxygen consumption by B.licheniformis using D-glucose or sodium succinate as substrate is shown in Figures 3.39-3.42. The determinations which were made using an oxygen electrode are expressed as % of the control. Both compounds show similar inhibitory pattern. The inhibitory effect on oxygen consumption is maximum (about 25% of the control) at the lowest concentration of the compounds studied (5 or 10 $\mu g.ml^{-1}$). However, with increasing concentration of the compound up to 100 $\mu g.ml^{-1}$ (50 $\mu g.ml^{-1}$ for dazomet in the presence of sodium succinate substrate), the inhibition is decreased and the oxygen consumption increases to about 50% of the control. At higher concentrations (up to 500 $\mu g.ml^{-1}$) very little change or a slight decrease in oxygen consumption is observed.

The inhibitory activity of dazomet and Na-MDC on oxygen consumption by S.cerevisiae using D-glucose as substrate is shown in Figures 3.48-3.49. Again both compounds show maximum inhibitory activity (about 15-20% of the control) at the lowest concentration studied (5 $\mu g.ml^{-1}$). At slightly higher concentration (10 $\mu g.ml^{-1}$ for dazomet and 20 $\mu g.ml^{-1}$ for Na-MDC), the inhibitory activity decreased sharply and the oxygen consumption increased to about 75% of the control. The decrease was more pronounced compared to similar observations against B.lichenformis (Figures 3.39 and 3.41). The effect of the compounds at higher concentrations, also varied compared to B.licheniformis. With concentrations up to

100 $\mu g.ml^{-1}$, the inhibitory effect became progressively greater and oxygen consumption decreased to about 20% of the control. Further inhibition was observed at higher concentrations up to 500 $\mu g.ml^{-1}$.

The effect of MIT against B.licheniformis using D-glucose and sodium succinate as substrate is shown in Figures 3.43 - 3.44. The nature of inhibitory activity in the presence of both substrates was very similar. Thus, at low concentrations up to 20 $\mu g.ml^{-1}$, oxygen consumption was affected very little, or a slight stimulation was observed. At higher concentrations up to 50 $\mu g.ml^{-1}$, the inhibitory effect was progressively larger and the oxygen consumption decreased to about 45% and 65% of the control in the presence of D-glucose and sodium succinate, respectively. MIT had a similar effect against S.cerevisiaeusing D-glucose as substrate (Figure 3.51). However, the stimulatory effect at low concentrations was more pronounced (about 140% of the control). At higher concentrations of MIT, the inhibitory effect was greater, compared with the activity against B.licheniformis (Figure 3.43), and the oxygen consumption was reduced to about 20% of the control at 50 $\mu g.ml^{-1}$. The nature of inhibitory effect of MIT against B.licheniformis and S.cerevisiae was similar to the effect of 2,4-dinitrophenol (DNP) against B.licheniformis using D-glucose as substrate (Figure 3.45).

The inhibitory effect of formaldehyde at various concentrations was different using the two substrates. Figure 3.46 shows the effect of formaldehyde against B.licheniformis using D-glucose.

The degree of inhibition increased with increasing concentrations of formaldehyde, and oxygen consumption was reduced to about 30% of the control at 70 μg.ml^{-1}, but changed very little at higher concentrations. However, a slight stimulation of oxygen consumption was observed at concentrations of formaldehyde below 50 μg.ml^{-1} when sodium succinate was used as a substrate (Figure 3.47), after an initial inhibition of oxygen consumption (about 95% of the control) at 5 μg.ml^{-1}. At higher concentrations, the degree of inhibition increased and the oxygen consumption decreased to about 60% of the control at 100 μg.ml^{-1}. The overall inhibitory effect was, however, much less than where D-glucose was used as substrate (Figure 3.46). The effect of formaldehyde against S.cerevisiae using D-glucose is shown in Figure 3.50 and is essentially similar to that on the oxygen consumption by B.licheniformis, using the same substrate (Figure 3.46). However, the extent of inhibition was more pronounced and the oxygen consumption was reduced to about 10% of the control at 70 μg.ml^{-1}. Little change was observed at higher concentrations.

The effect of L-cysteine on oxygen consumption inhibition by dazomet, Na-MDC, MIT and formaldehyde against B.licheniformis is shown in Figures 3.54-3.58,respectively. The figures show a typical oxygen electrode run under each condition.

Figure 3.54 shows the effect of L-cysteine (0.625 mM) on the inhibitory activity of 0.05 mM (8.33μg.ml^{-1}) dazomet. When dazomet was added to cells respiring on D-glucose, oxygen consumption decreased to about 30% of the control, but when L-cysteine was added to the medium after further incubation, the degree of inhibition was reduced and the oxygen consumption increased to about 77% of the control.

In the presence of 0.065 mM (8.33 $\mu g.ml^{-1}$) Na-MDC (Figure 3.55), the inhibitory effect on oxygen consumption was much less (about 55% of the control) compared with the effect under dazomet (Figure 3.54). When L-cysteine was added to the incubation mixture, the inhibitory effect was reduced and the oxygen consumption increased to about 90% of the control.

The effect of substrate on the inhibitory activity of Na-MDC against B.licheniformis is shown in Figure 3.56. In the absence of substrate, 0.129 mM (16.67 $\mu g.ml^{-1}$) Na-MDC had no effect on endogeneous respiration. The addition of D-glucose after a few minutes incubation had no effect on oxygen consumption also. When L-cysteine was added after further incubation, the inhibitory effect was reduced and the oxygen consumption increased from 27% to about 60% of the control. A similar effect was not observed with dazomet.

The effect of L-cysteine on the inhibitory activity of MIT is shown in Figure 3.57. At 1.82 mM MIT, the oxygen consumption decreased to about 30% of the control. When L-cysteine was added after a few minutes incubation, the inhibitory activity was reduced and the oxygen consumption increased to about 45% of the control.

In the presence of 5.55 mM formaldehyde (Figure 3.58), the effect of L-cysteine was minimal, resulting in only a slight increase in oxygen consumption from about 42% to about 46% of the control.

Oxygen consumption was also studied using Warburg apparatus. The effect of Na-MDC and dazomet on oxygen consumption by B.licheniformis using D-glucose is shown in Figures 3.52 and 3.53,respectively. The compounds were inhibitory on substrate oxidation at all concentrations studied, but had no effect on endogeneous respiration. The lowest concentration (10 $\mu g.ml^{-1}$) of the compounds had the maximum effect initially, and inhibited substrate oxidation almost completely. However, after longer incubation periods (60 and 90 minutes for dazomet and Na-MDC, respectively), the inhibitory activity was reduced slightly and an increase in oxygen consumption was observed. At higher concentrations of 50, 100 and 500 $\mu g.ml^{-1}$, both compounds showed increased oxygen consumption in that order. However Na-MDC (Figure 3.52) showed more inhibitory effect initially compared with dazomet (Figure 3.53), but its activity was reduced with longer incubation periods.

3.2.4 Effect of predegraded dazomet on oxygen consumption by B.licheniformis

The effect of 50 and 100 μg.ml^{-1} solutions of predegraded dazomet on oxygen consumption by B.licheniformis is shown in Figures 3.59 and 3.60, respectively. The solutions which were preincubated in minimal salts media at 37°C before being assayed for activity against B.licheniformis, showed varying toxicity according to the age of the solutions. Hence, fresh solutions of dazomet in minimal salts media at 50 μg.ml^{-1} (Figure 3.59) inhibited oxygen consumption to about 42% of the control. After 15minutes preincubation at 37°C, the solution showed increased inhibitory activity and the oxygen consumption decreased to about 23% of the control. Further sampling of the solution up to 120 minutes showed very little change. However after 180 minutes, the inhibitory activity of the solution was reduced and the oxygen consumption increased to about 33% of the control, with a further increase to about 50% after 240 minutes. Very little change was observed with further incubation.

The observed inhibitory activity of dazomet after various preincubation times was expected to be related to the amount of dazomet still remaining in the solution. The degradation of dazomet in minimal salts media at 37°C was studied in Section 3.1.9. From Figure 3.29, the amount of dazomet in the solution could be determined at any given time. The final concentrations (corrected after incubating with bacterial suspensions) are shown in Table 3.7. The inhibitory effect of dazomet at these concentrations was determined from Figure 3.40, which shows the effect of various concentrations of freshly prepared dazomet on oxygen consumption by B.licheniformis. The corresponding values of oxygen consumption (% of the control) at the determined concentrations of dazomet are also shown in Table 3.7. These points (theoretical values) are plotted in Figure 3.59 for comparison with experimental points. The overall pattern of the two inhibitory curves is very similar, with the largest differences between the two curves observed after 180 minutes. For the theoretical curve the expected inhibitory activity increases from an initial value of about 42% to about 25% of the control after 60 minutes. Very little change in oxygen consumption is observed with further incubation up to 120 minutes, after which the inhibitory activity decreases and the oxygen consumption increases to about 82% of the control after 240 minutes and remains the same at 300 minutes.

The inhibitory activity of a 100 $\mu g.ml^{-1}$ solution of dazomet in minimal salts media was also studied (Figure 3.60). A fresh solution of dazomet inhibited oxygen consumption by about 57% of the control. With increasing incubation time, the inhibitory activity of the solutions increased and the oxygen consumption decreased to about 43% of the control after 120 minutes. Further sampling of the solution showed very little change up to 300 minutes. The amount of dazomet remaining in solution at any given preincubation time was determined from Figure 3.26. The corresponding values of oxygen consumption (% of the control) were determined from Figure 3.40. The individual values of these points (theore-

TABLE 3.7

Estimation of the oxygen consumption inhibition due to dazomet alone in predegraded solutions against *B.licheniformis*

Initial concn.[1] (μg.ml^{-1})+	50 (.31)		100 (.62)	
Time (mins)	Final concn.[2] (μg.ml^{-1})+	% Inhibition[3]	Final concn.[2] (μg.ml^{-1})+	% Inhibition[3]
0	30.0 (.19)	42	60.0 (.37)	56
15	21 (.13)	40	48.0 (.30)	49
30	15.6 (.10)	34	41.0 (.25)	48
45	13.0 (.08)	30	35.0 (.22)	47
60	10.0 (.06)	25	30.0 (.19)	44
90	8.6 (.05)	24	23.0 (.14)	42
120	5.0 (.03)	22	17.0 (.11)	37
180	2.4 (.02)	63	10.0 (.06)	27
240	1.2 (.01)	82	7.0 (.04)	25
300	1.2 (.01)	82	5.0 (.03)	22

1. Initial concentration of dazomet in minimal salts media
2. Final concentration of predegraded samples of dazomet after dilution with bacterial suspension
3. Mean value (% of control) obtained for corresponding concentrations of dazomet from Figure 3.40

+ Figures in brackets are concentrations in mM

tical values) are shown in Table 3.7. These points are also plotted in Figure 3.60 for comparison with the experimental points. The shapes of both curves are very similar with the largest differences observed after 180 minutes. The theoretical curve also shows an initial oxygen consumption inhibition of about 57% of the control. With larger preincubation times, the expected toxicity of the solution increases and the oxygen consumption decreases to about 38% of control after 120 minutes, with further decrease to about 30% of the control after 180 minutes. Very little change is observed with further incubation.

3.2.5 Assessment of the activity of dazomet, Na-MDC, MIT and formaldehyde against *B.licheniformis* by spectrophotometric measurement of growth

The effect of various concentrations of dazomet against *B.licheniformis* is shown in Figure 3.61. At 5 and 10 $\mu g.ml^{-1}$, complete inhibition of growth was observed. At higher concentrations of the compound (50, 100, 250 and 500 $\mu g.ml^{-1}$), the curves were very similar in shape, with no change or only a slight increase in OD up to about 45 minutes. After further incubation period, the OD increased up to about 120 minutes and, in each case, decreased with time thereafter. The maximum OD reached was at 500 $\mu g.ml^{-1}$ and decreased in the order of decreasing concentrations. The apparent increase in turbidity of the suspensions at higher concentrations was similar to the effect observed with MIC tubes (Plate 3.3).

Microscopic examination revealed the presence of precipitate in tubes at concentrations above the MIC values.

The effect of various concentrations of Na-MDC is shown in Figure 3.62. At both 5 and 10μg.ml^{-1}, maximum inhibition of growth was observed up to 210 minutes, after which a slight increase was observed at 5 μg.ml^{-1}. At higher concentrations (50, 100, 250 and 500 μg.ml^{-1}) of Na-MDC the nature of the curves were very similar to those in the presence of dazomet (Figure 3.61). Following the initial period of little change, an increase in OD was observed reaching a maximum value after about 60 minutes. With further incubation however, a decrease was observed. The only exception was incubation mixtures containing 500 μg.ml^{-1} Na-MDC in which a decrease in OD was observed after 180 minutes. As in the case of dazomet (Figure 3.61), maximum OD was observed at the highest concentration and decreased in the order of decreasing concentration of Na-MDC. However, the apparent increase in the turbidity of the suspensions at higher concentrations, was more pronounced than in the presence of dazomet. This effect was also observed in the MIC tubes (Plate 3.4) at concentrations above MIC values. Microscopic examination of the tubes revealed the presence of precipitate.

The activity of MIT and formaldehyde at various concentrations against B.licheniformis is shown in Figures 3.63 and 3.64, respectively. The log of OD versus time plots at various concentrations were approximately linear for both, with the maximum inhibitory effect observed at the highest concentration studied. Formaldehyde caused complete inhibition and also a decrease in cell growth at concentrations of 20 $\mu g.ml^{-1}$ and above. MIT was less toxic and did not inhibit cell growth completely, even at 50 $\mu g.ml^{-1}$.

The activity of dazomet, Na-MDC and MIT at concentrations causing maximum inhibition of growth, was in each case reduced in the presence of 0.625 mM L-cysteine (Figures 3.65-3.67, respectively). However, the effect of L-cysteine on the inhibitory activity of formaldehyde was minimal (Figure 3.68). The log of OD versus time plots for each of the compounds at concentrations which caused maximum inhibition, and in the presence of L-cysteine at these concentrations, are approximately linear.

FIGURE 3.1

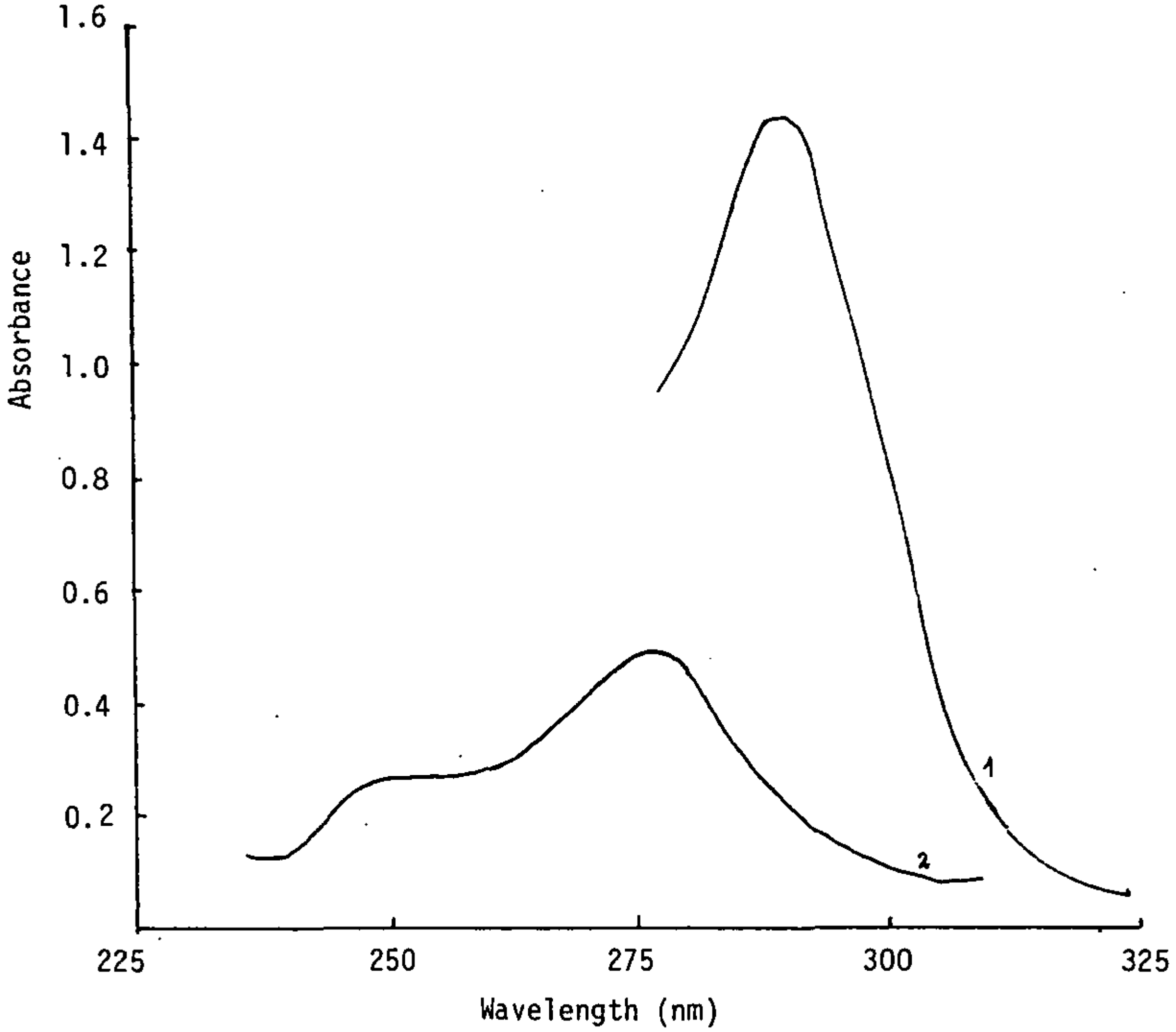
UV Spectra of dazomet

Curve 1: in carbon tetrachloride

Curve 2: fresh solution in distilled water

FIGURE 3.2

UV spectra of Na-MDC in distilled water


Absorbance
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
225
250
275
300
325
Wavelength (nm)
1
2

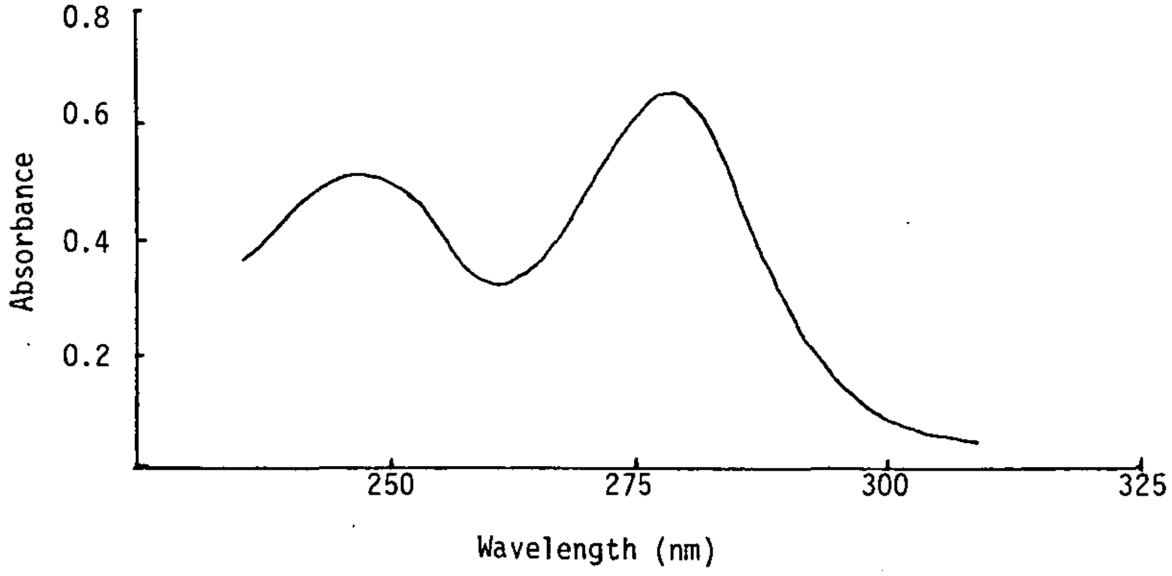

Absorbance
0.8
0.6
0.4
0.2
250
275
300
325
Wavelength (nm)

FIGURE 3.3

UV spectra of dazomet in distilled water at 25°C

Curve 1: fresh solution

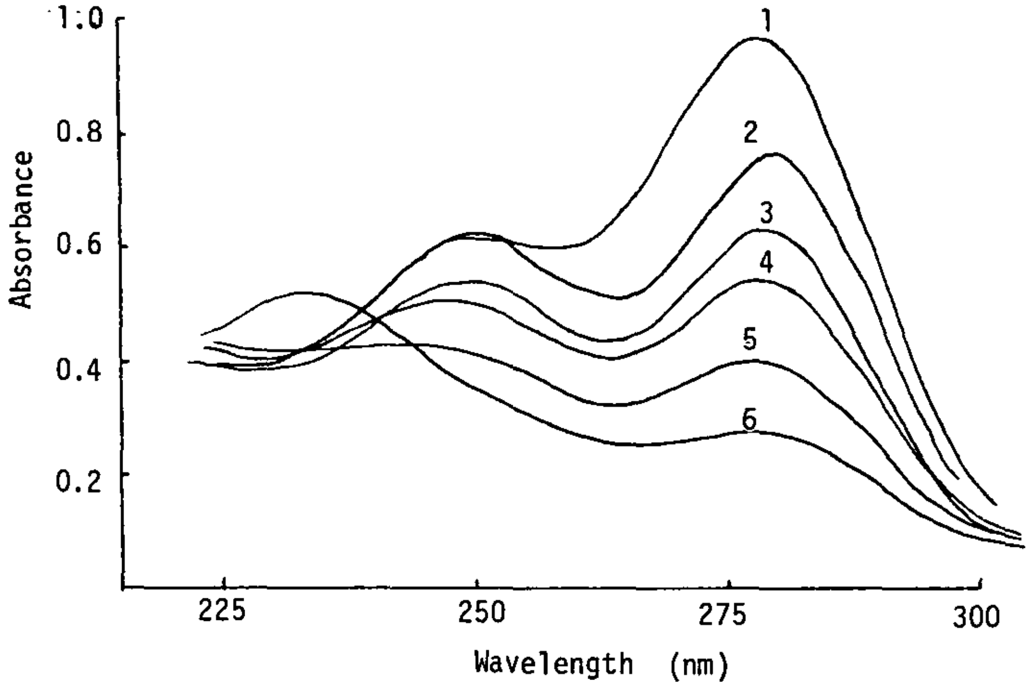
Curves 2-6: after 1, 2, 3, 4 and 5 days incubation, respectively

FIGURE 3.4

UV spectra of dazomet in distilled water at 37°C

Curve 1: fresh solution

Curves 2-6: after 1, 2, 3, 4 and 5 days, respectively


1.0
0.8
0.6
0.4
0.2
Absorbance
225
250
275
300
Wavelength (nm)
1
2
3
4
5
6

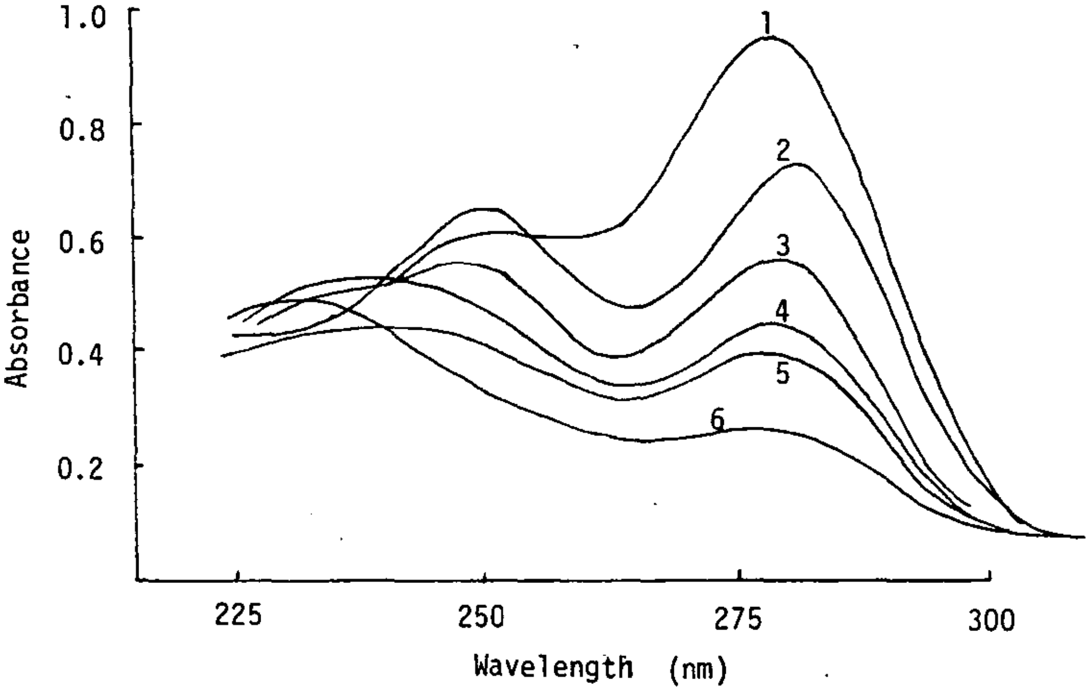

1.0
0.8
0.6
0.4
0.2
Absorbance
225
250
275
300
Wavelength (nm)
1
2
3
4
5
6

FIGURE 3.5

UV spectra of dazomet in distilled water at 45^{o}C

Curve 1: fresh solution

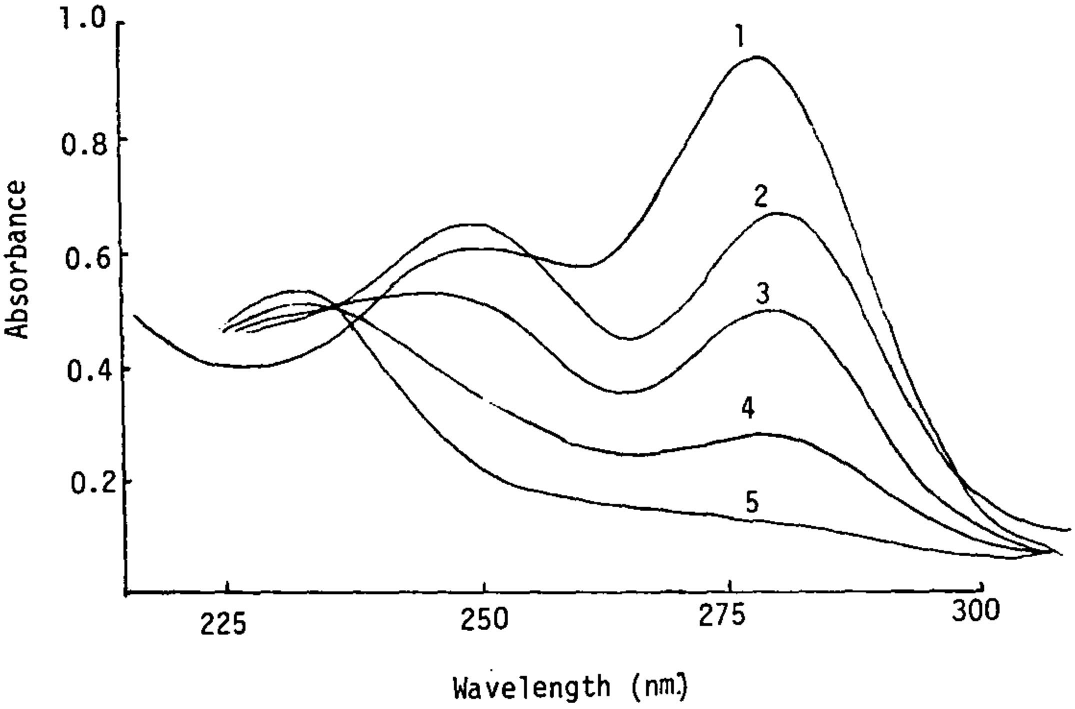
Curves 2-5: after 1, 2, 3, 4 and 5 days incubation, respectively

FIGURE 3.6

UV spectra of dazomet in distilled water at 55^{o}C

Curve 1: fresh solution

Curves 2-5: after 1, 2,3, 4 and 5 days incubation, respectively


1.0
0.8
0.6
0.4
0.2
Absorbance
225
250
275
300
Wavelength (nm)
1
2
3
4
5

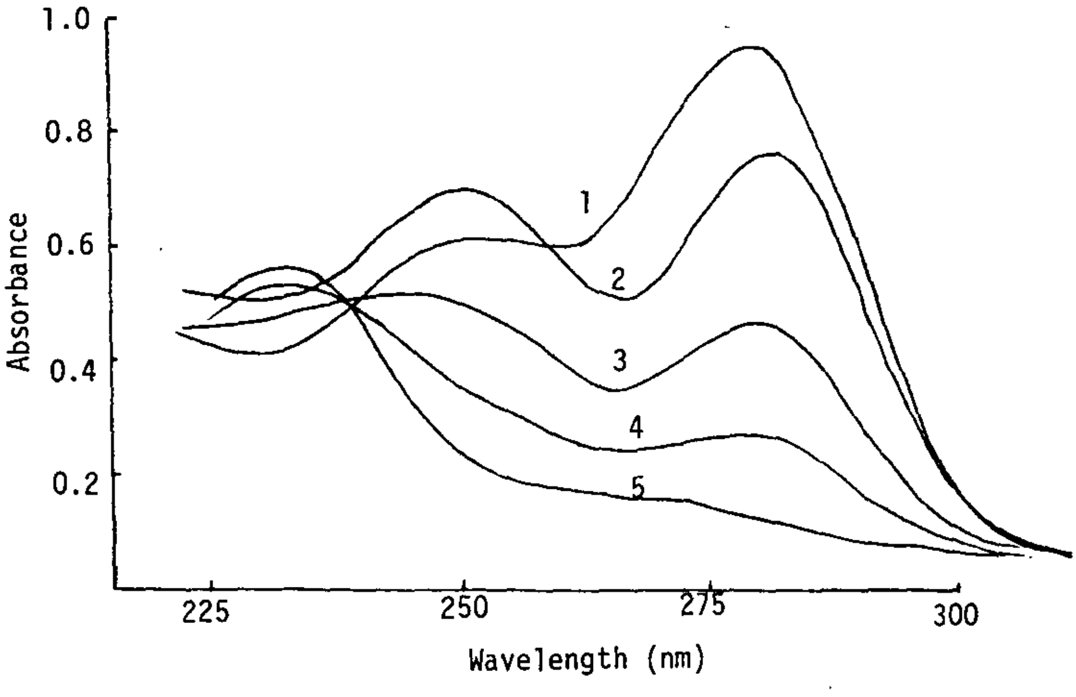

1.0
0.8
0.6
0.4
0.2
Absorbance
225
250
275
300
Wavelength (nm)
1
2
3
4
5

FIGURE 3.7

UV spectra of dazomet in distilled water at 70°C

Curve 1: fresh solution

Curves 2-8: after 0.5, 1, 2, 3, 4, 5 and 7 hours incubation, respectively

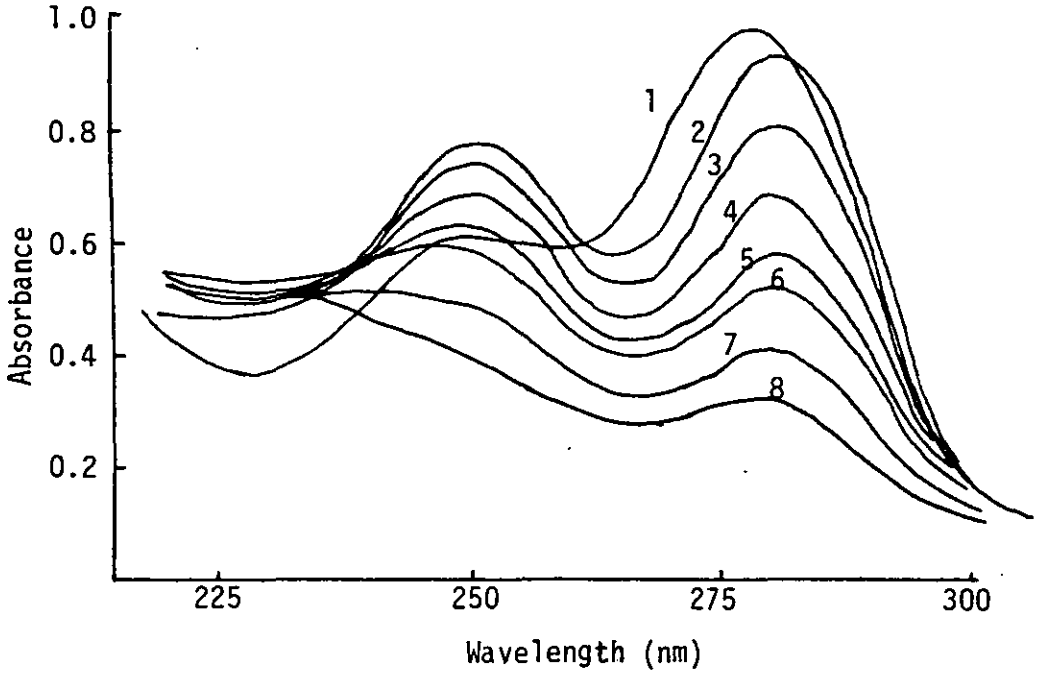
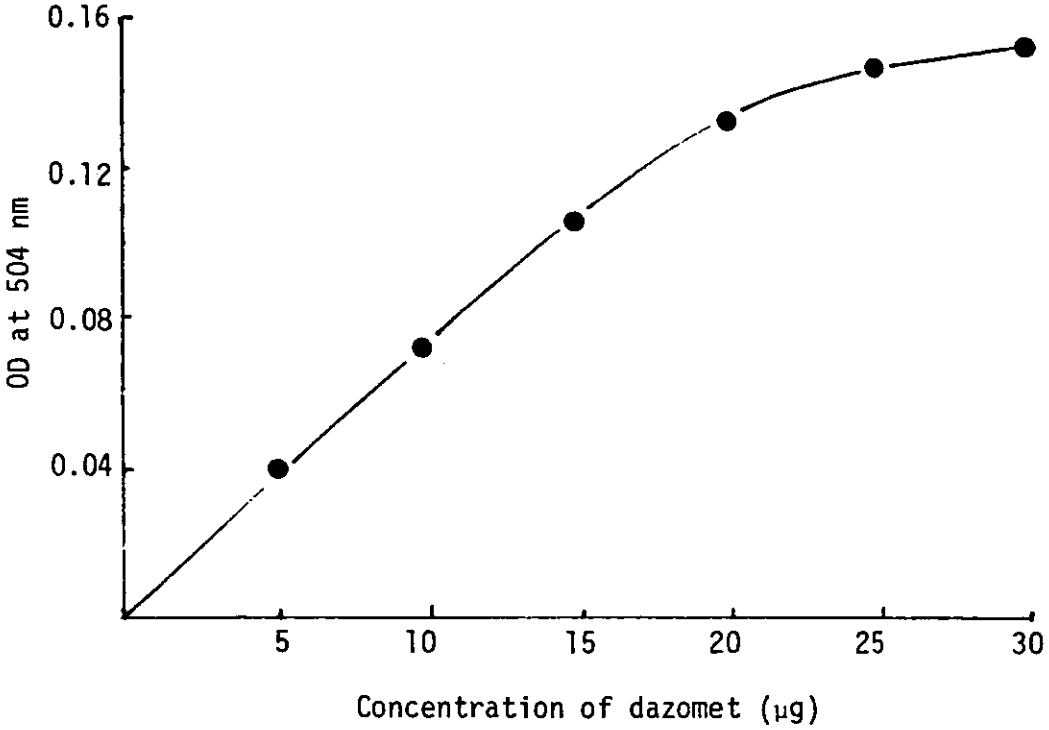

1.0
0.8
0.6
0.4
0.2
Absorbance
225
250
275
300
Wavelength (nm)
1
2
3
4
5
6
7
8

FIGURE 3.8

Calibration graph for the determination of dazomet by colorimetric procedure using paper chromatography

FIGURE 3.9

Degradation study of dazomet in distilled water at 55°C by colorimetric procedure using paper chromatography


0.16
0.12
0.08
0.04
OD at 504 nm
5
10
15
20
25
30
Concentration of dazomet (µg)

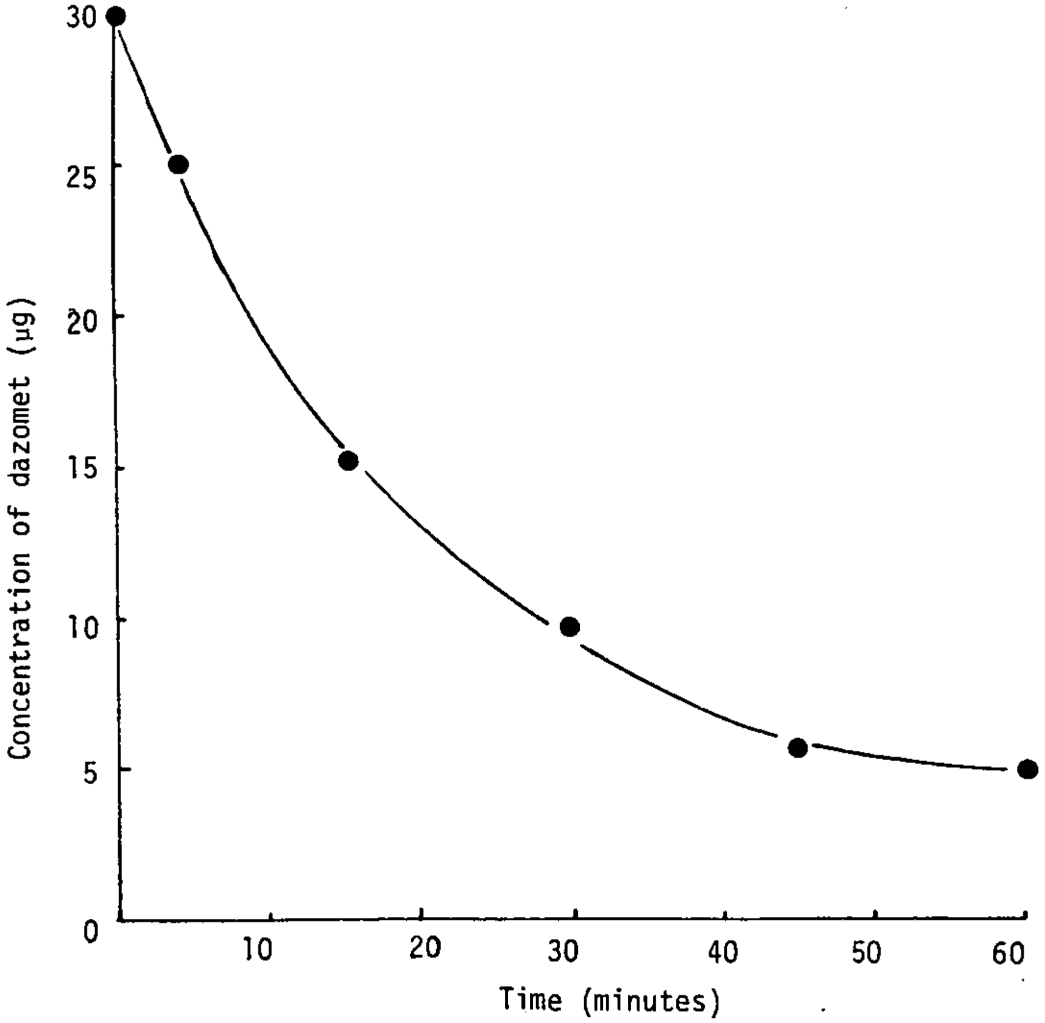

30
25
20
15
10
5
0
Concentration of dazomet (µg)
10
20
30
40
50
60
Time (minutes)

FIGURE 3.10

Calibration graph for the determination of formaldehyde using Nash's reagent

Slope = 0.222 OD units/μg.ml^{-1}

Correlation coefficient = 0.9998

Standard deviation = 0.010 μg.ml^{-1}

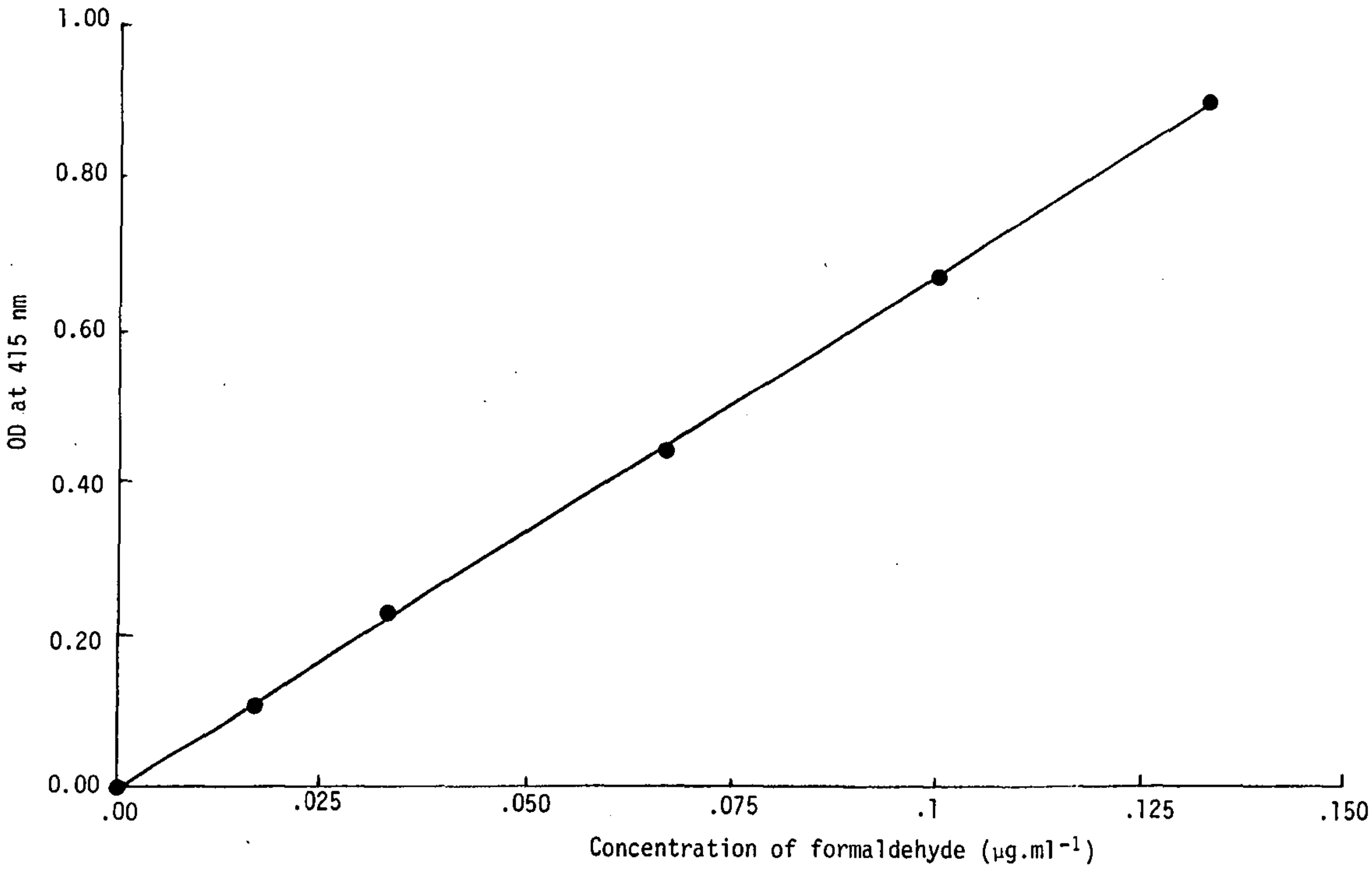

1.00
0.80
0.60
0.40
0.20
0.00
OD at 415 nm
.00
.025
.050
.075
.1
.125
.150
Concentration of formaldehyde (μg.ml^{-1})

FIGURE 3.11

Determination of formaldehyde in predegraded sample of dazomet using Nash's reagent

Curve A: standard formaldehyde solution ($4\ \mu g.ml^{-1}$; 0.133 mM)

Curve B: 0.133 mM solution of dazomet in distilled water predegraded at 55°C for 1½ hours

Incubation temperature = 55°C

Final absorbance readings: standard formaldehyde solution = 1.09;

predegraded dazomet solution = 1.865

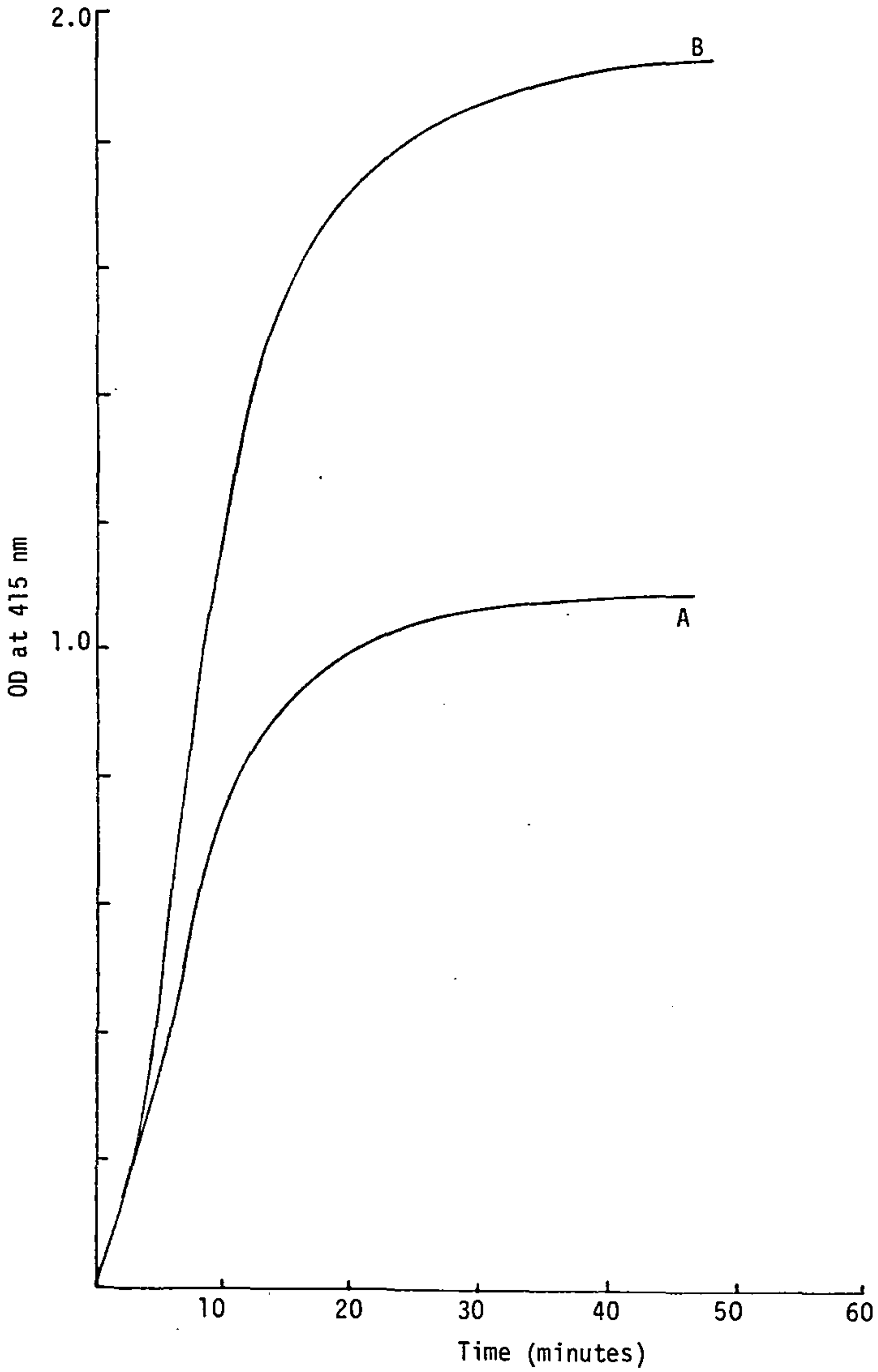

2.0
1.0
OD at 415 nm
B
A
10
20
30
40
50
60
Time (minutes)

FIGURE 3.12

Calibration graph for the determination of methylamine by fluorimetry

Excitation λ = 396 nm

Emission λ = 480 nm

Slope = 191.73 scale units/μg.ml^{-1}

Correlation coefficient = 0.9998

Standard deviation = 0.0047 μg.ml^{-1}

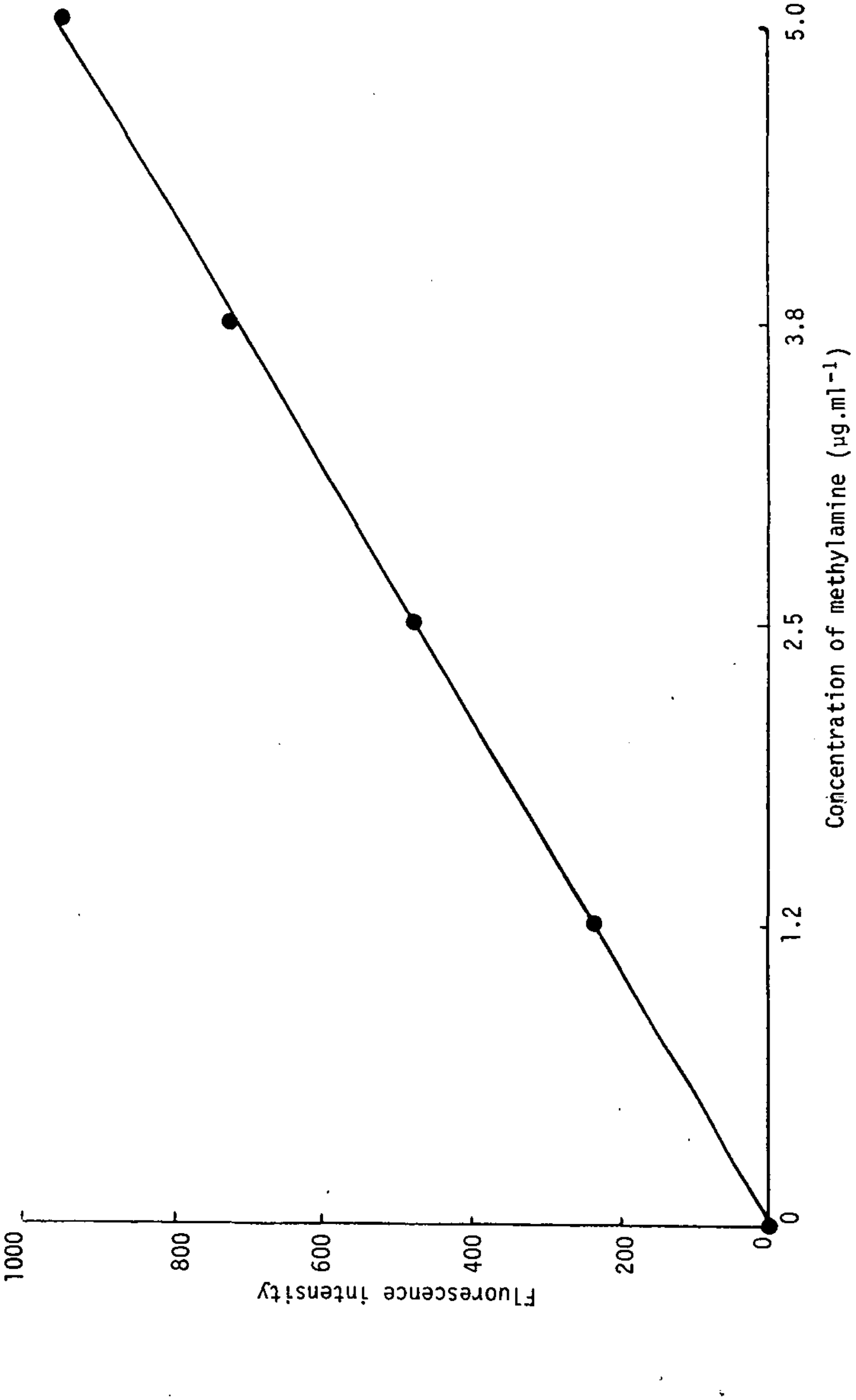

Fluorescence intensity
0
200
400
600
800
1000
Concentration of methylamine ($\mu g.ml^{-1}$)
0
1.2
2.5
3.8
5.0

FIGURE 3.13

Determination of dazomet (1000 µg.ml^{-1}; 6.173 mM) and its degradation products by HPLC using 30 + 70 methanol - 0.05M phosphate buffer (pH 6)

Sample of dazomet degraded in phosphate buffer (pH 7.0) at 55°C for 120 minutes

10 µl injection of sample solution containing caffeine (400 µg.ml^{-1}) as injection standard

I: dazomet (1.11); II: caffeine (1.81) III: MIT (2.70)

Figures in brackets are K' values[+]

Solvent flow rate = 0.9 ml.min^{-1}

Attenuation = X64

[+] Capacity factor - see Appendix 6.1 for definition

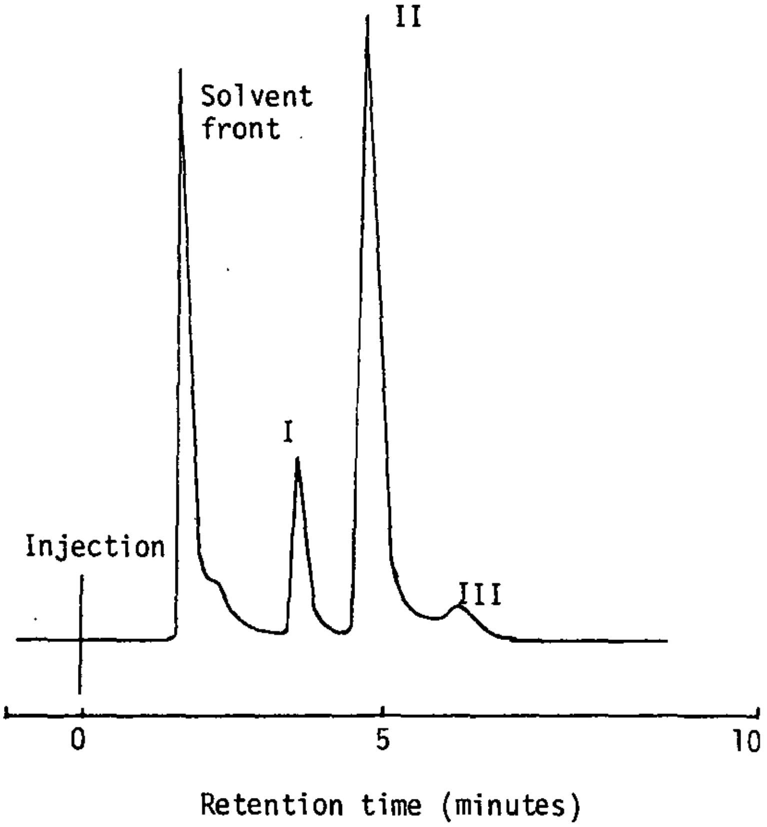

II
Solvent front
I
Injection
III
0
5
10
Retention time (minutes)

FIGURE 3.14

Determination of dazomet (1000 μg.ml^{-1}; 6.173 mM) and its degradation products by HPLC using 30 + 70 methanol - 0.05M phosphate buffer (pH 5.0) containing 0.1% cobalt (11) nitrate

Sample of dazomet degraded in phosphate buffer (pH 7.0) at 55°C for 150 minutes

10 μl injection of sample solution containing caffeine (400 μg.ml^{-1}) as injection standard

Solvent flow rate = 1.5 ml min^{-1}

Attenuation = X64 except III = X04

I: dazomet (1.25); II: caffeine (2.0); III: MIT (3.13); IV: DMTD (13.25); V: MDC (18.88)

Figures in brackets are K' values[+]

[+] Capacity factor - see Appendix 6.1 for definition

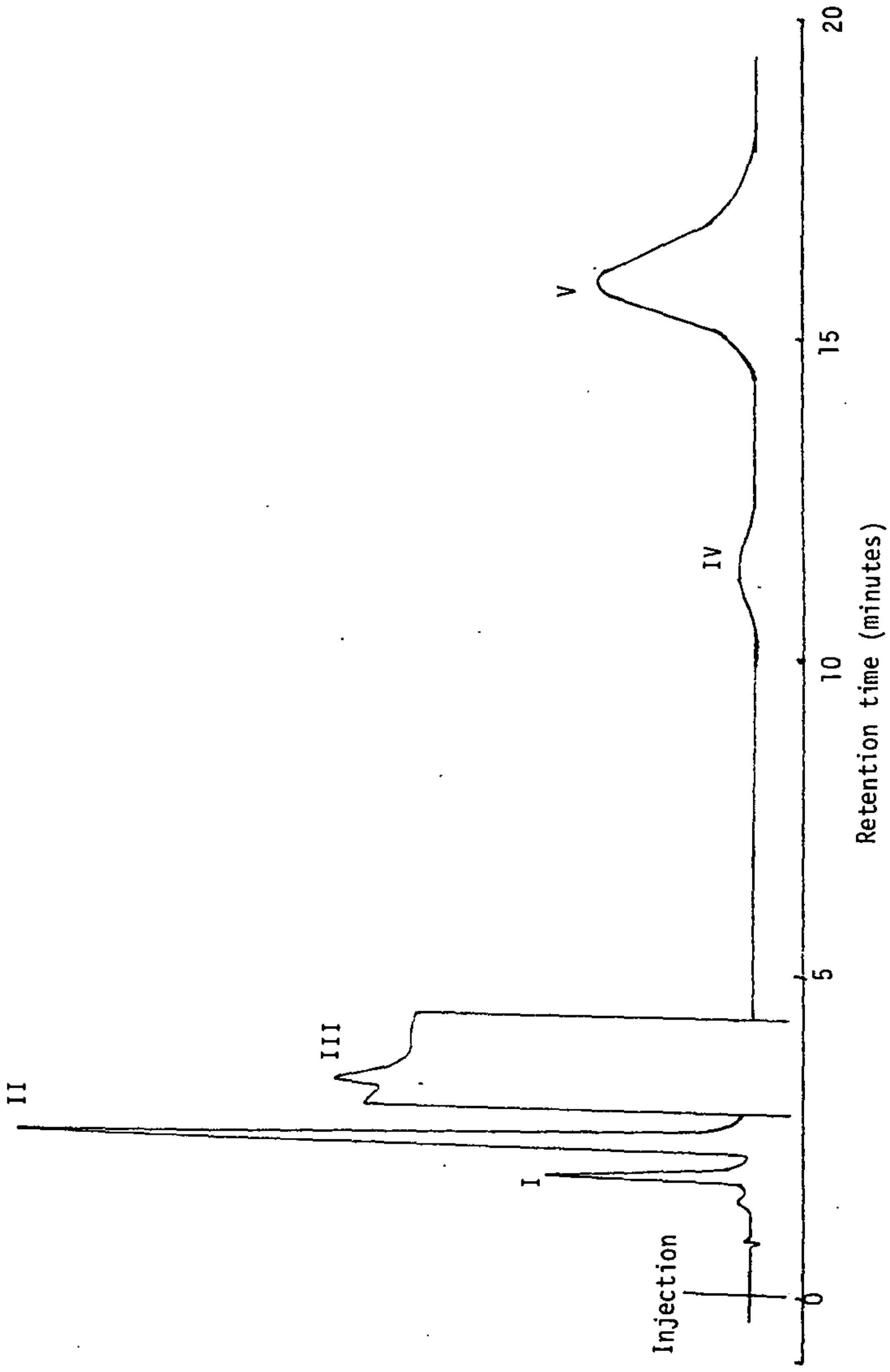

Injection
I
II
III
IV
V
0
5
10
15
20
Retention time (minutes)

FIGURE 3.15

Calibration graph for the determination of dazomet by HPLC using 30 + 70 methanol - 0.05M phosphate buffer (pH 5.0) containing 0.1% cobalt nitrate

Slope = 0.0045 scale units/$\mu g.ml^{-1}$

Correlation coefficient = 0.9998

Standard deviation = 2.696 $\mu g.ml^{-1}$

$^{+}$ 10 µl injection of sample containing caffeine (800 $\mu g.ml^{-1}$) in pH 7.0 phosphate buffer

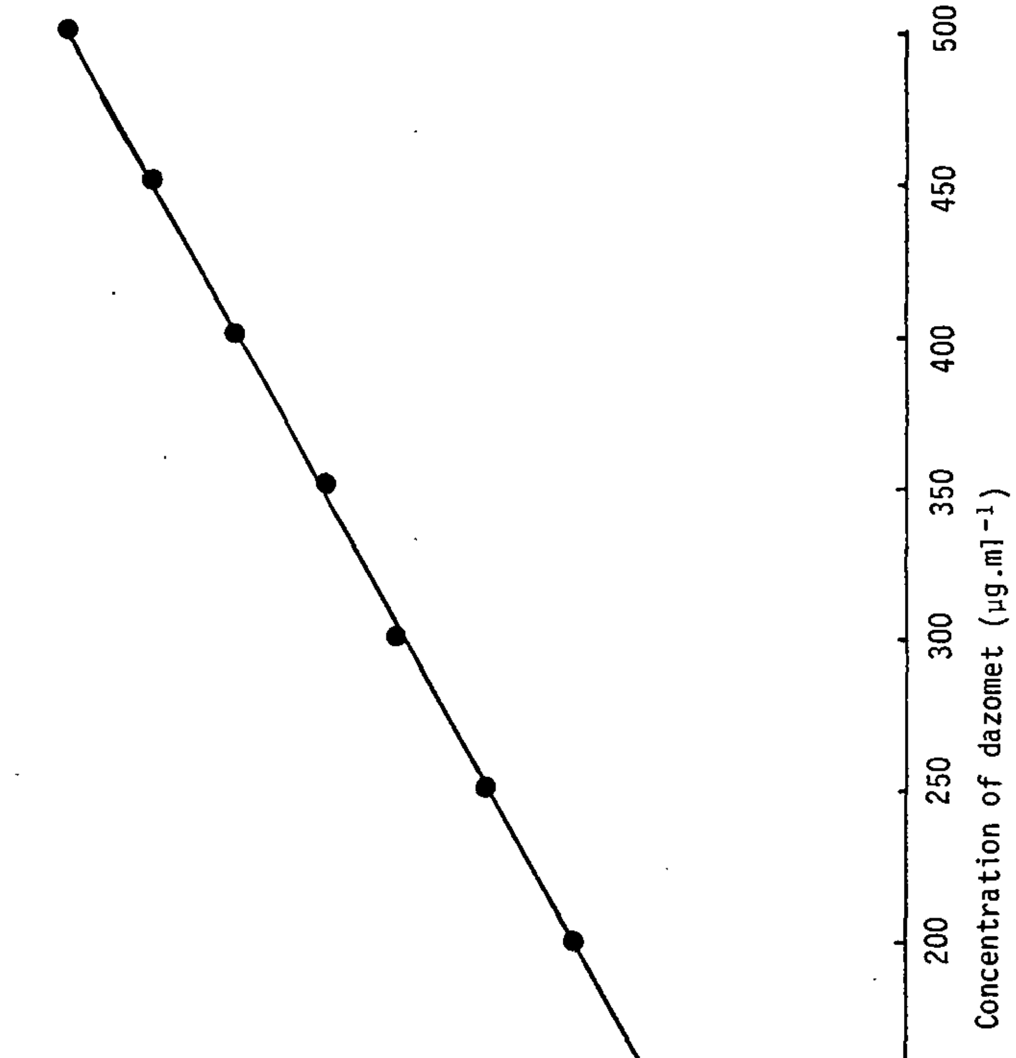

Peak height ratio $\left(\frac{\text{dazomet}}{\text{caffeine}}\right)$ +
0.00
0.50
1.00
1.50
2.00
2.50
Concentration of dazomet (μg.ml^{-1})
0
50
100
150
200
250
300
350
400
450
500

FIGURE 3.16

Calibration graph for the determination of MDC by HPLC using 30 + 70 methanol-0.05M phosphate buffer (pH 5.0) containing 0.1% cobalt nitrate

Slope = 0.0005 scale units/μg.ml^{-1}

Correlation coefficient = 0.9991

Standard deviation = 14.76 μg.ml^{-1}

Intercept = -0.0111 scale units

+ 10 μl injection of sample containing caffeine (800 μg.ml^{-1}) in pH 7.0 phosphate buffer

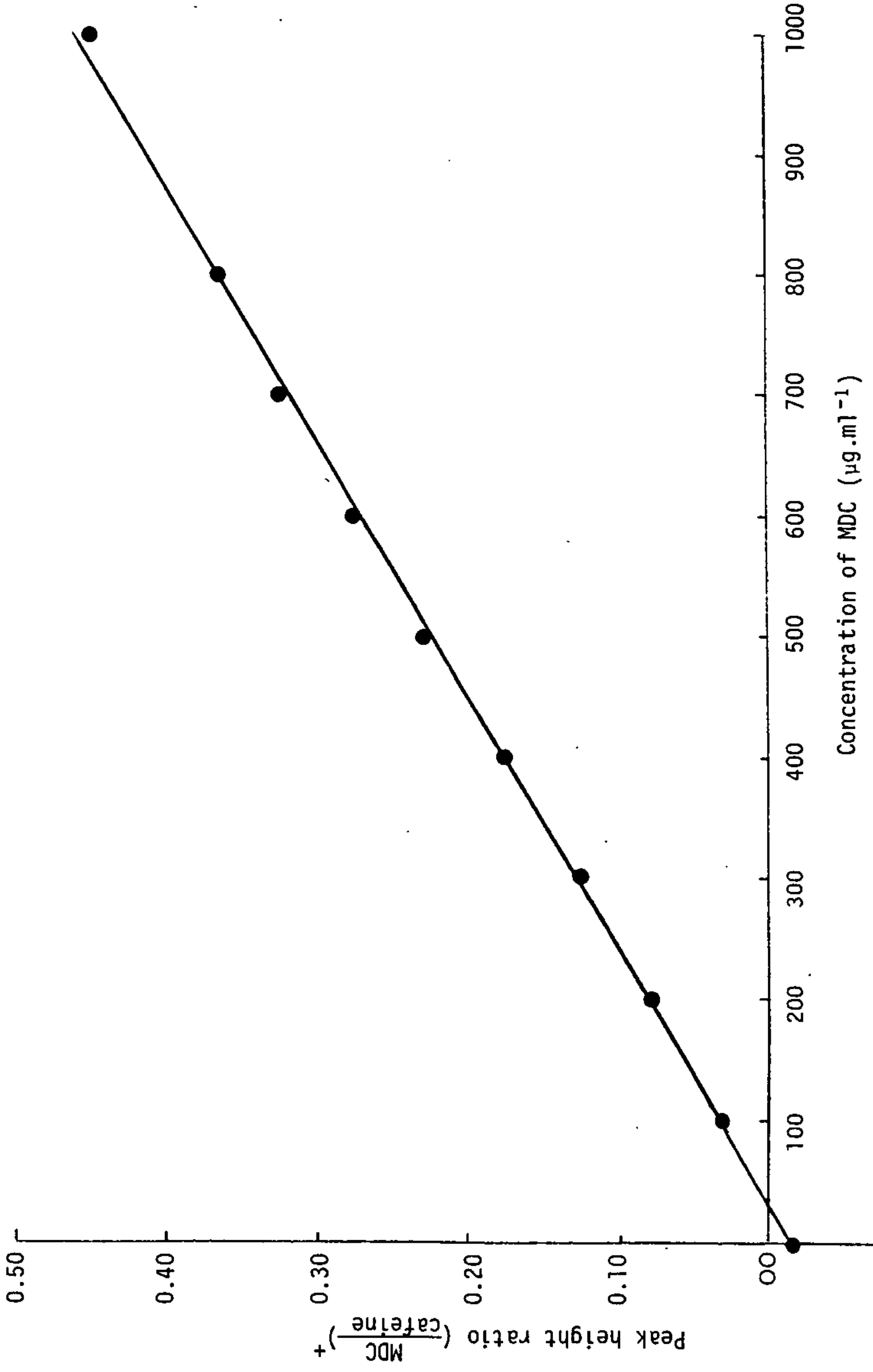

Peak height ratio $(\frac{MDC}{caffeine})^+$
0.50
0.40
0.30
0.20
0.10
OO
-0.10
100
200
300
400
500
600
700
800
900
1000
Concentration of MDC (μg.ml^{-1})

FIGURE 3.17

Calibration graph for the determination of MIT by HPLC using 30 + 70 methanol-0.05M phosphate buffer (pH 5.0) containing 0.1% cobalt nitrate

Slope = 0.00205 scale units/μg.ml^{-1}

Correlation coefficient = 0.9994

Standard deviation = 0.535 μg.ml^{-1}

+ 10 μl injection of sample containing caffeine (200 μg.ml^{-1}) in pH 7.0 phosphate buffer

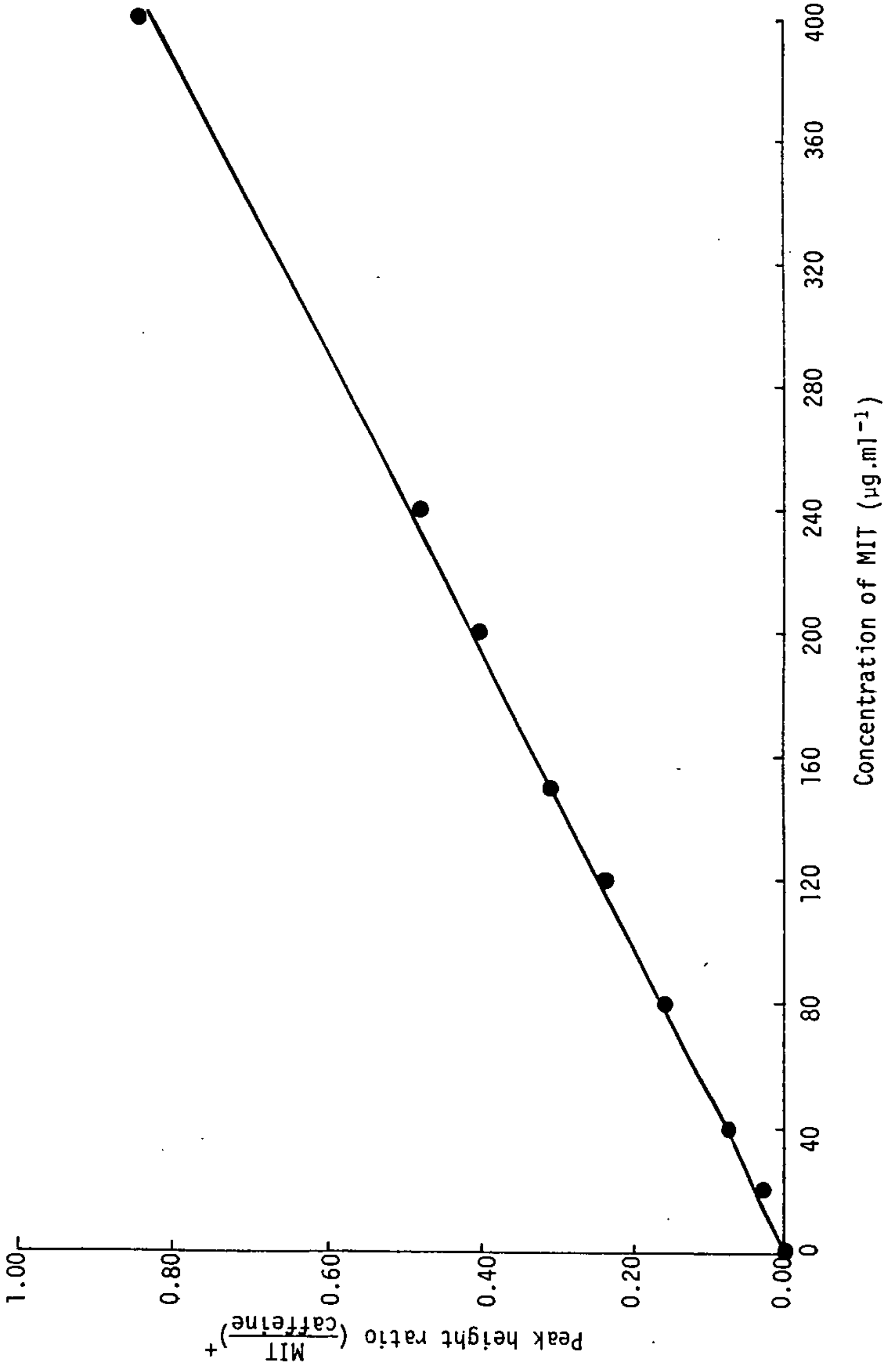

Peak height ratio $(\frac{MIT}{caffeine})^+$
0.00
0.20
0.40
0.60
0.80
1.00
Concentration of MIT ($\mu g.ml^{-1}$)
0
40
80
120
160
200
240
280
320
360
400

FIGURE 3.18

Degradation study of dazomet by HPLC in pH 5 phosphate buffer at 55°C using 30 + 70 methanol - 0.05M phosphate buffer (pH 5.0) containing 0.1% cobalt nitrate

○ dazomet

□ MDC

△ MIT

◇ methylamine$^+$

▽ D*

— solid line represents theoretical profiles using the kinetic model

* represents all other products not quantified

+ determined by fluorimetry

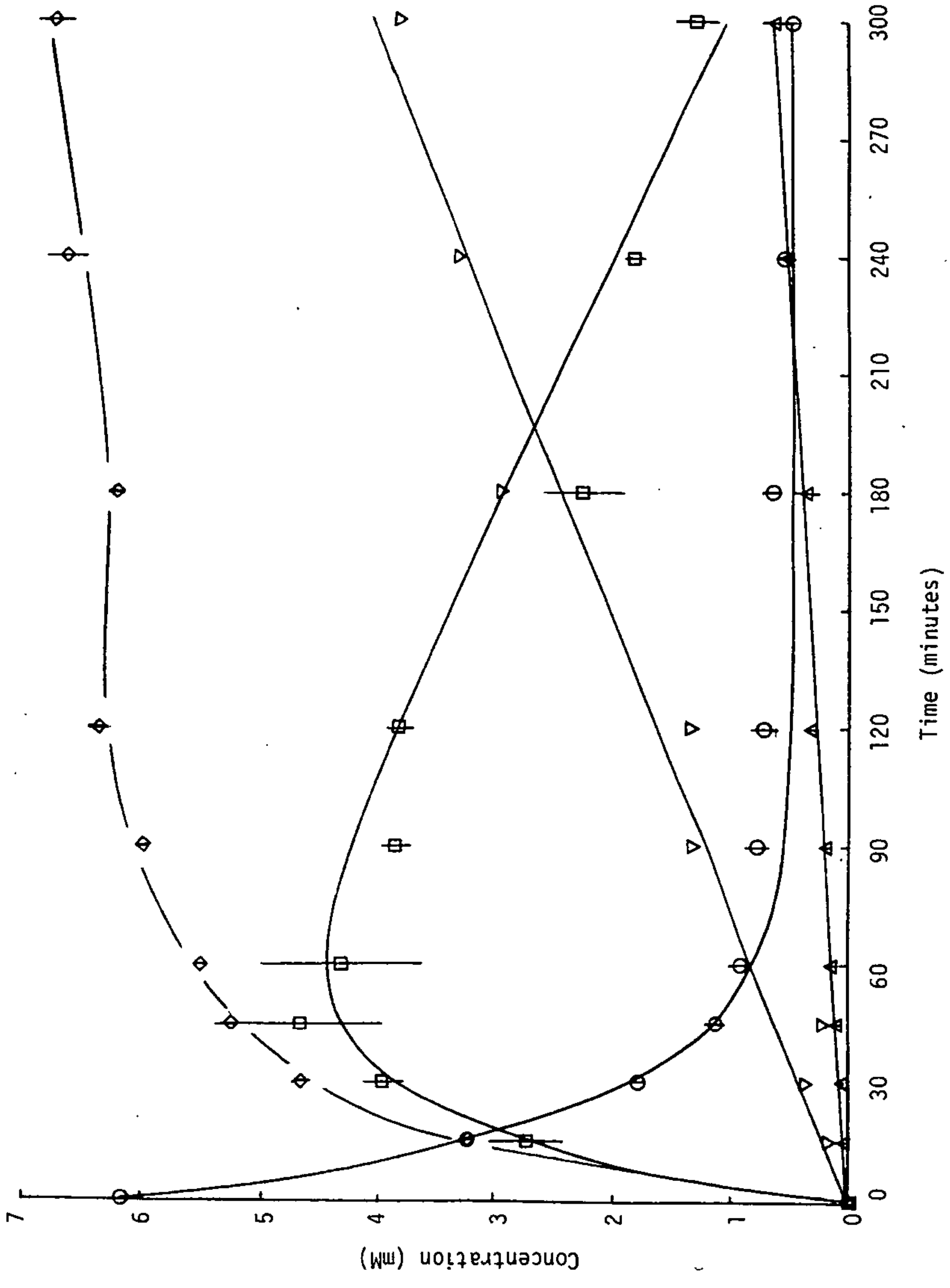

Concentration (mM)
0
1
2
3
4
5
6
7
Time (minutes)
0
30
60
90
120
150
180
210
240
270
300

FIGURE 3.19

Degradation study of dazomet by HPLC in pH 6 phosphate buffer at 55°C using 30 + 70 methanol - 0.05M phosphate buffer (pH 5.0) containing 0.1% cobalt nitrate

○ dazomet

□ MDC

△ MIT

◇ methylamine+

▽ D*

— solid line represents theoretical profiles using the kinetic model

* represents all other products not quantified

+ determined by fluorimetry

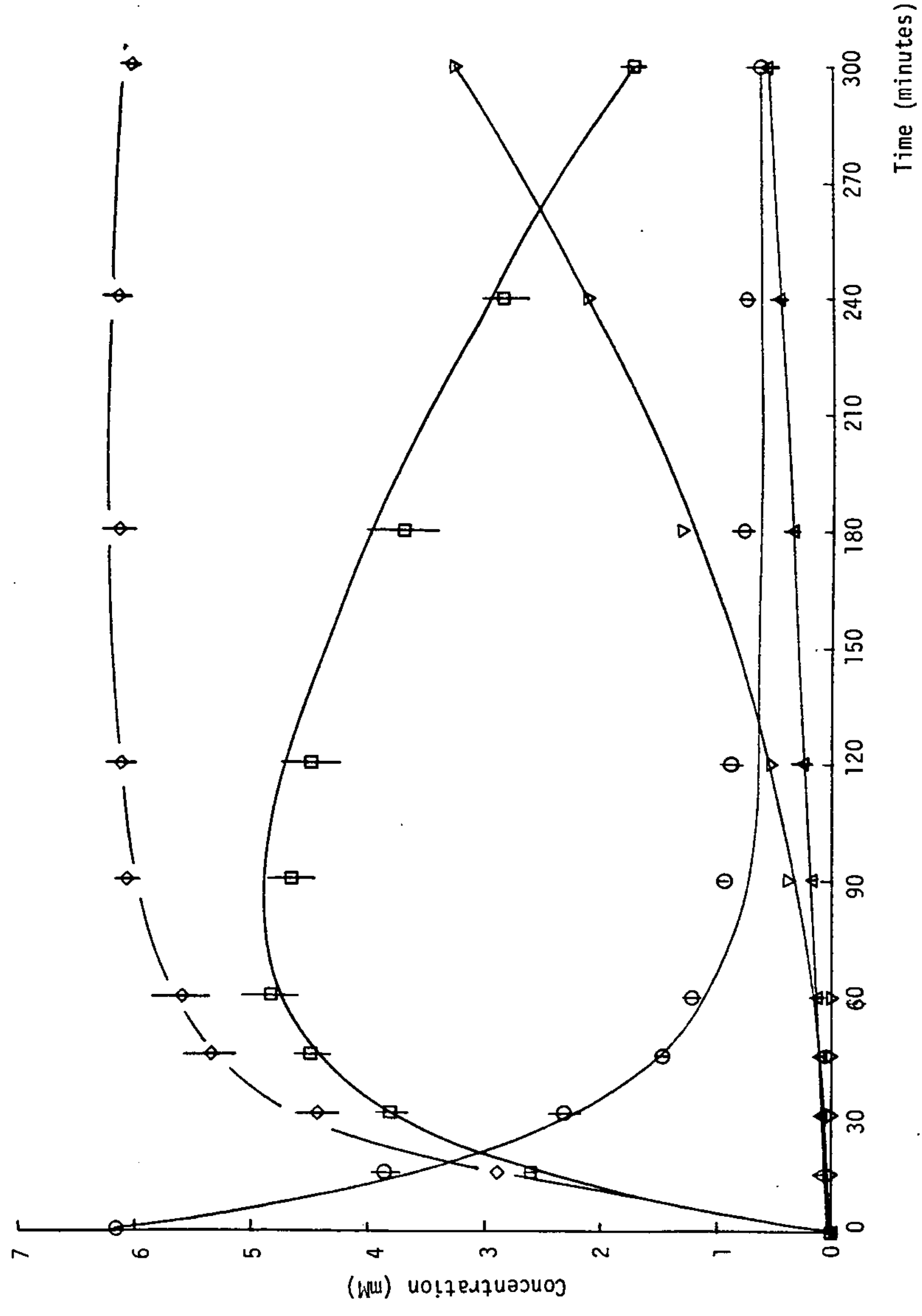

Concentration (mM)
0
1
2
3
4
5
6
7
Time (minutes)
0
30
60
90
120
150
180
210
240
270
300

FIGURE 3.20

Degradation study of dazomet by HPLC in pH 7 phosphate buffer at 55°C using 30 + 70 methanol - 0.05 M phosphate buffer (pH 5.0) containing 0.1% cobalt nitrate

○ dazomet

□ MDC

△ MIT

◇ methylamine+

▽ D*

— solid line represents theoretical profile using the kinetic model

* represents all other products not quantified

+ determined by fluorimetry

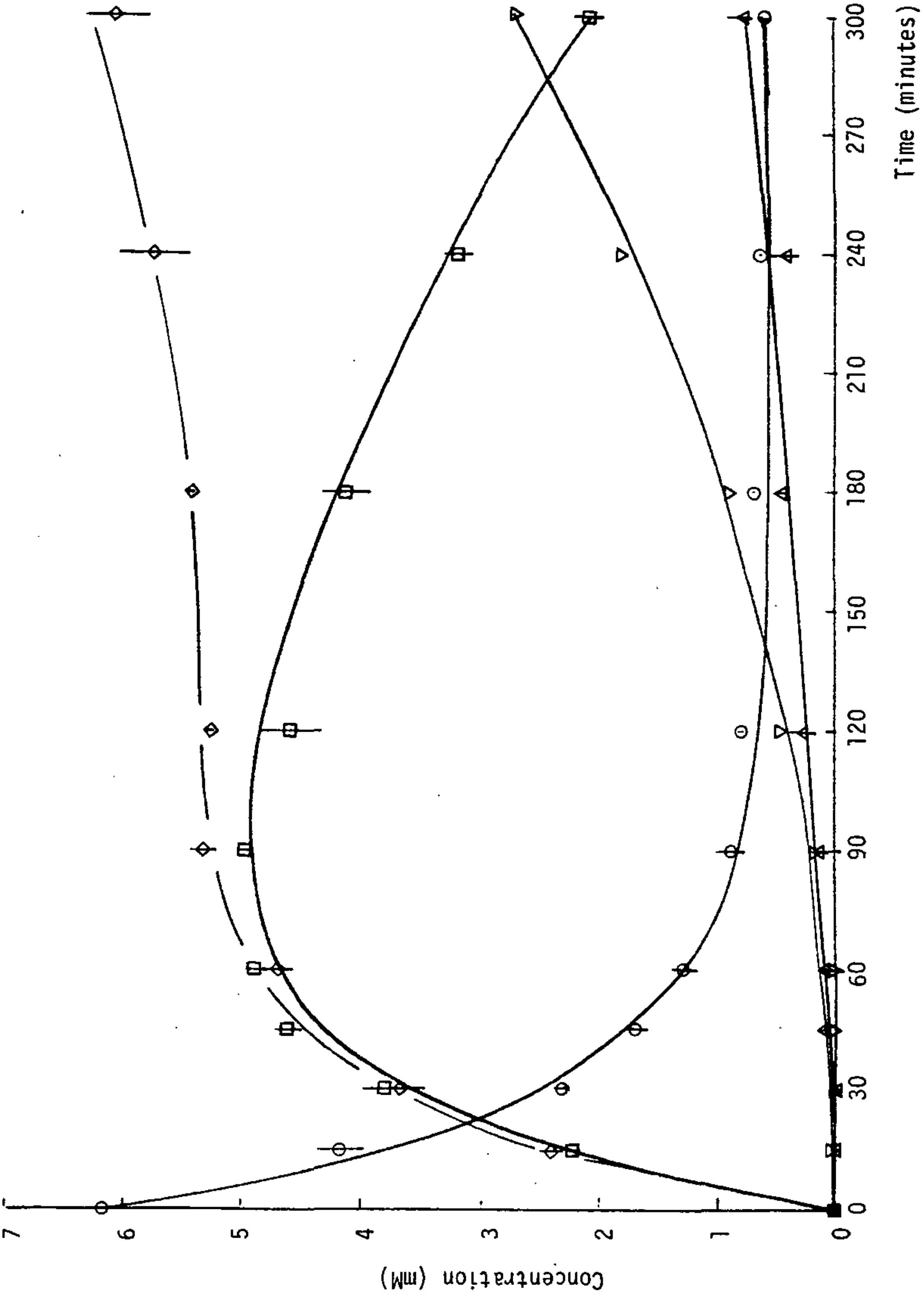

Concentration (mM)
0
1
2
3
4
5
6
7
Time (minutes)
0
30
60
90
120
150
180
210
240
270
300

FIGURE 3.21

Degradation study of dazomet by HPLC in pH 7.5 phosphate buffer at 55°C using 30 + 70 methanol - 0.05M phosphate buffer (pH 5.0) containing 0.1% cobalt nitrate

○ dazomet

□ MDC

△ MIT

◇ methylamine+

▽ D*

— solid line represents theoretical profile using the kinetic model

* represents all other products not quantified

+ determined by fluorimetry

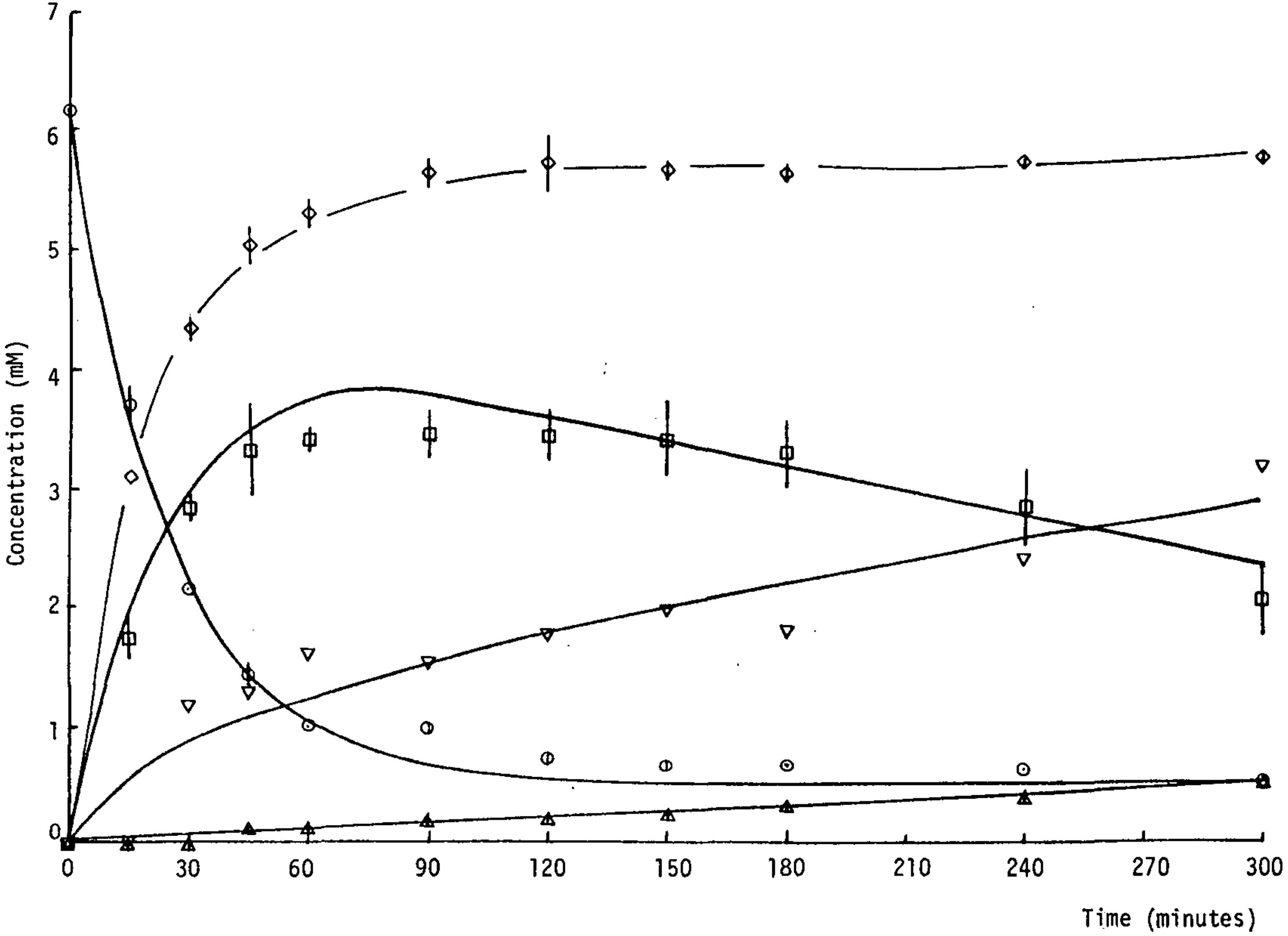

Concentration (mM)
0
1
2
3
4
5
6
7
0
30
60
90
120
150
180
210
240
270
300
Time (minutes)

FIGURE 3.22

Identification of sulphur in a degraded[+] sample of dazomet by UV spectroscopy

Curve 1: UV spectra of precipitate formed during degradation of dazomet

Curve 2: UV spectra of elemental sulphur

+ Degraded in pH 7 phosphate buffer for 5 hours at 55°C

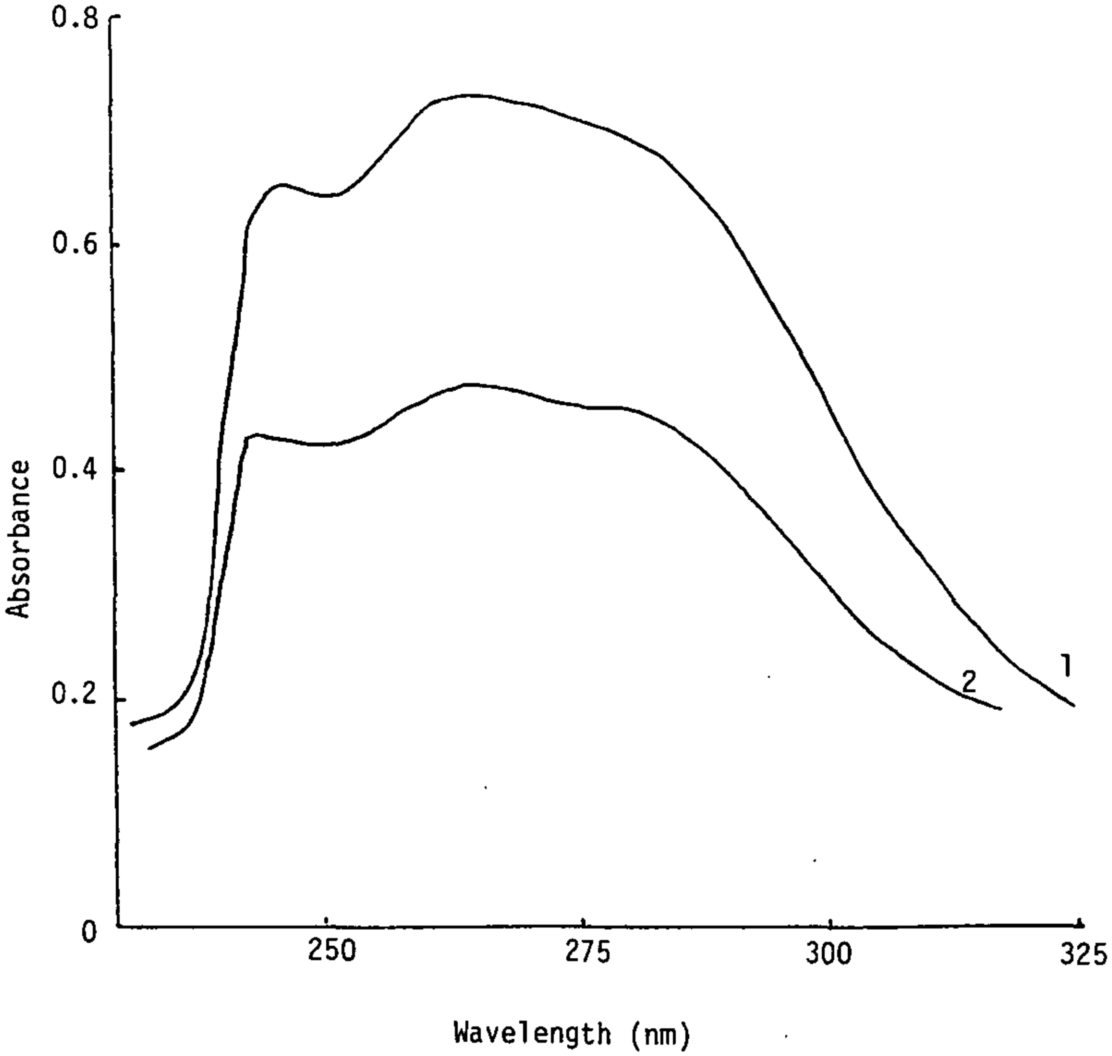

0.8
0.6
0.4
0.2
0
Absorbance
250
275
300
325
Wavelength (nm)
1
2

FIGURE 3.23

Degradation study of dazomet (500 µg.ml^{-1}; 3.086 mM) in minimal salts media at 37°C

●-● dazomet

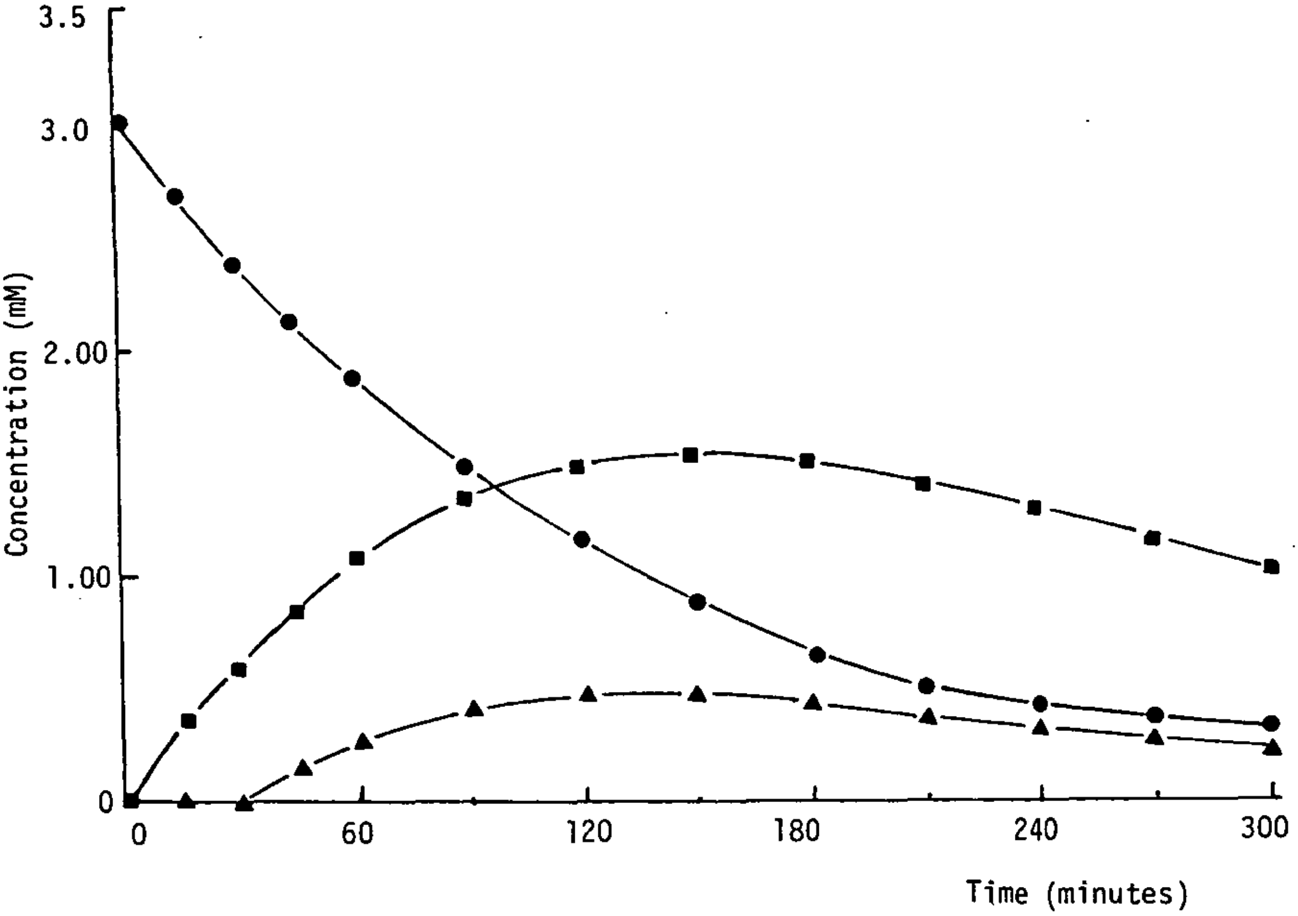
■-■ MDC

▲-▲ MIT

FIGURE 3.24

Degradation study of dazomet (500µg.ml^{-1}; 3.086 mM) in minimal salts media at 37°C in the presence of *B.licheniformis*

Symbols are the same as in Figure 3.23


3.5
3.0
2.00
1.00
0
Concentration (mM)
0
60
120
180
240
300
Time (minutes)

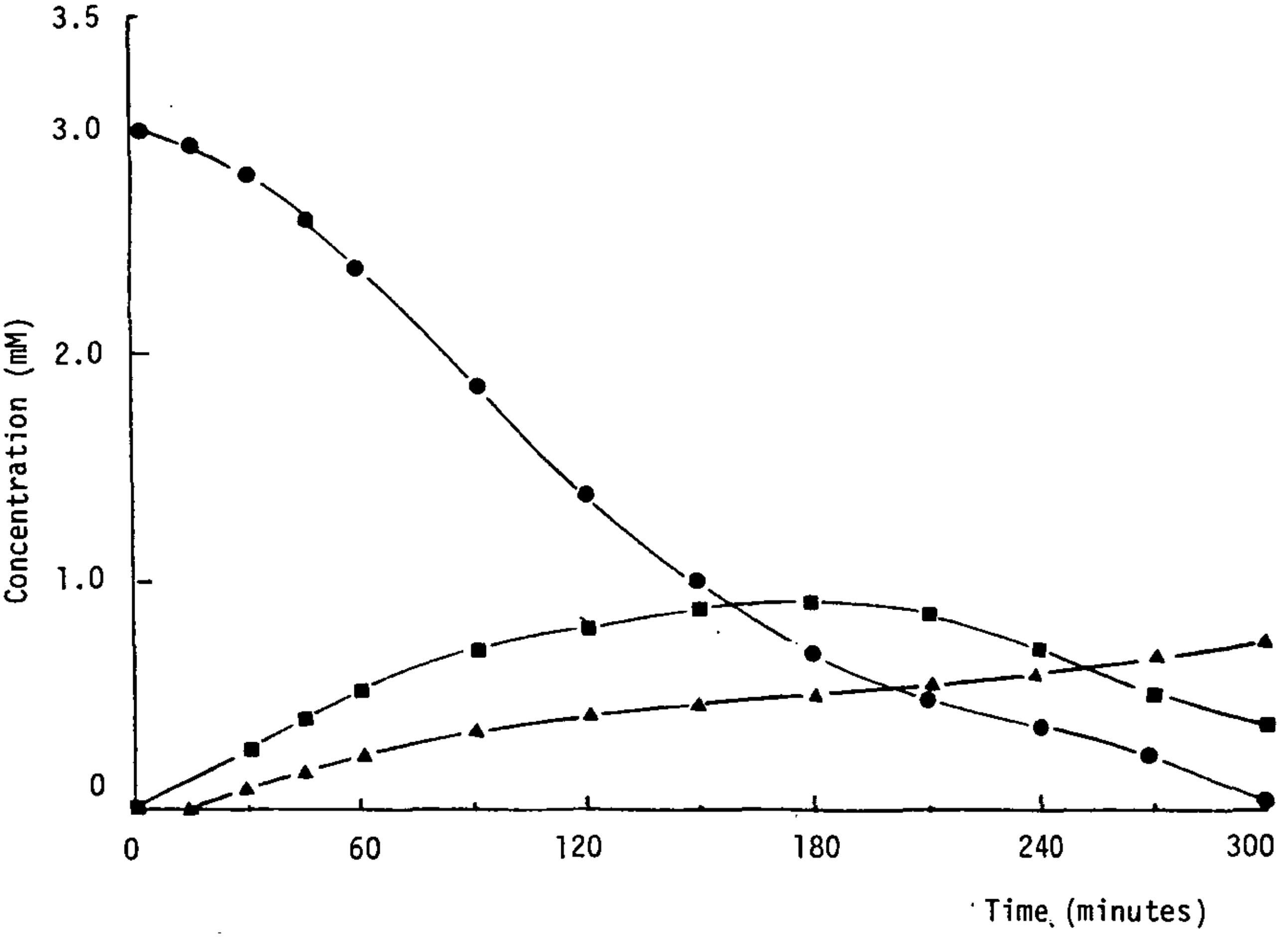

3.5
3.0
2.0
1.0
0
Concentration (mM)
0
60
120
180
240
300
Time (minutes)

FIGURE 3.25

Degradation study of dazomet (500 µg.ml^{-1}; 3.086 mM) in minimal salts media at 37°C in the presence of *P.aeruginosa* NCTC 6749

●-● dazomet

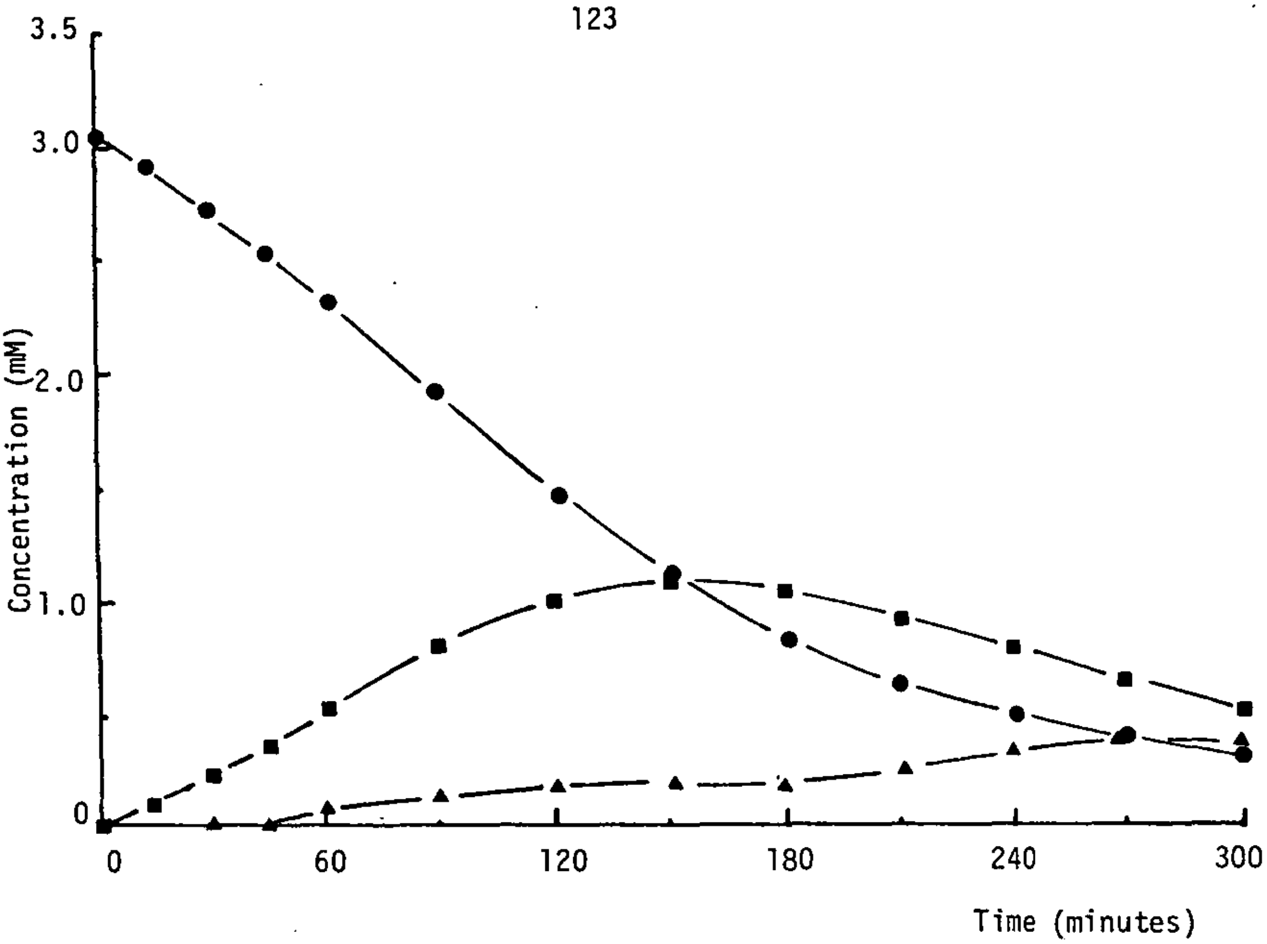
■-■ MDC

▲-▲ MIT

FIGURE 3.26

Degradation study of dazomet (100 µg.ml^{-1}; 0.617 mM) in minimal salts media at 37°C

Symbols are the same as in Figure 3.25


3.5
3.0
2.0
1.0
0
Concentration (mM)
0
60
120
180
240
300
Time (minutes)

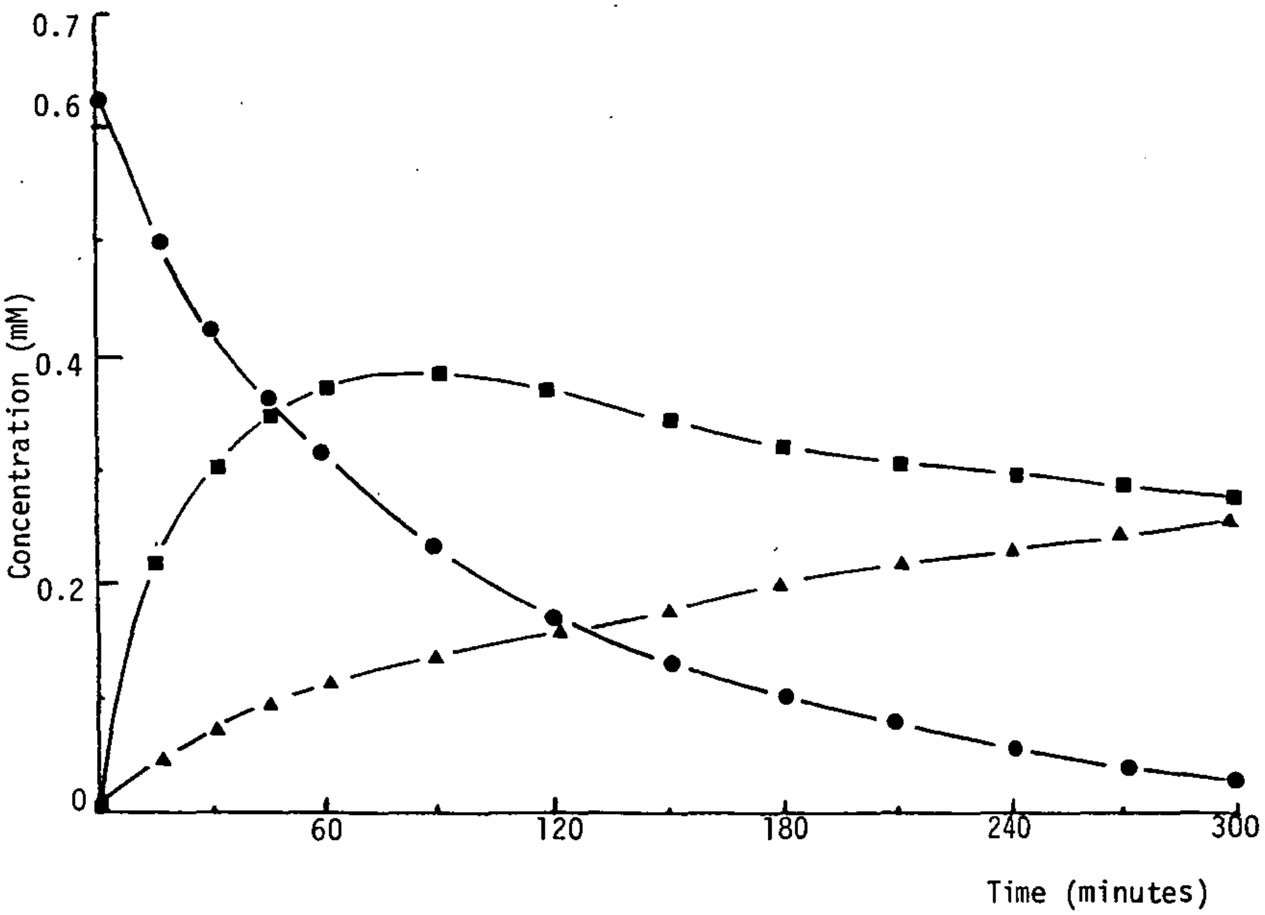

0.7
0.6
0.4
0.2
0
Concentration (mM)
60
120
180
240
300
Time (minutes)

FIGURE 3.27

Degradation study of dazomet (100 µg.ml^{-1}; 0.617 mM) in minimal salts media at 37°C in the presence of B.licheniformis

●-● dazomet

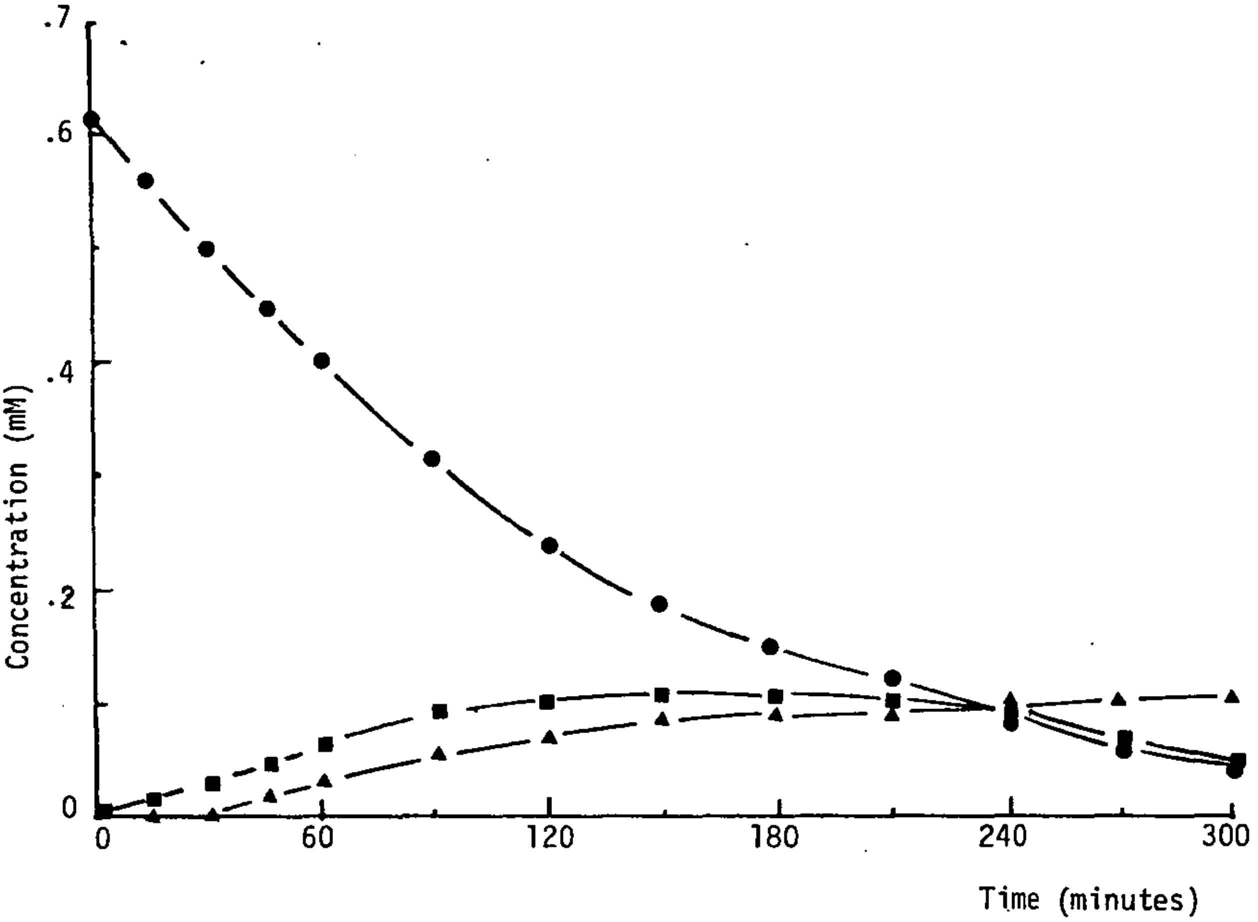
■-■ MDC

▲-▲ MIT

FIGURE 3.28

Degradation study of dazomet (100 µg.ml^{-1}; 0.617 mM) in minimal salts media at 37° in the presence of P.aeruginosa NCTC 6749

Symbols are the same as in Figure 3.27


Concentration (mM)
.7
.6
.4
.2
0
0
60
120
180
240
300
Time (minutes)

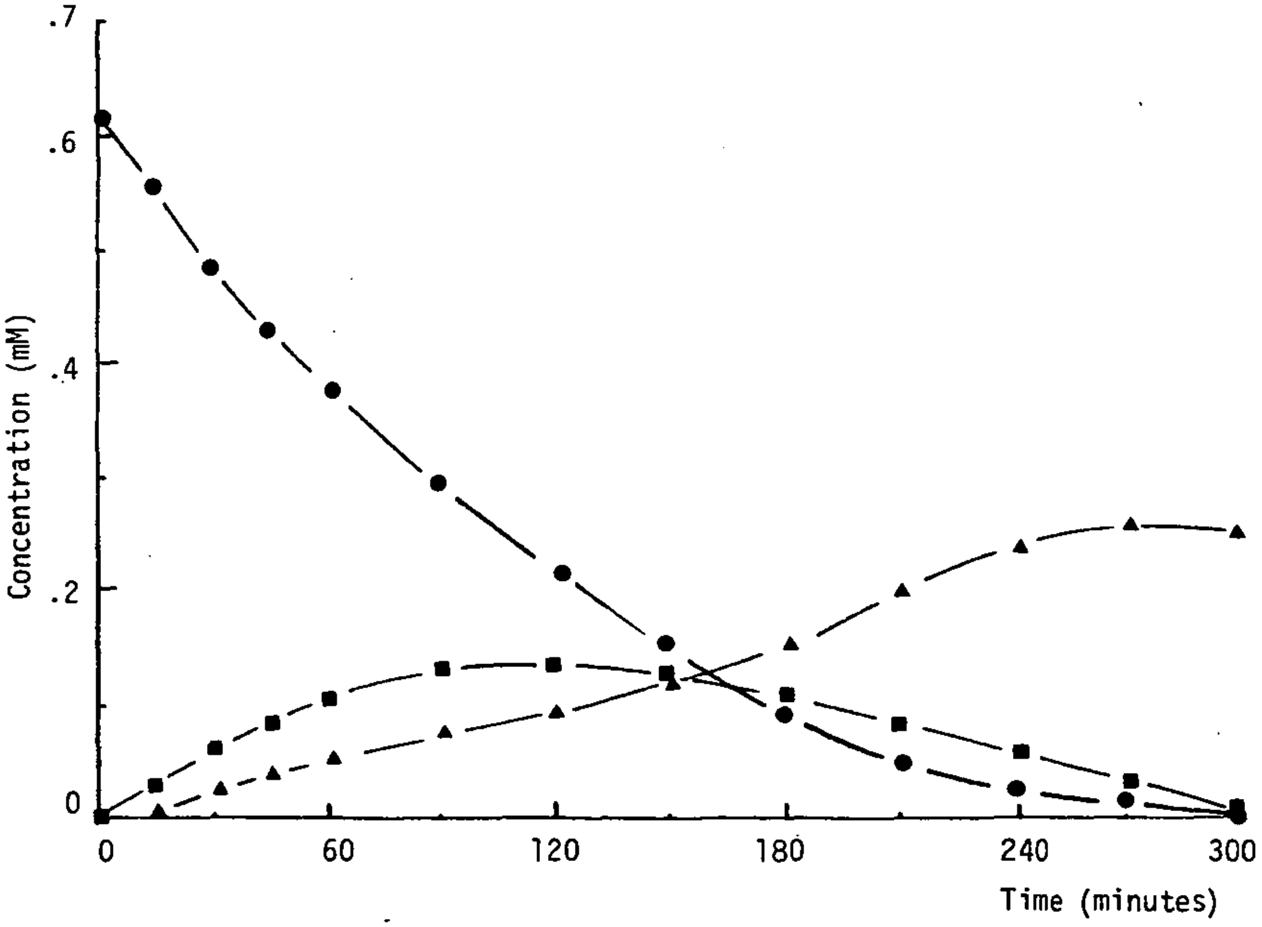

Concentration (mM)
.7
.6
.4
.2
0
0
60
120
180
240
300
Time (minutes)

FIGURE 3.29

Degradation study of dazomet (50 μg.ml^{-1}; 0.309 mM) in minimal salts media at 37^{o}C

●-● dazomet

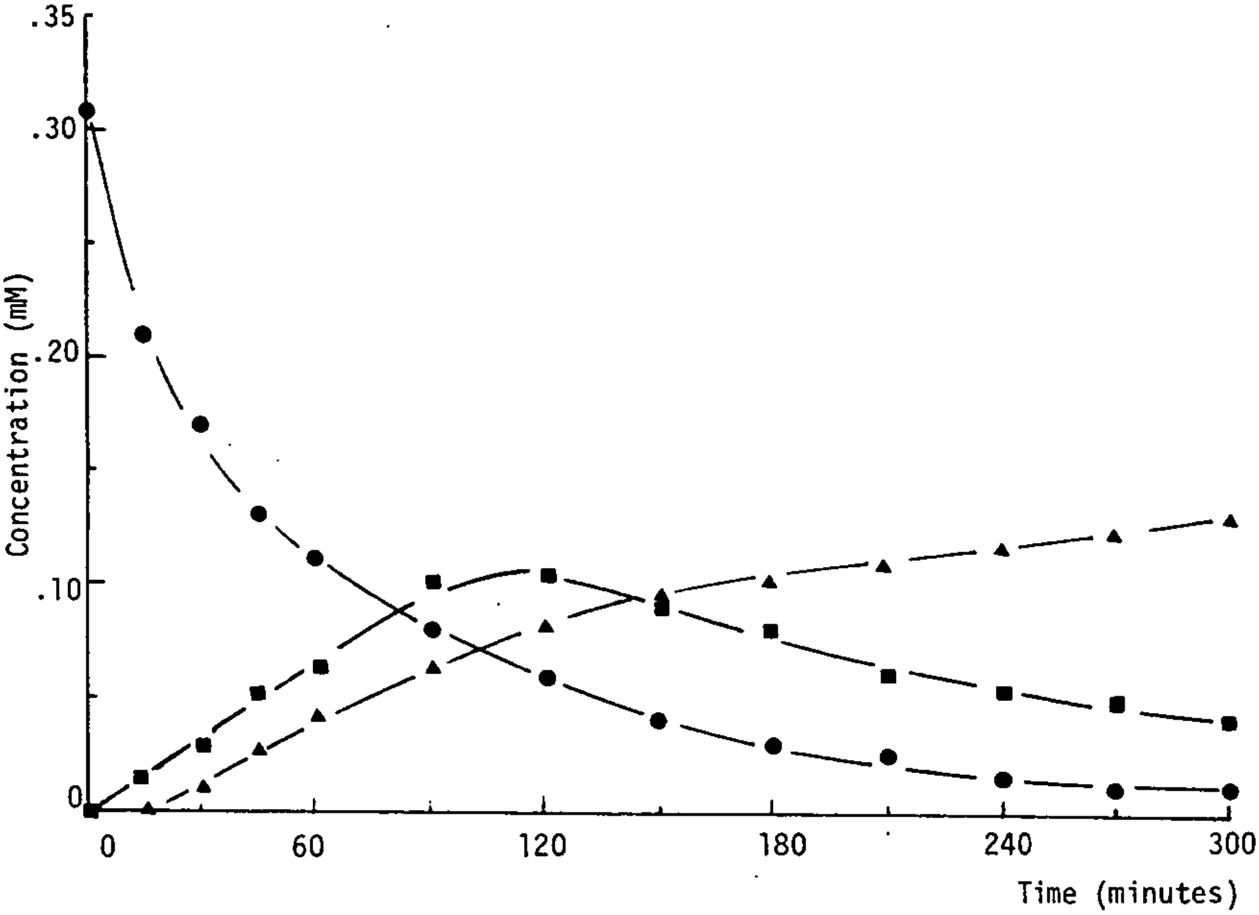
■-■ MDC

▲-▲ MIT

FIGURE 3.30

Degradation study of dazomet (50 μg.ml^{-1}; 0.309 mM) in minimal salts media in the presence of B.licheniformis

Symbols are the same as in Figure 3.29


.35
.30
.20
.10
0
Concentration (mM)
0
60
120
180
240
300
Time (minutes)

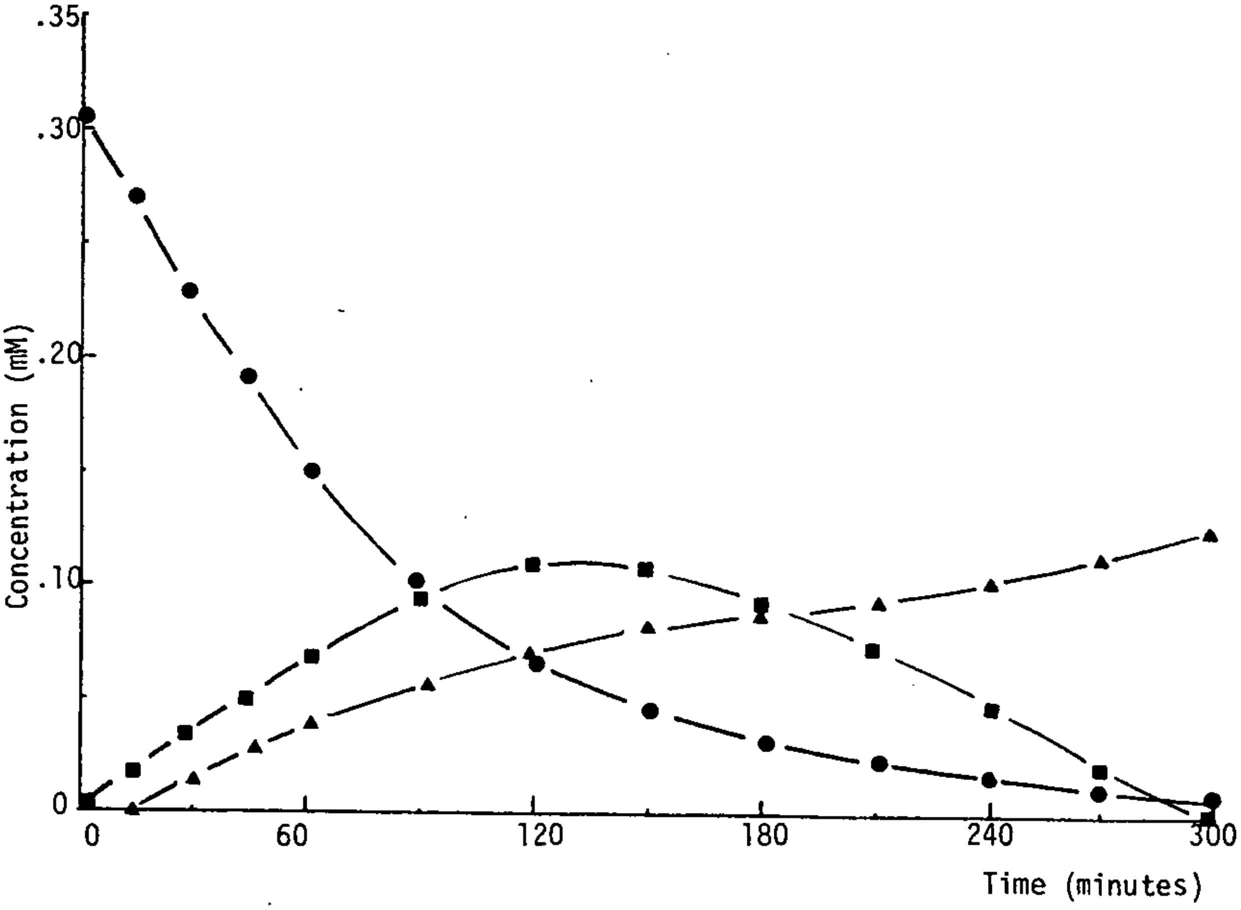

.35
.30
.20
.10
0
Concentration (mM)
0
60
120
180
240
300
Time (minutes)

FIGURE 3.31

Degradation study of dazomet (500 µg.ml^{-1}; 3.086 mM) in minimal salts media at 30°C

●-● dazomet

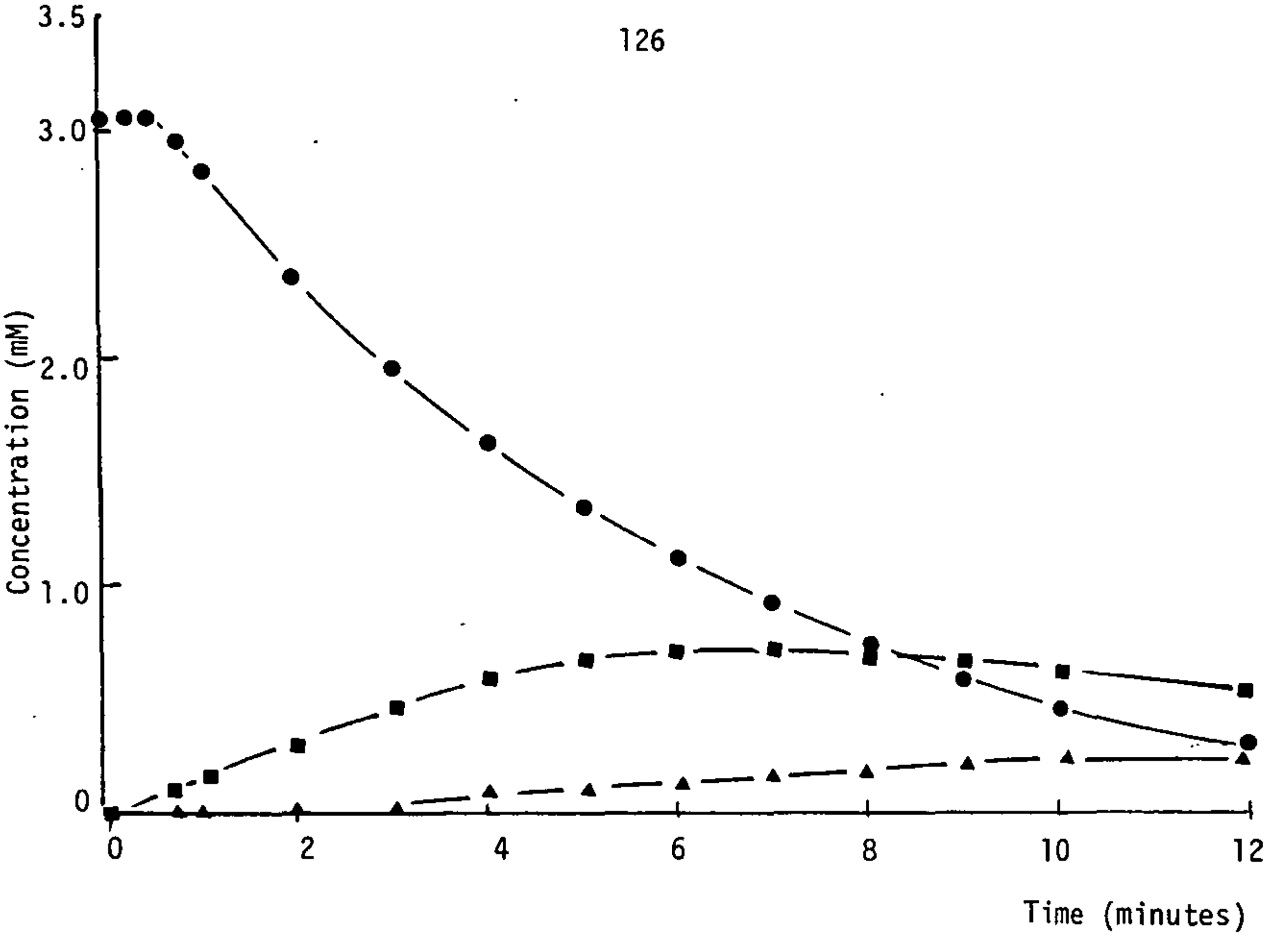
■-■ MDC

▲-▲ MIT

FIGURE 3.32

Degradation study of dazomet (500 µg.ml^{-1}; 3.086 mM) in minimal salts media at 30°C in the presence of S.cerevisiae

Symbols are the same as in Figure 3.31


3.5
3.0
2.0
1.0
0
Concentration (mM)
0
2
4
6
8
10
12
Time (minutes)

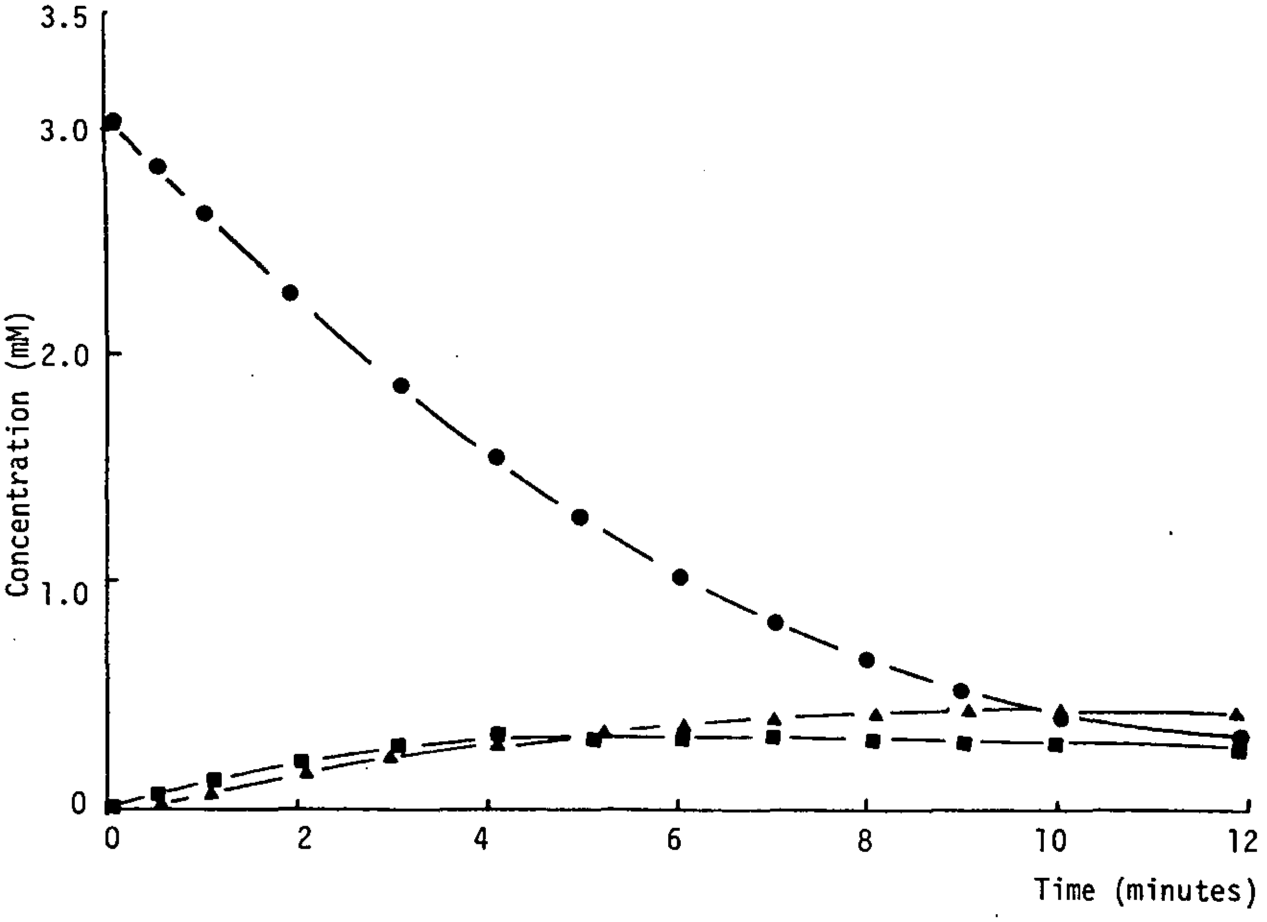

3.5
3.0
2.0
1.0
0
Concentration (mM)
0
2
4
6
8
10
12
Time (minutes)

FIGURE 3.33

Degradation study of dazomet (100 µg.ml^{-1}; 0.617 mM) in minimal salts media at 30°C

●-● dazomet

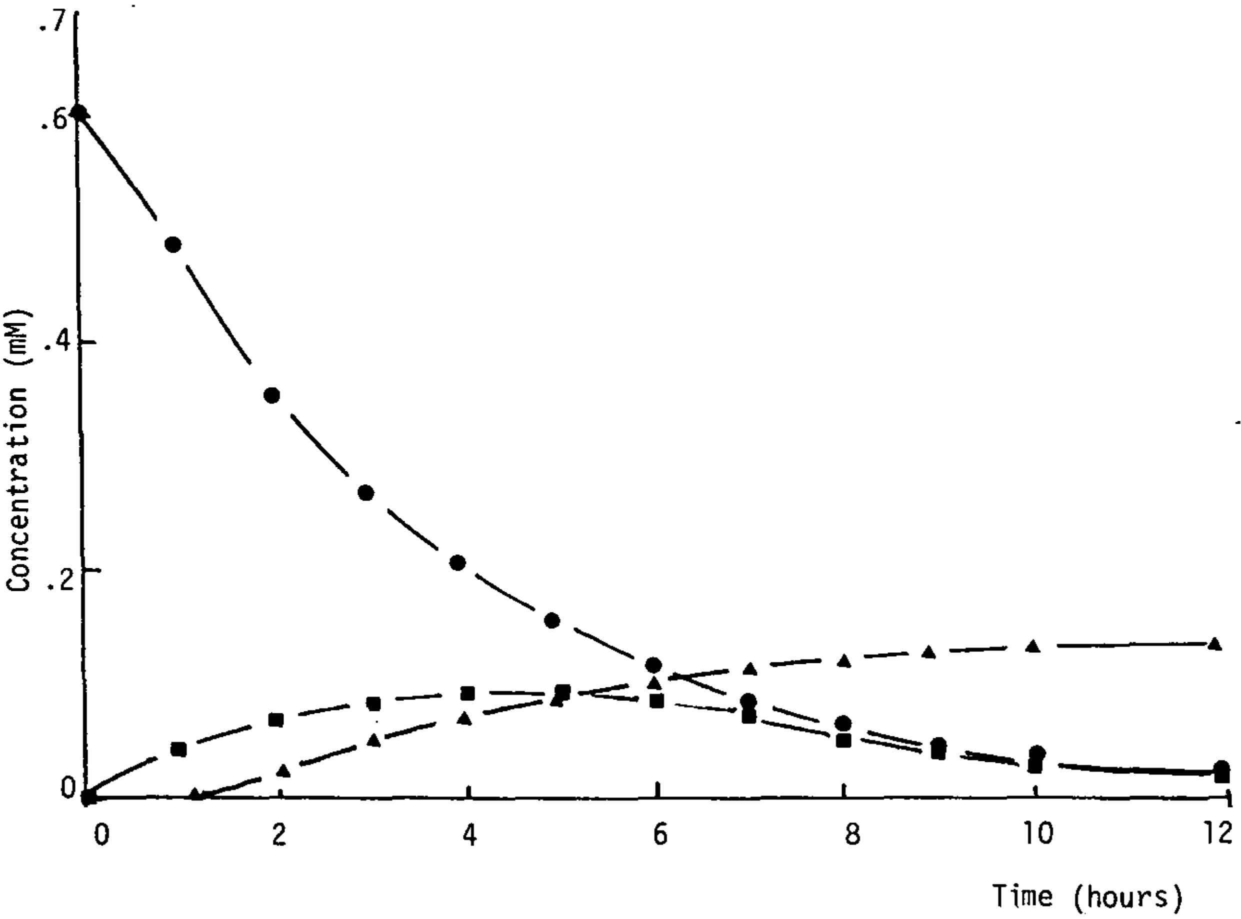
■-■ MDC

▲-▲ MIT

FIGURE 3.34

Degradation of dazomet (100 µg.ml^{-1}; 0.617 mM) in minimal salts media at 30°C in the presence of *S.cerevisiae*

Symbols are the same as in Figure 3.33


.7
.6
.4
.2
0
Concentration (mM)
0
2
4
6
8
10
12
Time (hours)

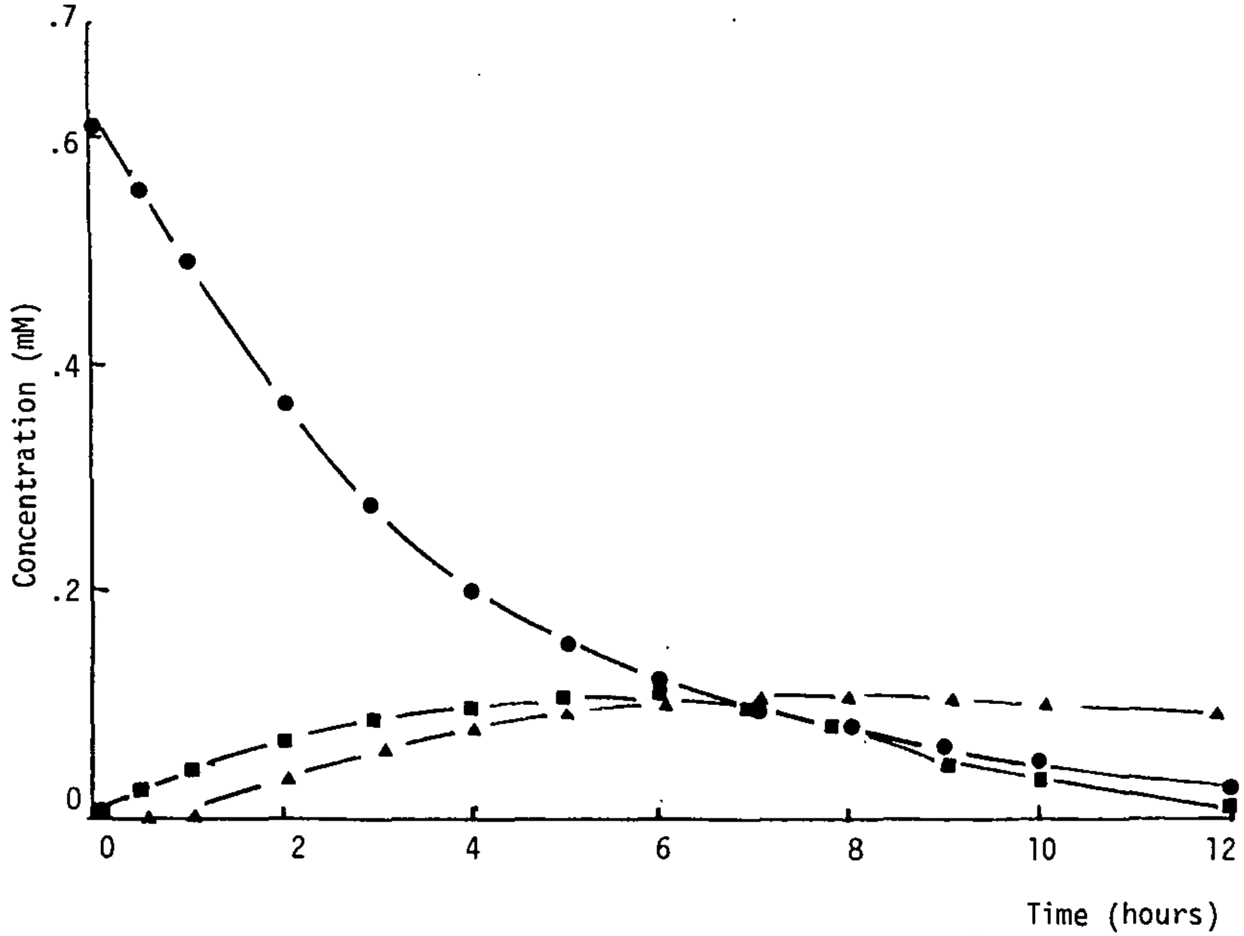

.7
.6
.4
.2
0
Concentration (mM)
0
2
4
6
8
10
12
Time (hours)

FIGURE 3.35

Degradation study of dazomet (500 μg.ml^{-1}; 3.086 mM) in nutrient broth at 30^{o}C

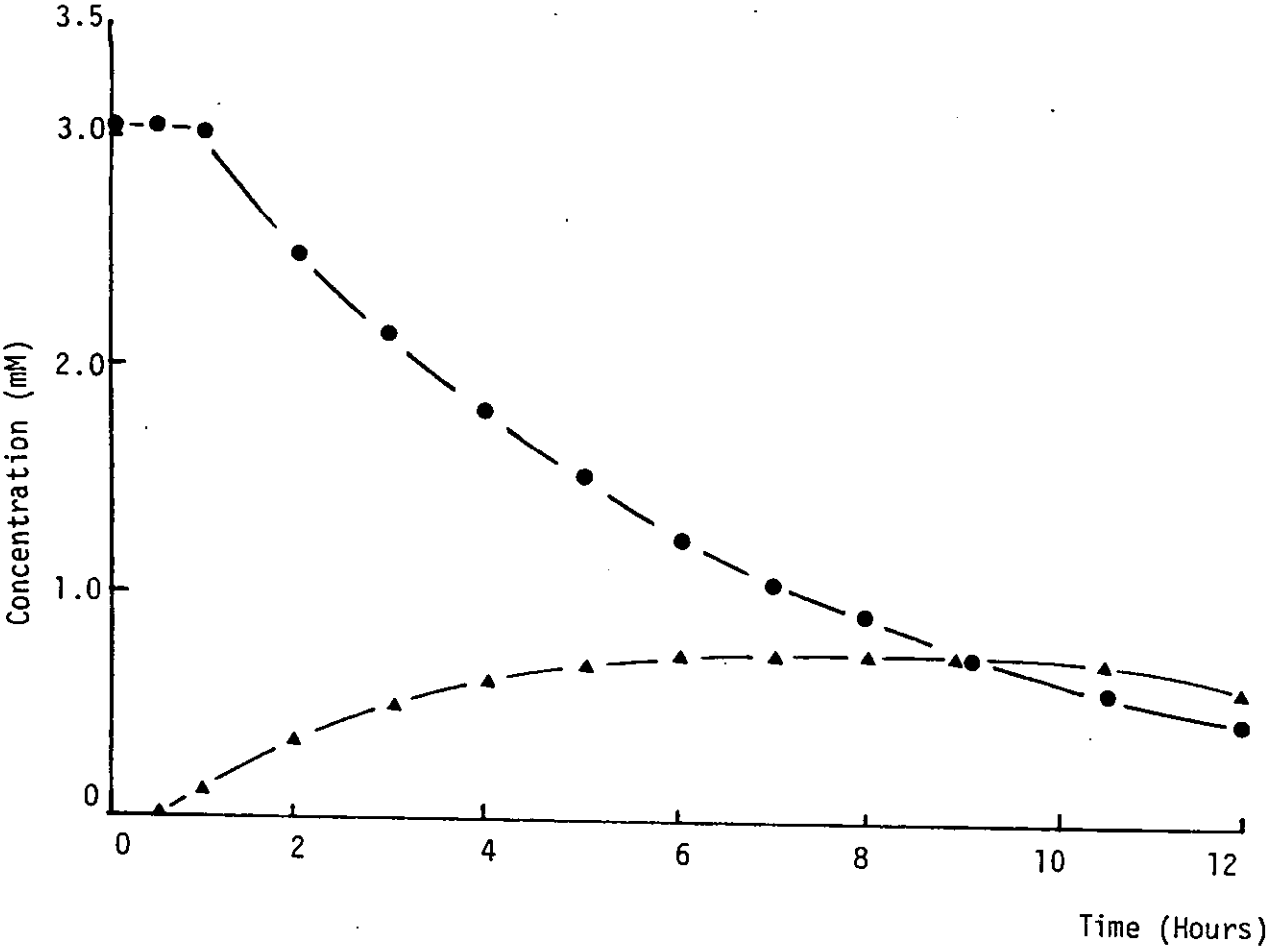
●-● dazomet

▲-▲ MIT

FIGURE 3.36

Degradation study of dazomet (500 μg.ml^{-1}; 3.086 mM) in nutrient broth at 30^{o}C in the presence of S.cerevisiae

Symbols are the same as in Figure 3.35


3.5
3.0
2.0
1.0
0
Concentration (mM)
0
2
4
6
8
10
12
Time (Hours)

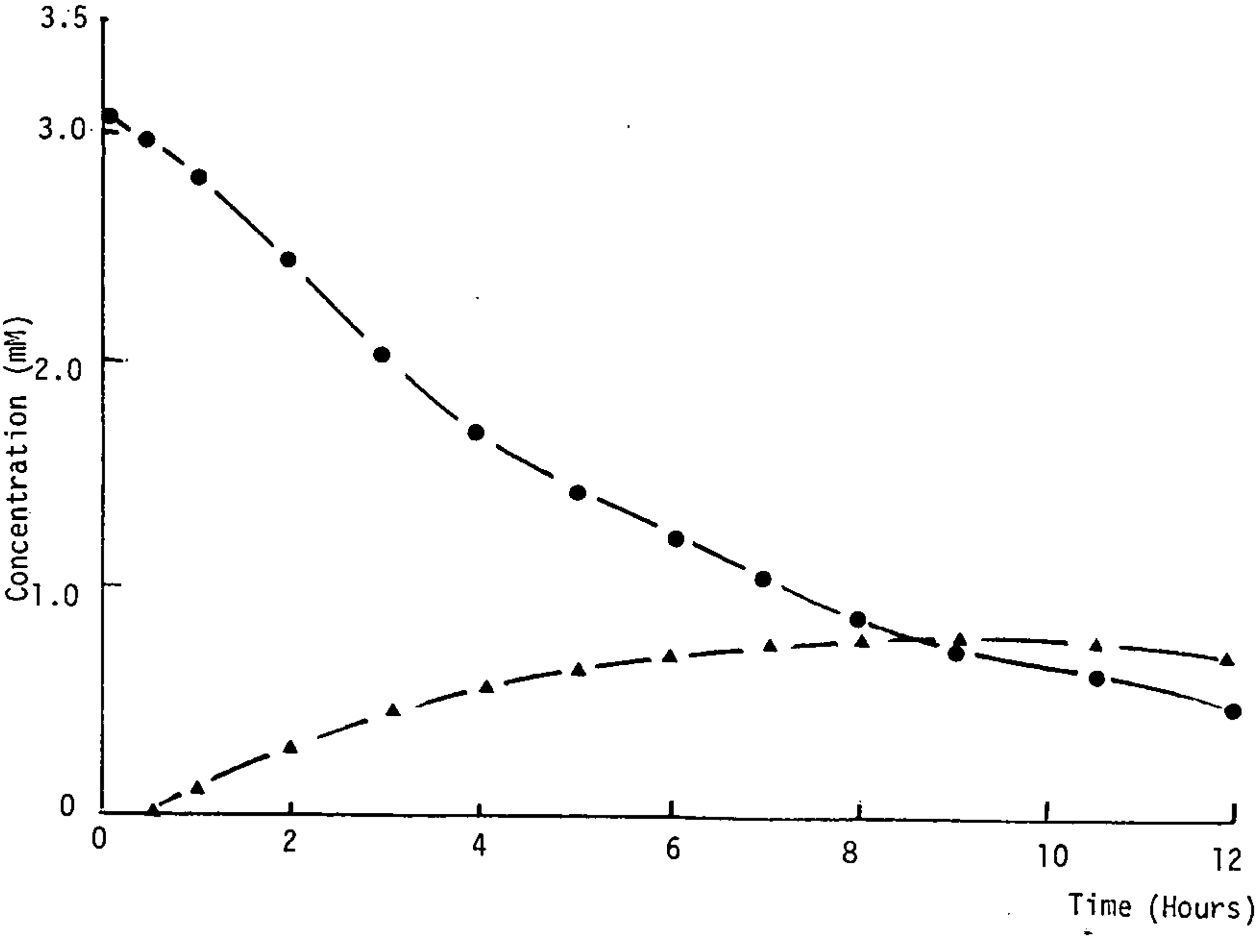

3.5
3.0
2.0
1.0
0
Concentration (mM)
0
2
4
6
8
10
12
Time (Hours)

FIGURE 3.37

Degradation study of dazomet (100 μg.ml^{-1}; 0.617 mM) in nutrient broth at 30°C

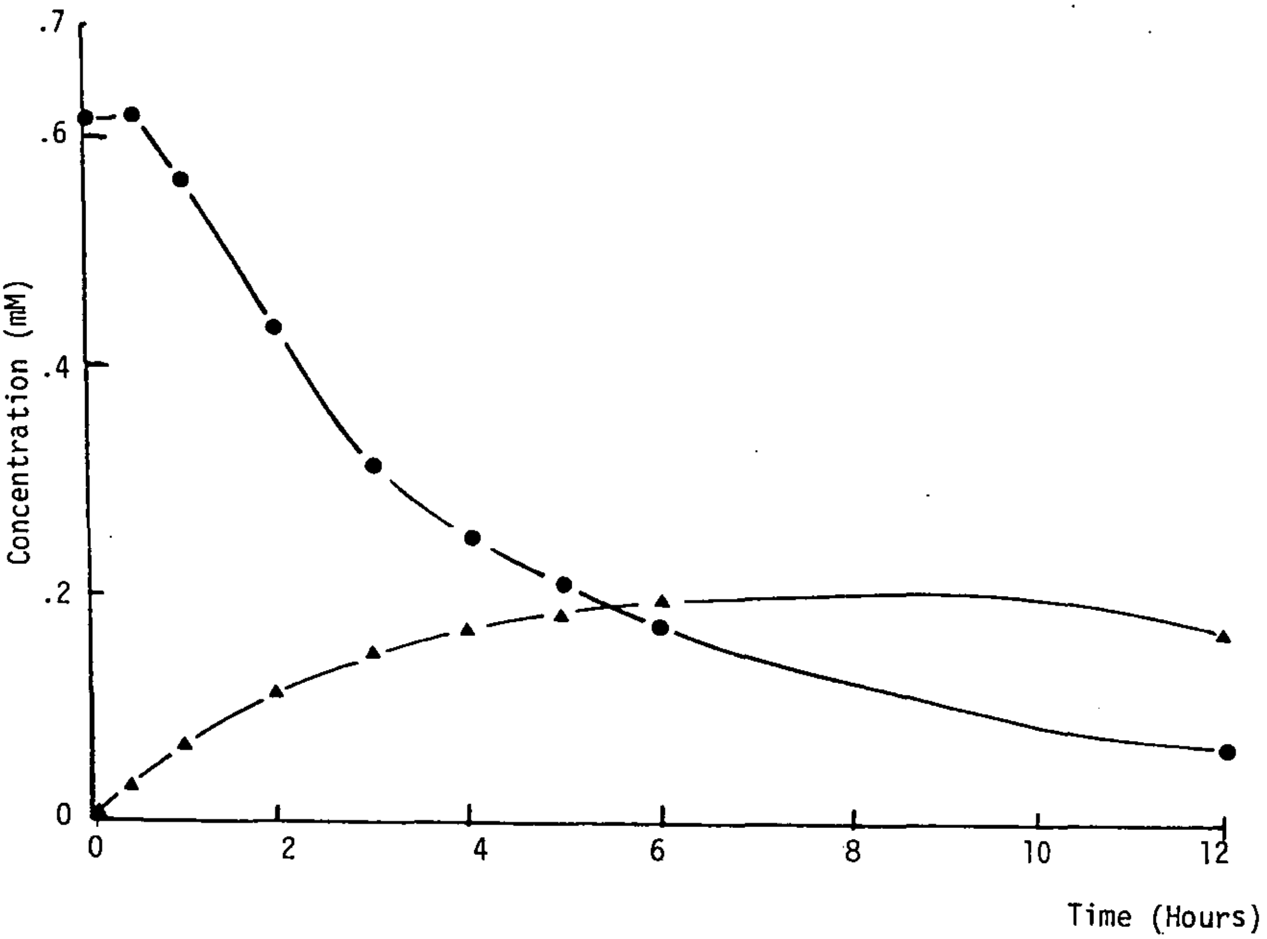
●-● dazomet

▲-▲ MIT

FIGURE 3.38

Degradation study of dazomet (100 μg.ml^{-1}; 0.617 mM) in nutrient broth at 30°C in the presence of S.cerevisiae

Symbols are the same as in Figure 3.37


.7
.6
.4
.2
0
Concentration (mM)
0
2
4
6
8
10
12
Time (Hours)

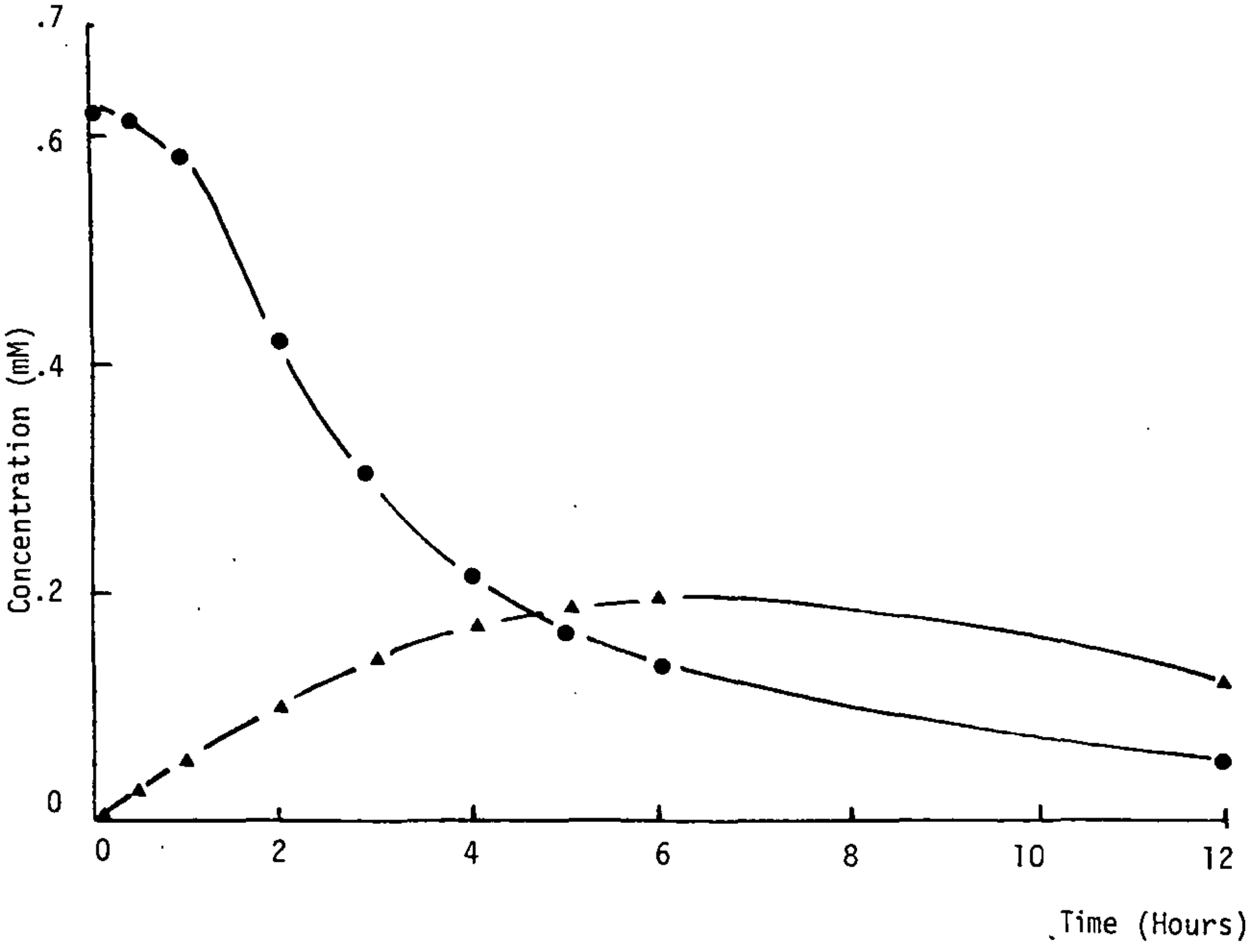

.7
.6
.4
.2
0
Concentration (mM)
0
2
4
6
8
10
12
Time (Hours)

FIGURE 3.39

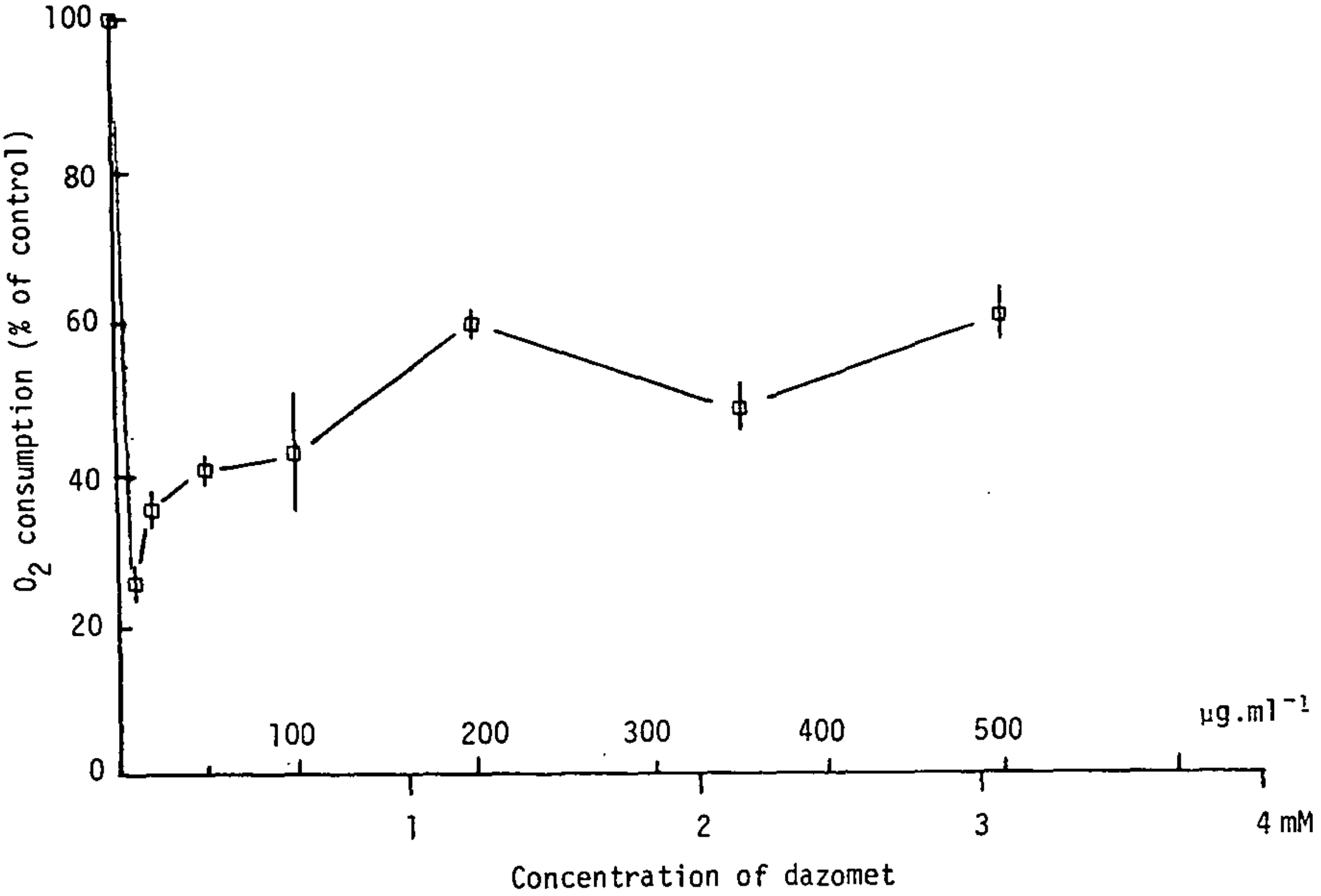
Effect of dazomet on oxygen consumption by B.licheniformis in minimal salts media at 37°C using an oxygen electrode

Substrate: D-glucose (25 mM)

FIGURE 3.40

Effect of dazomet on oxygen consumption by B.licheniformis in minimal salts media at 37°C using an oxygen electrode

Substrate: sodium succinate (25 mM)


100
80
60
40
20
0
O$_2$ consumption (% of control)
100
200
300
400
500
µg.ml^{-1}
1
2
3
4 mM
Concentration of dazomet

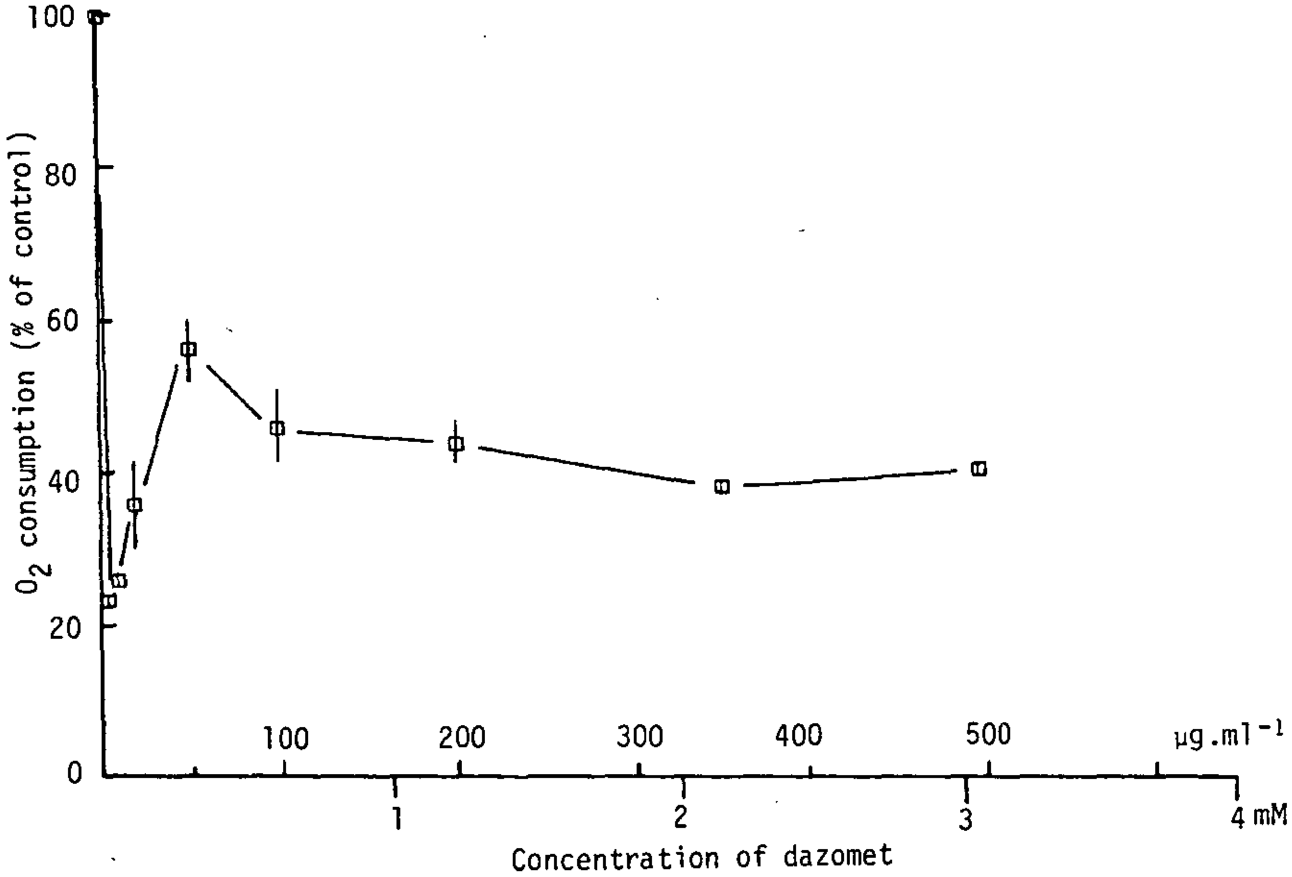

100
80
60
40
20
0
O$_2$ consumption (% of control)
100
200
300
400
500
µg.ml^{-1}
1
2
3
4 mM
Concentration of dazomet

FIGURE 3.41

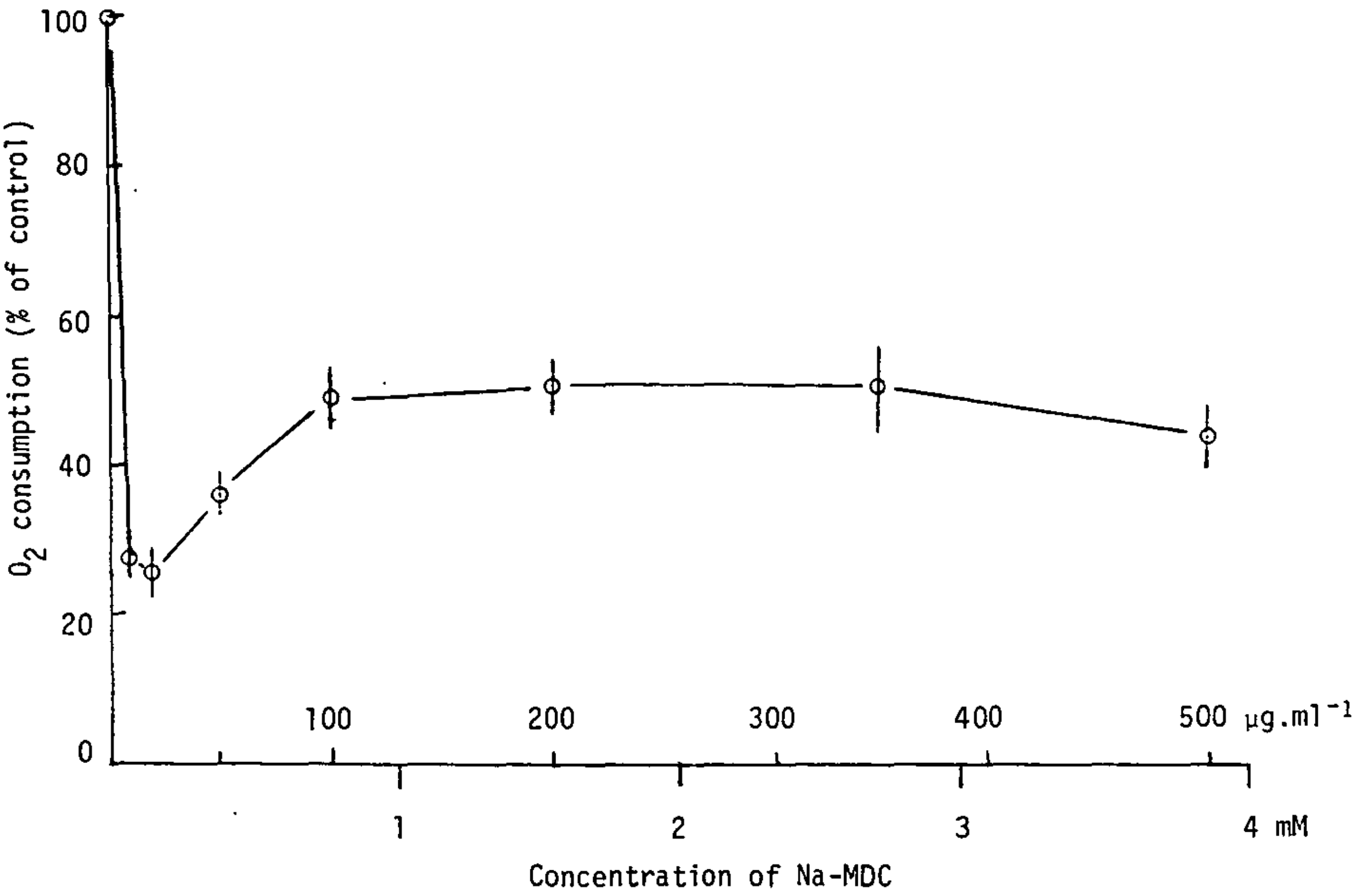
Effect of Na-MDC on oxygen consumption by B.licheniformis in minimal salts media at 37°C using an oxygen electrode

Substrate: D-glucose (25 mM)

FIGURE 3.42

Effect of Na-MDC on oxygen consumption by B.licheniformis in minimal salts media at 37°C using an oxygen electrode

Substrate: sodium succinate (25 mM)


100
80
60
40
20
0
O_2 consumption (% of control)
100
200
300
400
500 µg.ml^{-1}
1
2
3
4 mM
Concentration of Na-MDC

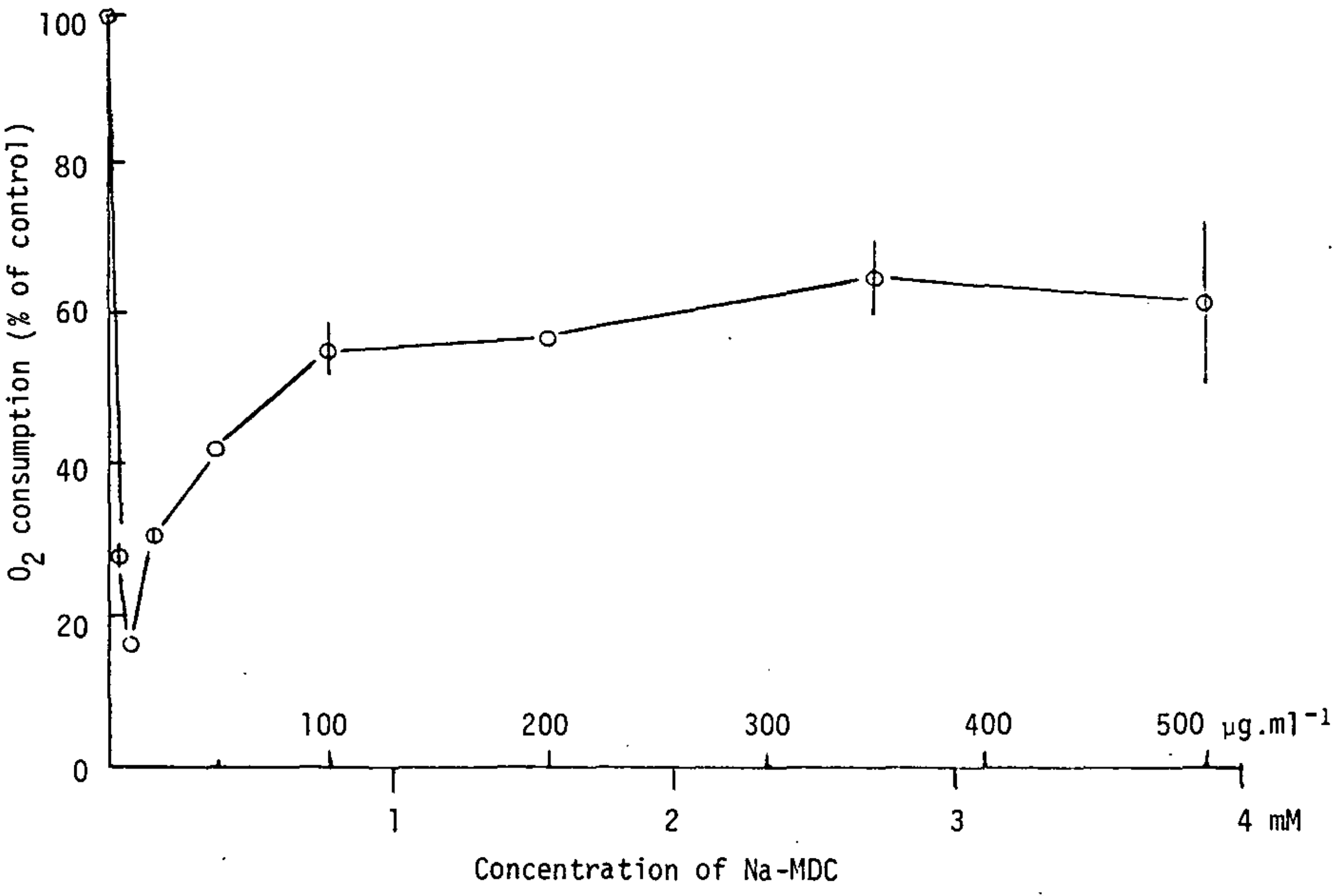

100
80
60
40
20
0
O_2 consumption (% of control)
100
200
300
400
500 µg.ml^{-1}
1
2
3
4 mM
Concentration of Na-MDC

FIGURE 3.43

Effect of MIT on oxygen consumption by *B.licheniformis* in minimal salts media at 37°C using an oxygen electrode

Substrate: D-glucose (25 mM)

FIGURE 3.44

Effect of MIT on oxygen consumption by *B.licheniformis* in minimal salts media at 37°C using an oxygen electrode

Substrate: sodium succinate (25 mM)

FIGURE 3.45

Effect of DNP on oxygen consumption by *B.licheniformis* in minimal salts media at 37°C using an oxygen electrode

Substrate: D-glucose (25 mM)

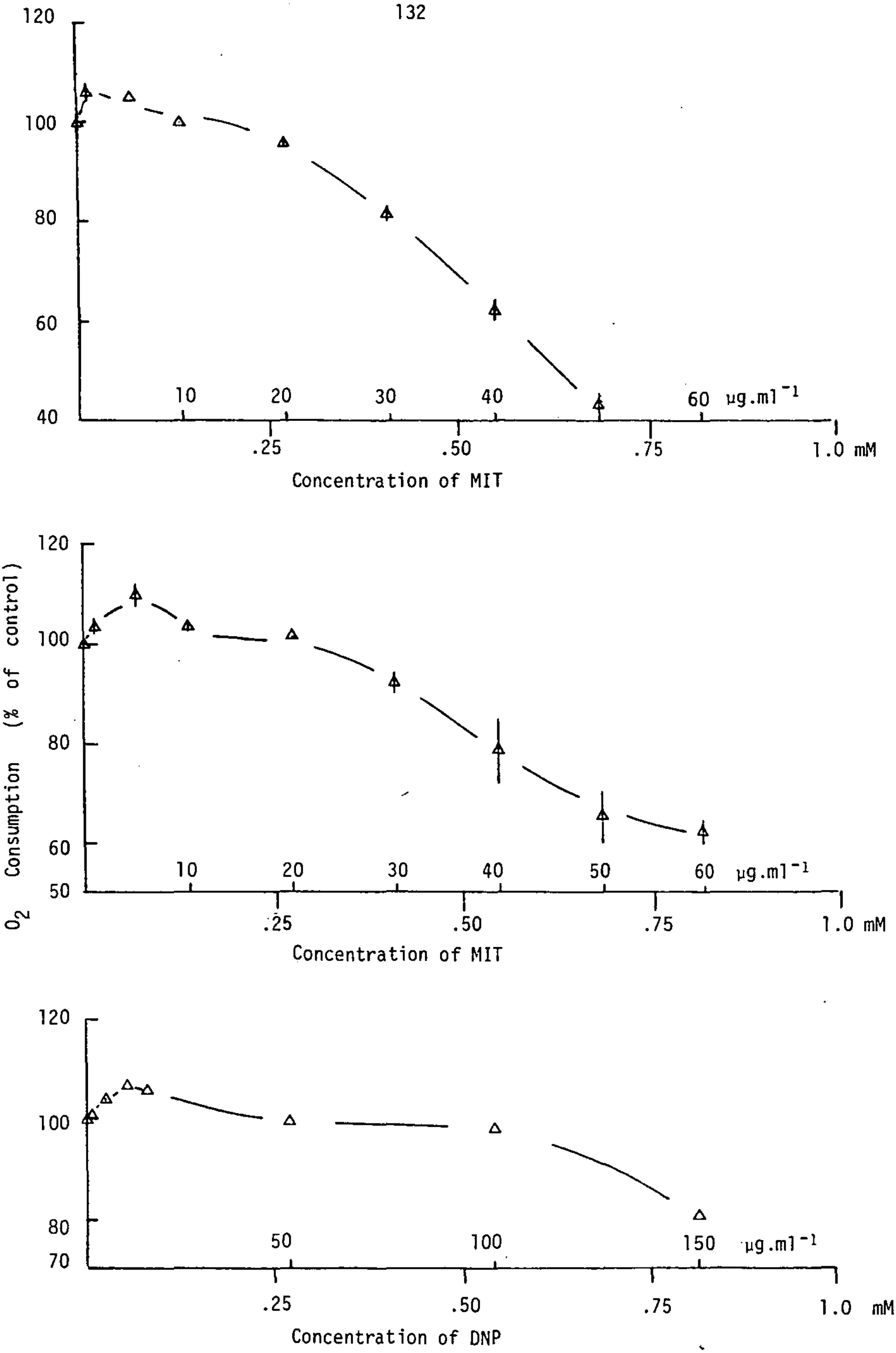

120
100
80
60
40
10
20
30
40
60 μg.ml^{-1}
.25
.50
.75
1.0 mM
Concentration of MIT
O_2 Consumption (% of control)
120
100
80
60
50
10
20
30
40
50
60 μg.ml^{-1}
.25
.50
.75
1.0 mM
Concentration of MIT
120
100
80
70
50
100
150 μg.ml^{-1}
.25
.50
.75
1.0 mM
Concentration of DNP

FIGURE 3.46

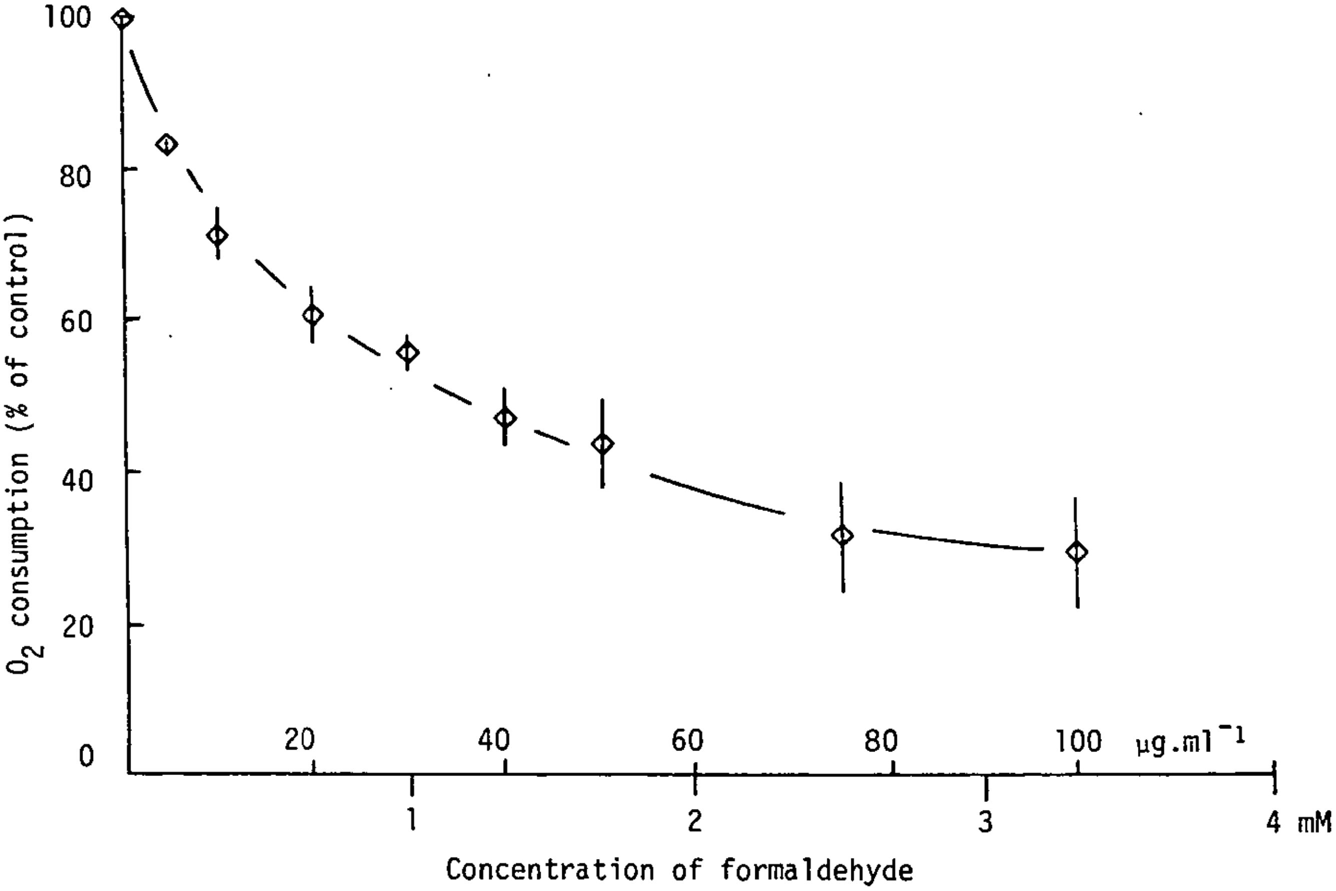
Effect of formaldehyde on oxygen consumption by B.licheniformis in minimal salts media at 37°C using an oxygen electrode

Substrate: D-glucose (25 mM)

FIGURE 3.47

Effect of formaldehyde on oxygen consumption by B.licheniformis in minimal salts media at 37°C using an oxygen electrode

Substrate: sodium succinate (25 mM)


100
80
60
40
20
0
O_2 consumption (% of control)
20
40
60
80
100
µg.ml^{-1}
1
2
3
4 mM
Concentration of formaldehyde

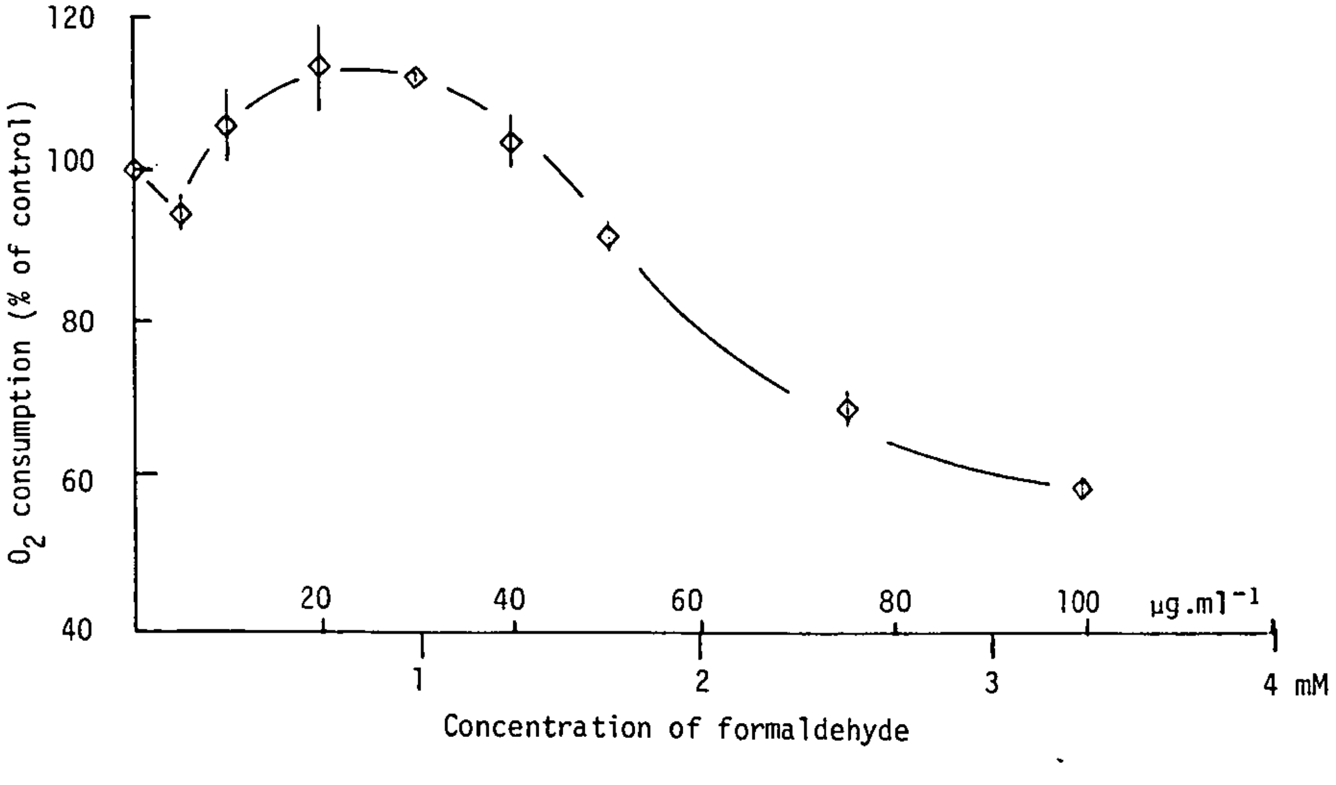

120
100
80
60
40
O_2 consumption (% of control)
20
40
60
80
100
µg.ml^{-1}
1
2
3
4 mM
Concentration of formaldehyde

FIGURE 3.48

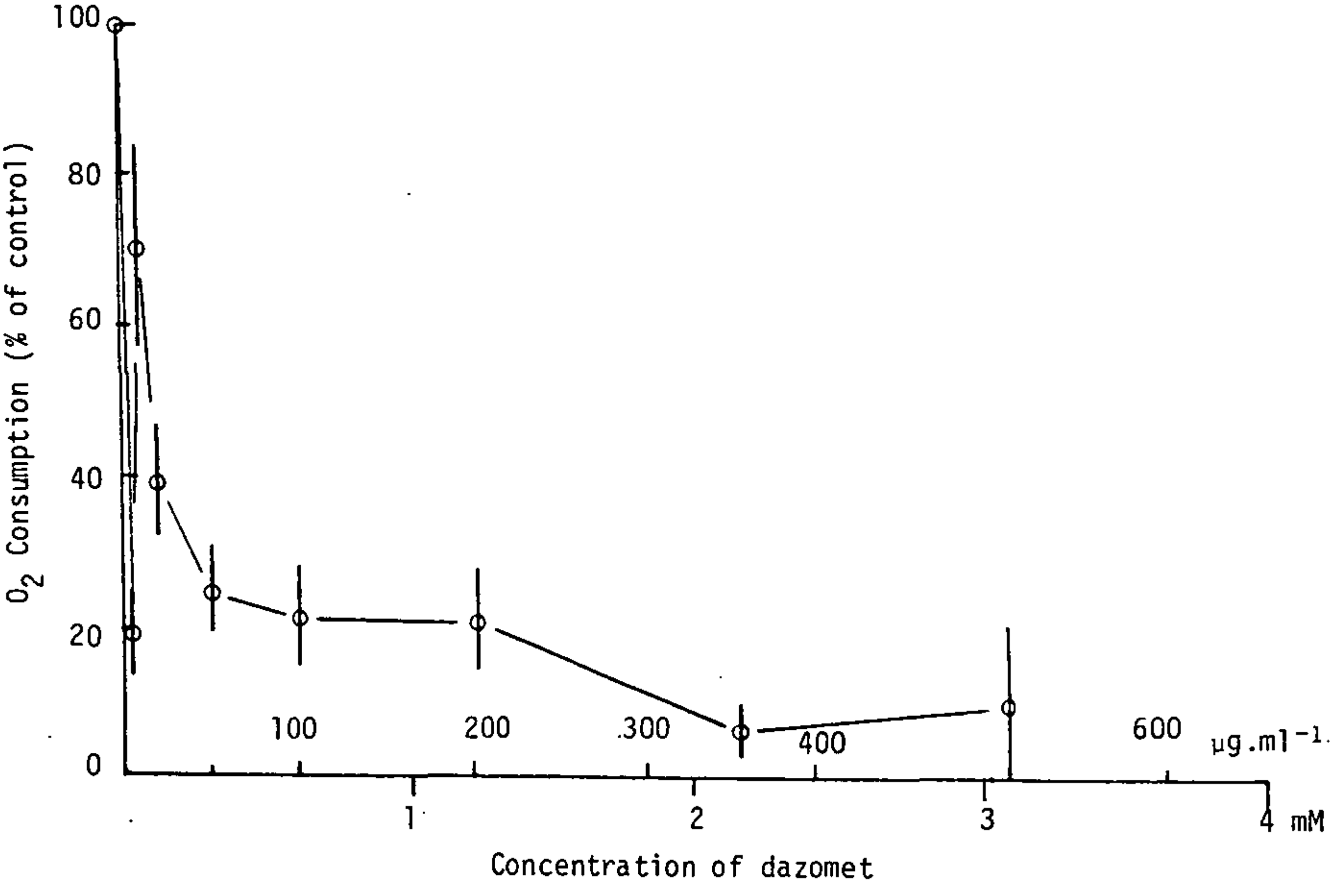
Effect of dazomet on oxygen consumption by *S.cerevisiae* in minimal salts media at 30^{o}C using an oxygen electrode

Substrate: D-glucose (25 mM)

FIGURE 3.49

Effect of Na-MDC on oxygen consumption by *S.cerevisiae* in minimal salts media at 30^{o}C using an oxygen electrode

Substrate: D-glucose (25 mM)


O$_2$ Consumption (% of control)
100
80
60
40
20
0
100
200
300
400
600
µg.ml^{-1}
1
2
3
4 mM
Concentration of dazomet

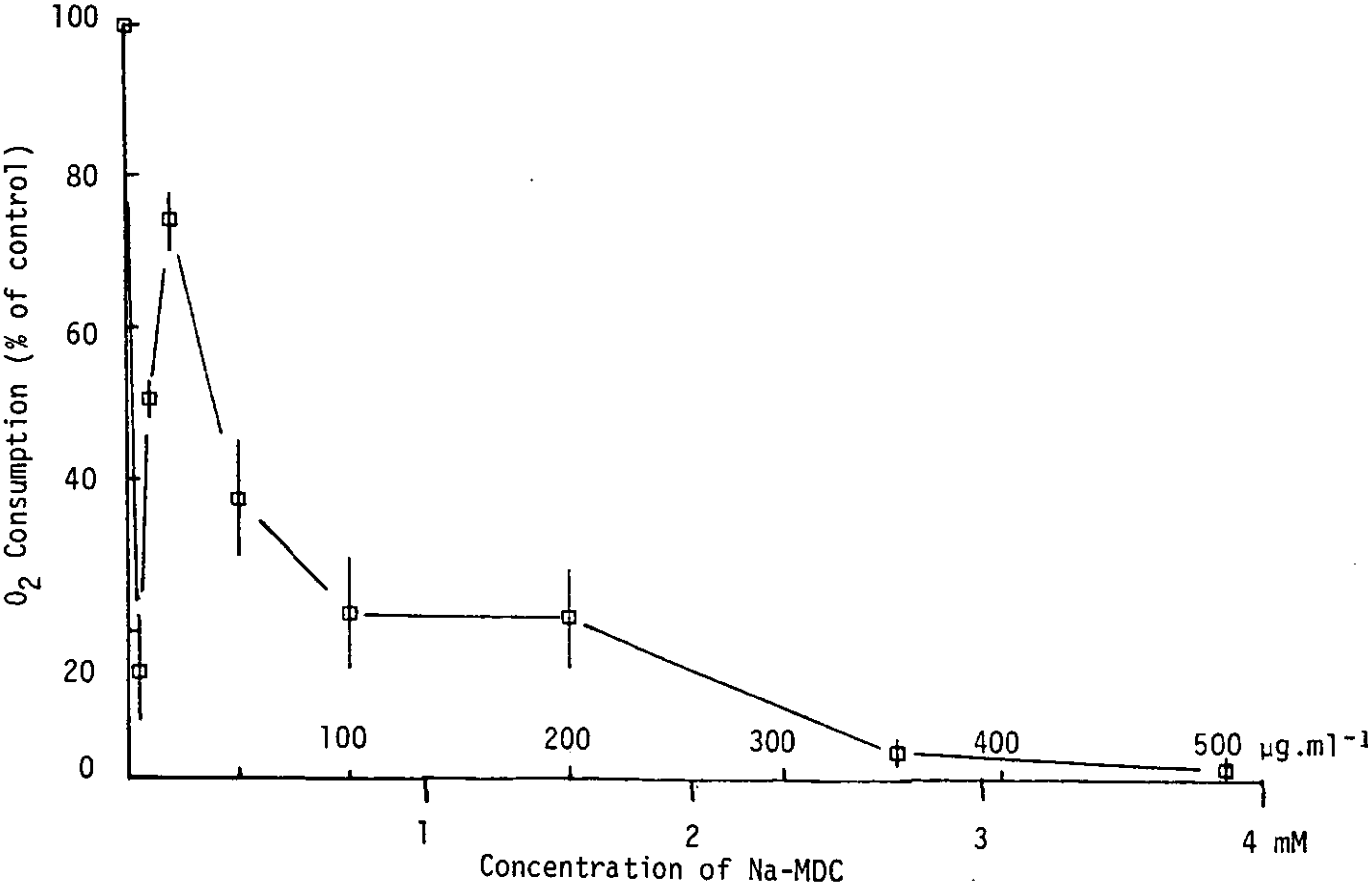

O$_2$ Consumption (% of control)
100
80
60
40
20
0
100
200
300
400
500
µg.ml^{-1}
1
2
3
4 mM
Concentration of Na-MDC

FIGURE 3.50

Effect of formaldehyde on oxygen consumption by *S.cerevisiae* in minimal salts media at 37°C using an oxygen electrode

Substrate: D-glucose (25 mM)

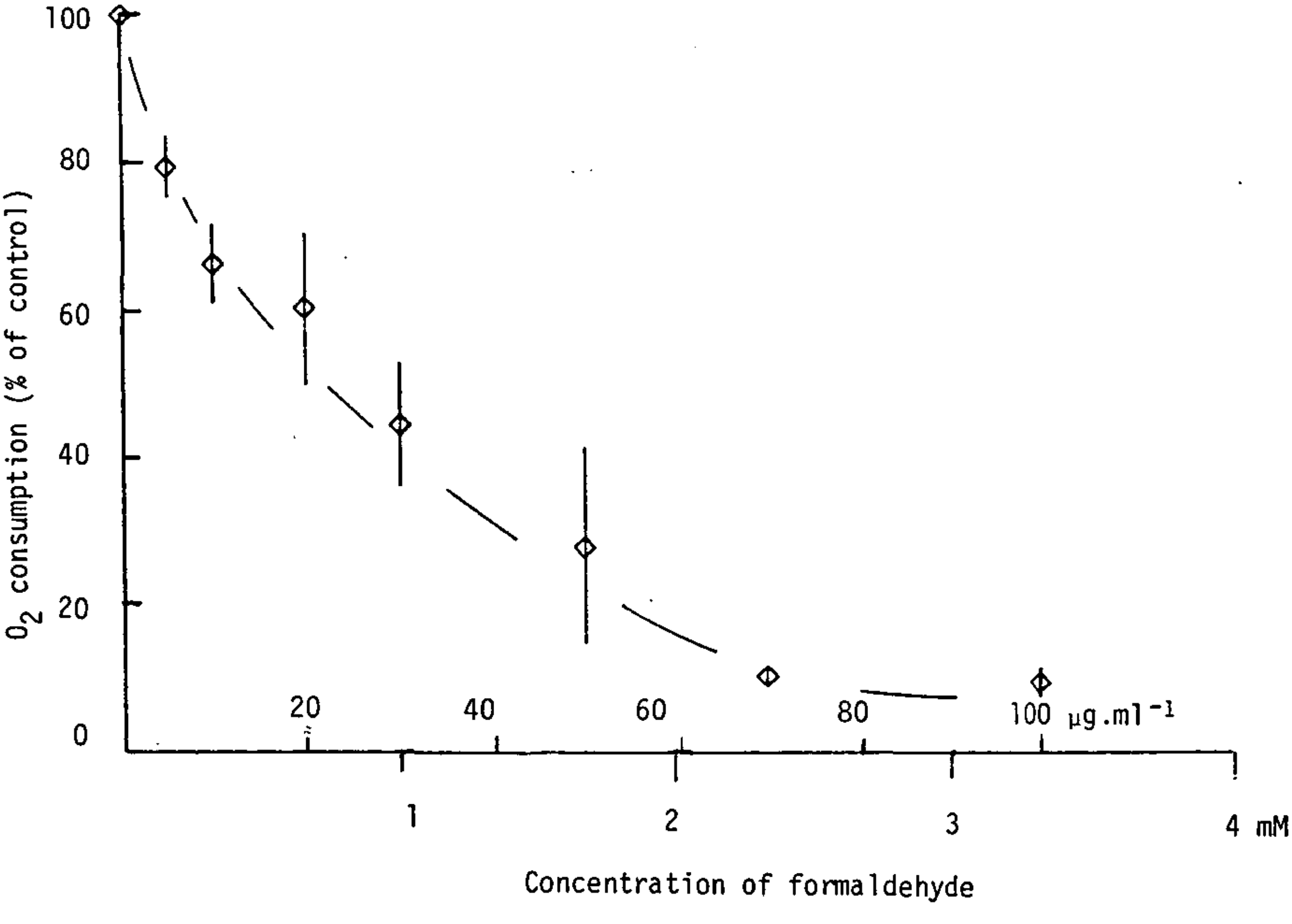

100
80
60
40
20
0
O_2 consumption (% of control)
20
40
60
80
100 μg.ml^{-1}
1
2
3
4 mM
Concentration of formaldehyde

FIGURE 3.51

Effect of MIT on oxygen consumption by S.cerevisiae in minimal salts media at 30^{o}C using an oxygen electrode

Substrate: D-glucose (25 mM)

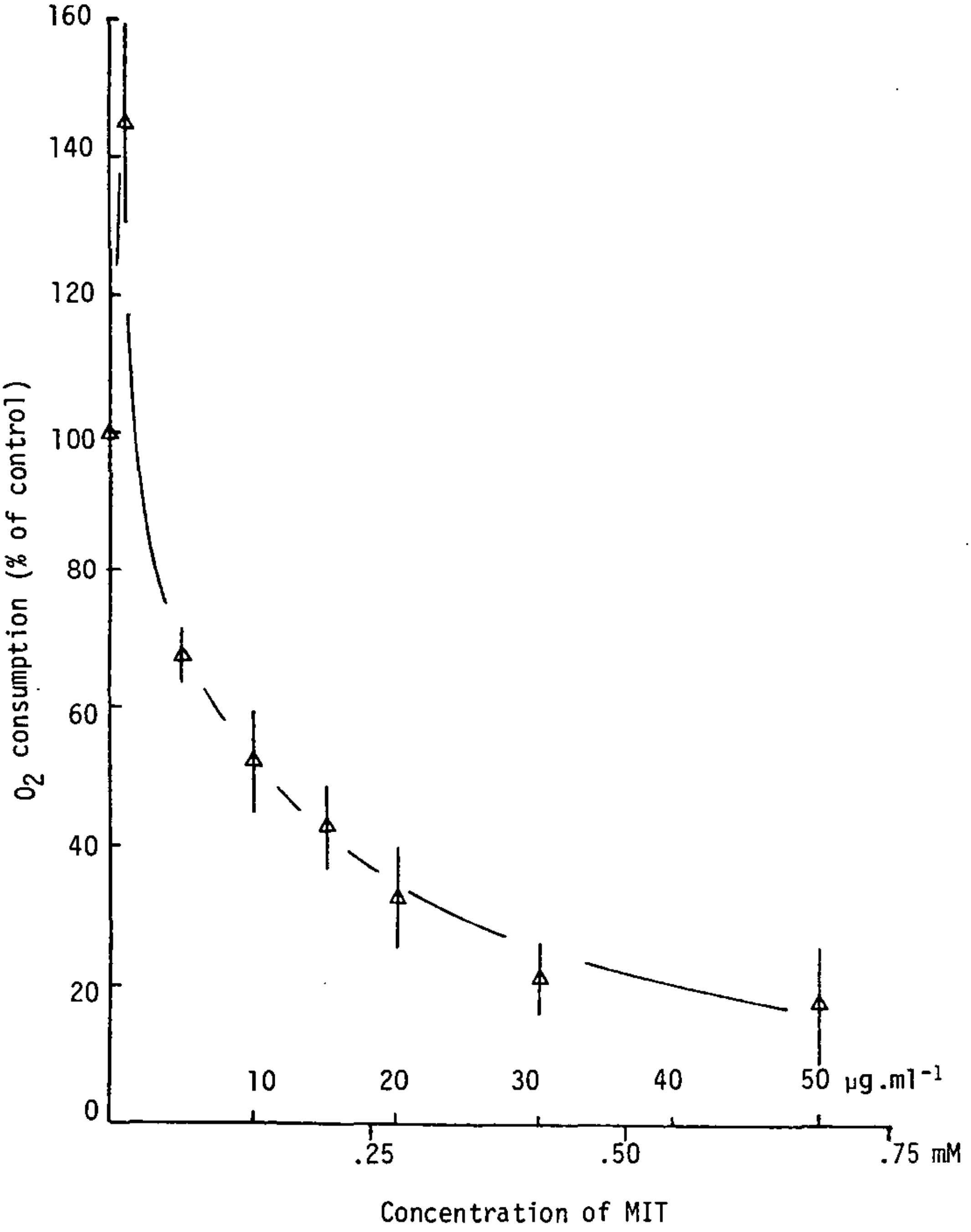

160
140
120
100
80
60
40
20
0
O_2 consumption (% of control)
10
20
30
40
50 µg.ml^{-1}
.25
.50
.75 mM
Concentration of MIT

FIGURE 3.52

Effect of Na-MDC on oxygen consumption by *B.licheniformis* in minimal salts media at 30^{o}C using Warburg apparatus

Substrate: D-glucose (25 mM)

▼-▼ 10 μg.ml^{-1}

□-□ 50 "

△-△ 200 "

○-○ 500 "

●-● Control

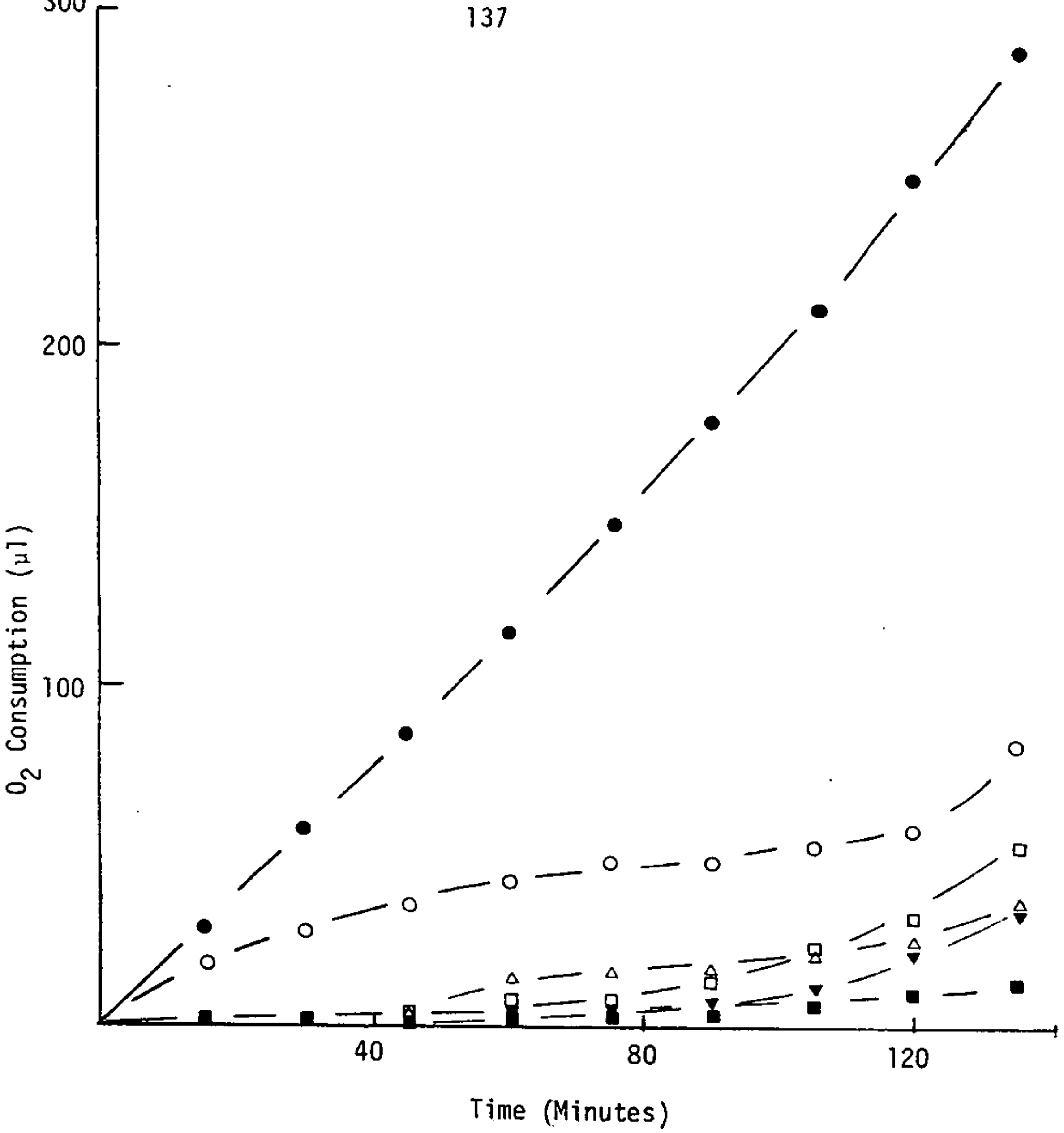
■-■ Endogeneous respiration

FIGURE 3.53

Effect of dazomet on oxygen consumption by *B.licheniformis* in minimal salts media at 30^{o}C using Warburg apparatus

Substrate: D-glucose (25 mM)

Symbols are the same as in Figure 3.52


300
200
100
O_2 Consumption (μl)
40
80
120
Time (Minutes)

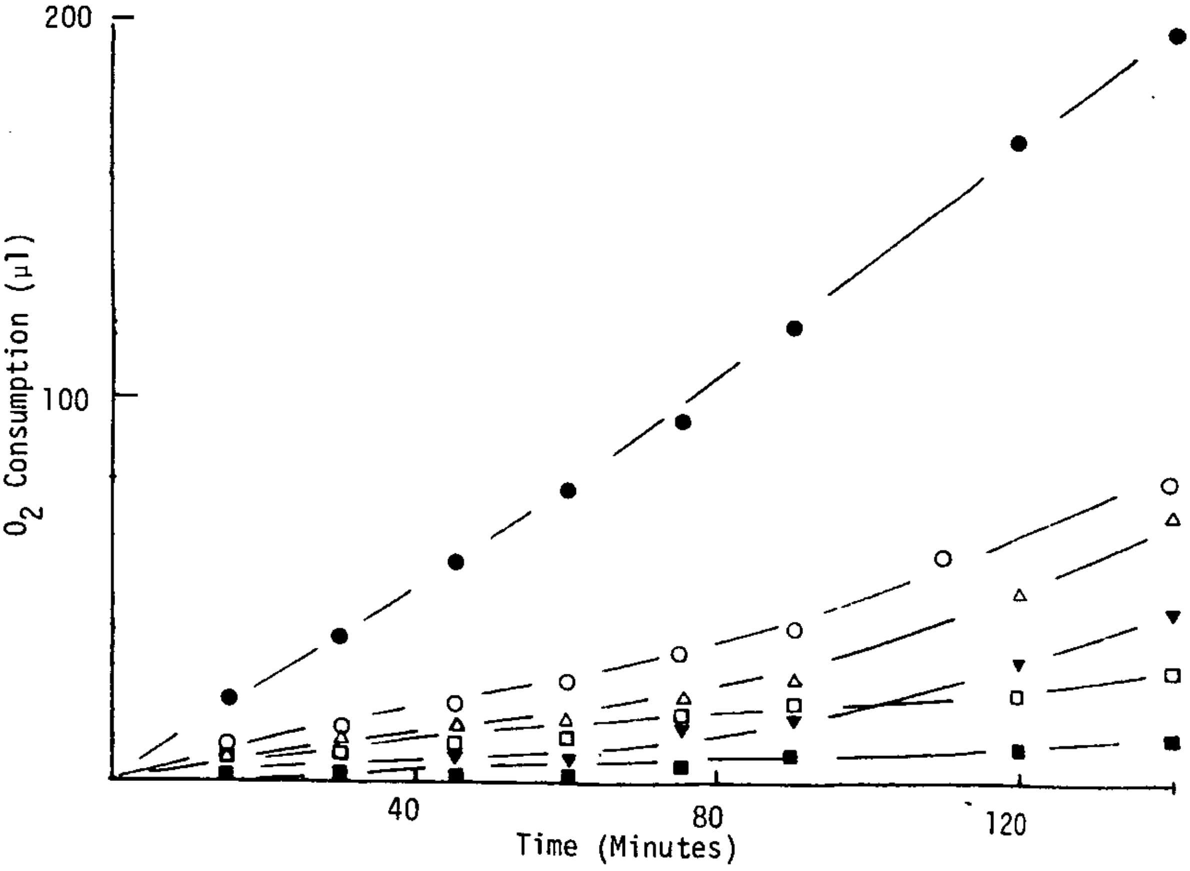

200
100
O_2 Consumption (μl)
40
80
120
Time (Minutes)

FIGURE 3.54

Effect of dazomet on oxygen consumption by B.licheniformis in minimal salts media at 37°C in the presence of L-cysteine

Arrow indicates additions to the electrode vessel

Final concentrations:

D-glucose = 25 mM

Dazomet = 0.05 mM (8.33μg.ml^{-1})

L-cysteine = 0.625 mM

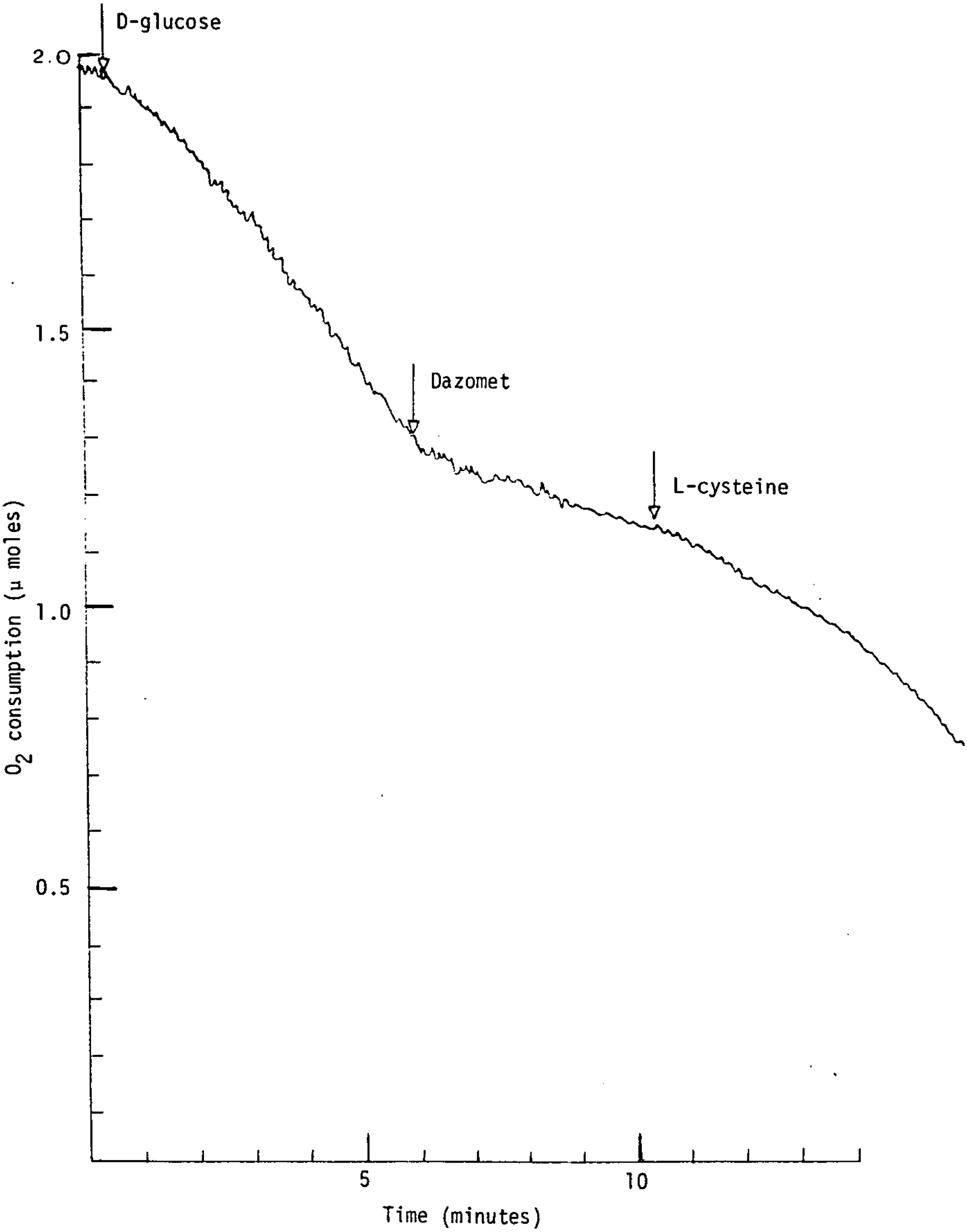

D-glucose
Dazomet
L-cysteine
2.0
1.5
1.0
0.5
O_2 consumption (μ moles)
5
10
Time (minutes)

FIGURE 3.55

Effect of Na-MDC on oxygen consumption by *B.licheniformis* in minimal salts media at 37°C in the presence of L-cysteine (substrate effect)

Arrow indicates additions to the electrode vessel

Final concentrations:

D-glucose = 25 mM

Na-MDC = 0.065 mM (8.33 $\mu g.ml^{-1}$)

L-cysteine = 0.625 mM

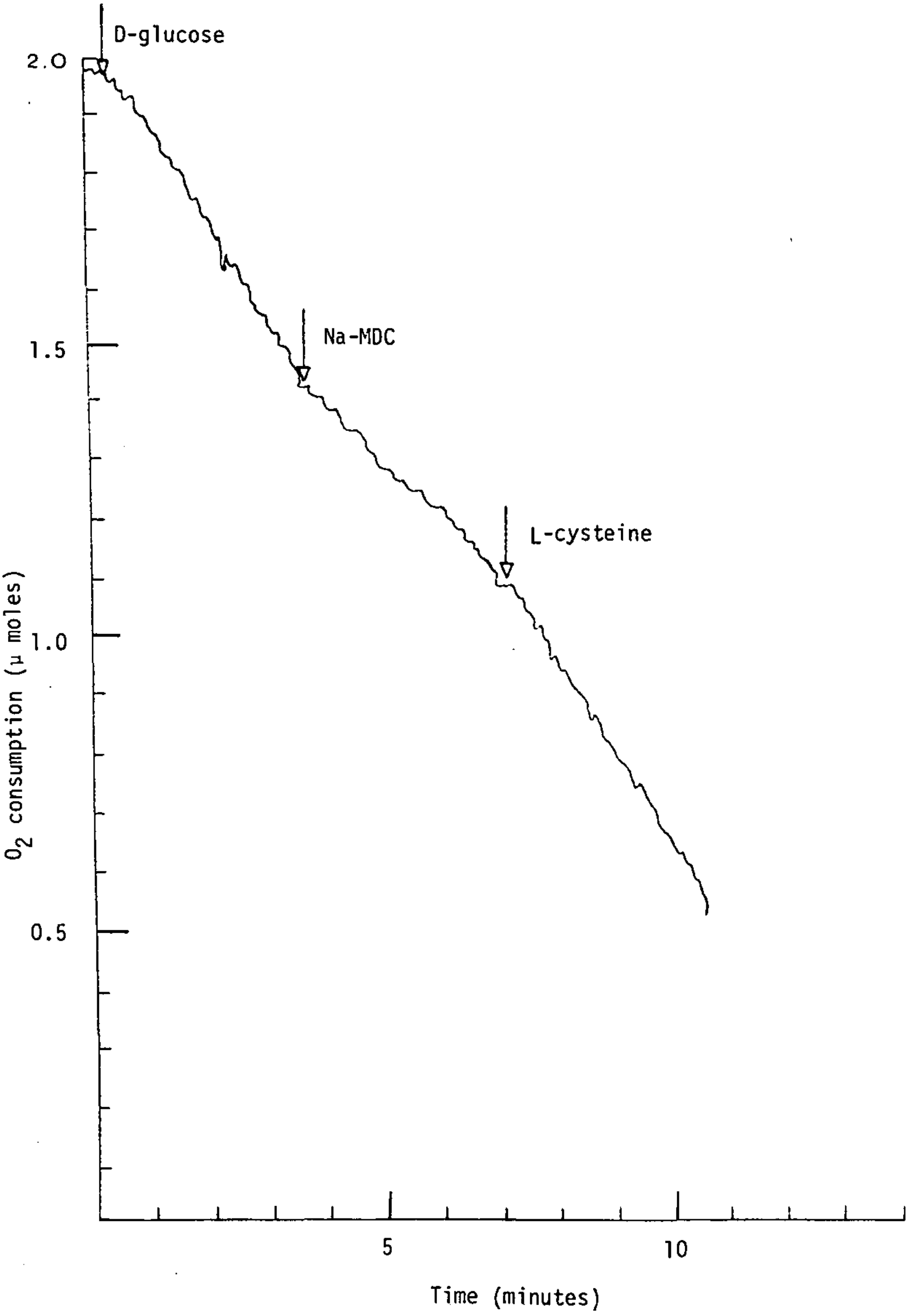

D-glucose
Na-MDC
L-cysteine
2.0
1.5
1.0
0.5
O$_2$ consumption (μ moles)
5
10
Time (minutes)

FIGURE 3.56

Effect of Na-MDC on oxygen consumption by *B.licheniformis* in minimal salts media at 37°C in the presence of L-cysteine

Arrow indicates additions to the electrode vessel

Broken line is control

Final concentrations:

D-glucose	=	25 mM
Na-MDC	=	0.129 mM (16.67 μg.ml^{-1})
L-cysteine	=	0.625 mM

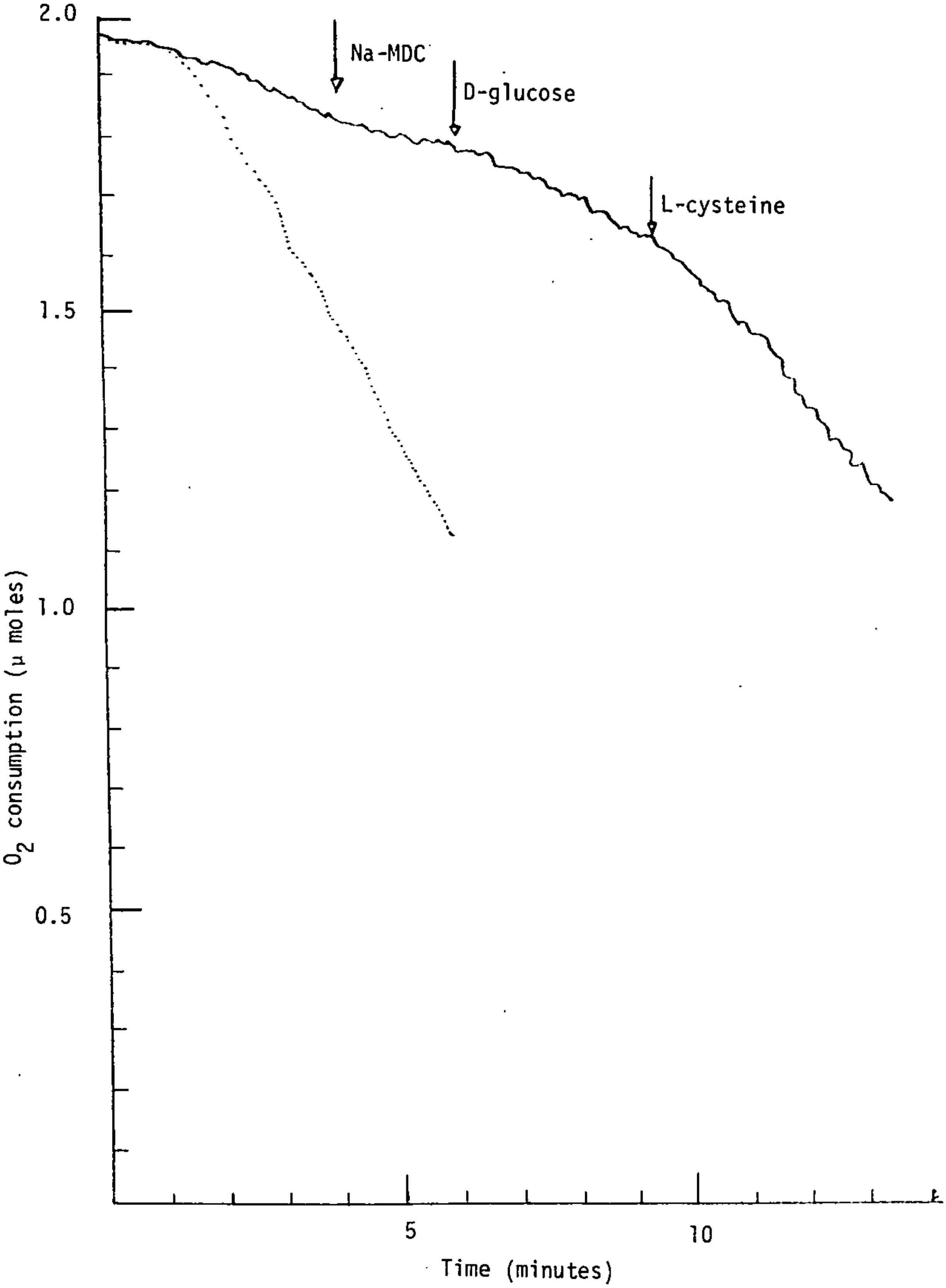

2.0
1.5
1.0
0.5
O_2 consumption (μ moles)
Na-MDC
D-glucose
L-cysteine
5
10
Time (minutes)

FIGURE 3.57

Effect of MIT on oxygen consumption by *B.licheniformis* in minimal salts media at 37°C in the presence of L-cysteine

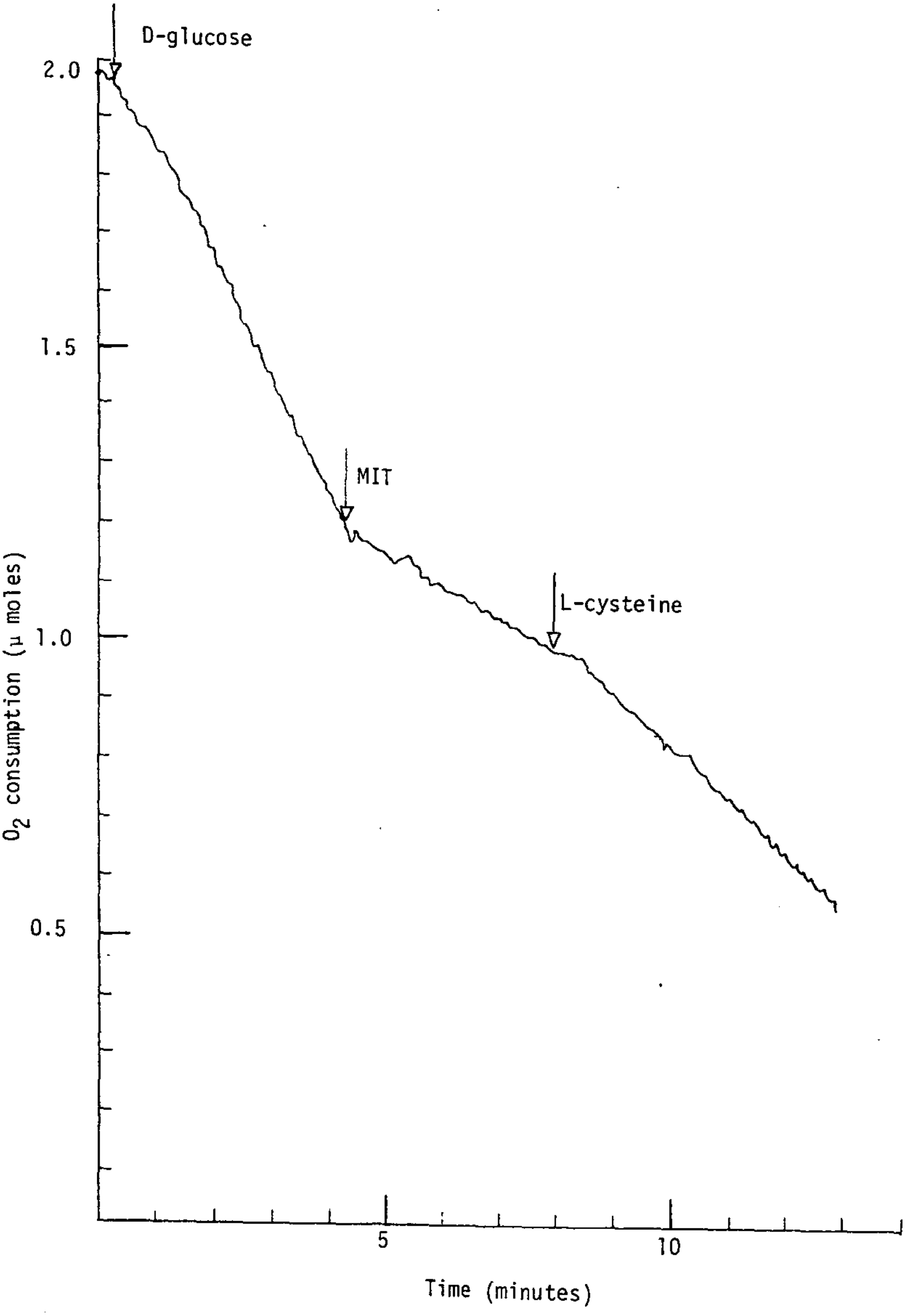
Arrow indicates additions to the electrode vessel

Final concentrations:

D-glucose = 25 mM

MIT = 0.182 mM

L-cysteine = 0.625 mM



D-glucose
MIT
L-cysteine
2.0
1.5
1.0
0.5
O_2 consumption (μ moles)
5
10
Time (minutes)

FIGURE 3.58

Effect of formaldehyde on oxygen consumption by *B.licheniformis* in minimal salts media at 37^{o}C in the presence of L-cysteine

Arrow indicates additions to the electrode vessel

Final concentrations:

D-glucose = 25 mM

Formaldehyde = 5.55 mM

L-cysteine = 0.625 mM

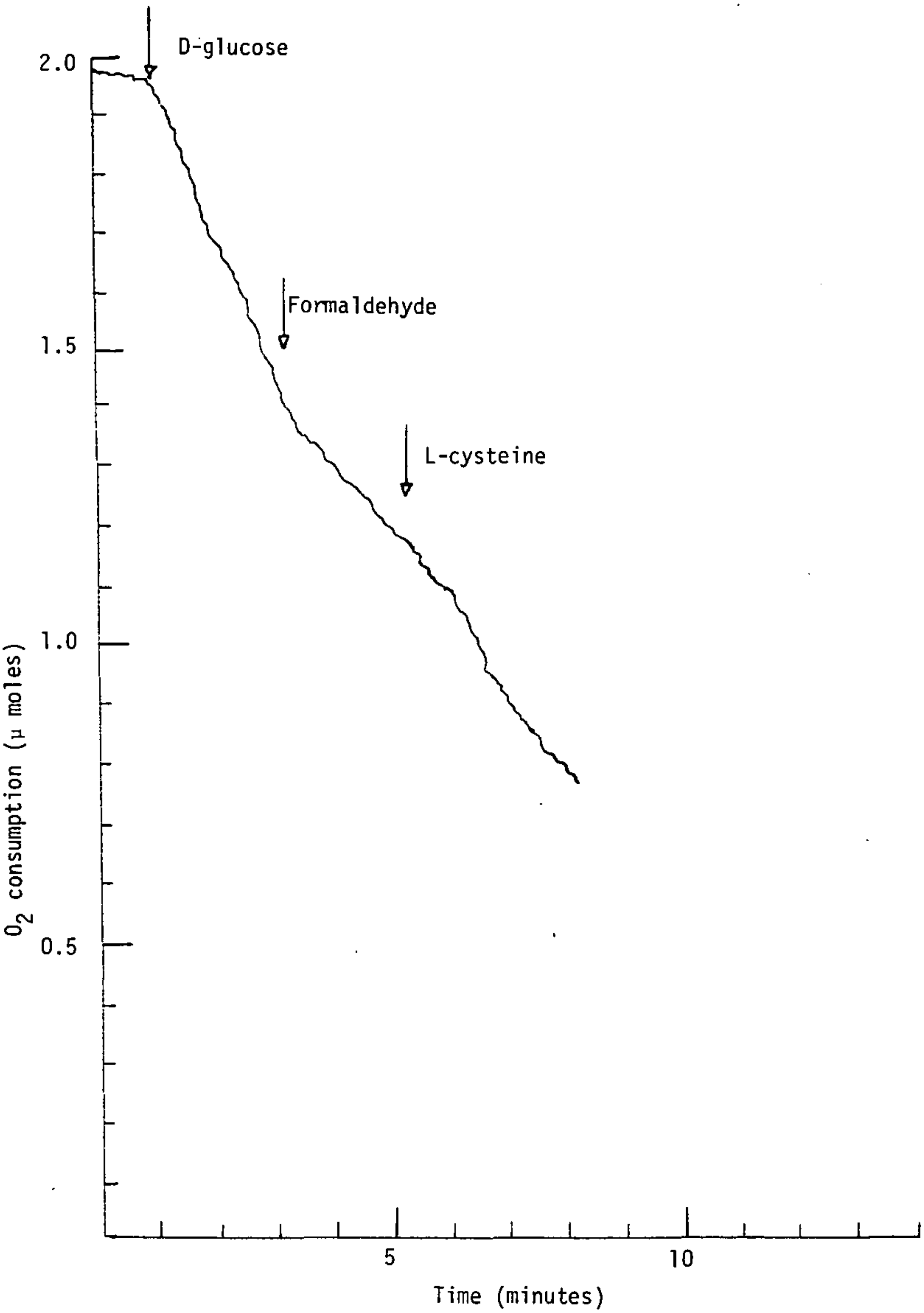

D-glucose
Formaldehyde
L-cysteine
2.0
1.5
1.0
0.5
O_2 consumption (μ moles)
5
10
Time (minutes)

FIGURE 3.59

Effect of predegraded dazomet (50 μg.ml^{-1}; 0.309 mM) on oxygen consumption by B.licheniformis in minimal salts media at 37^{o}C using an oxygen electrode

Substrate: sodium succinate (25 mM)

▽-▽ experimental values

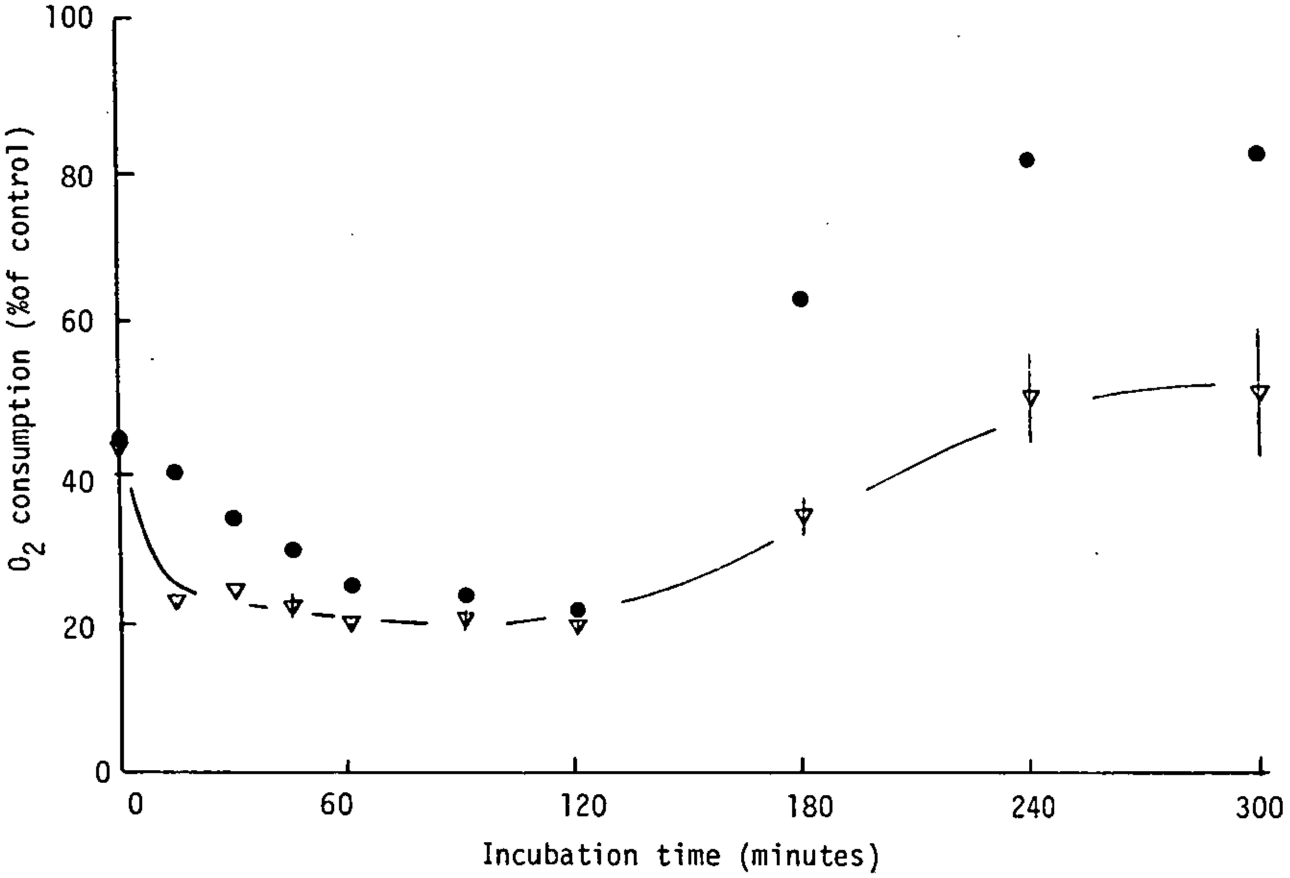
●-● estimated values due to dazomet alone in the predegraded mixture

FIGURE 3.60

Effect of predegraded dazomet (100 μg.ml^{-1}; 0.617 mM) on oxygen consumption by B.licheniformis in minimal salts media at 37^{o}C using an oxygen electrode

Substrate: sodium succinate (25 mM)

Symbols are the same as in Figure 3.59


100
80
60
40
20
0
O_2 consumption (%of control)
0
60
120
180
240
300
Incubation time (minutes)

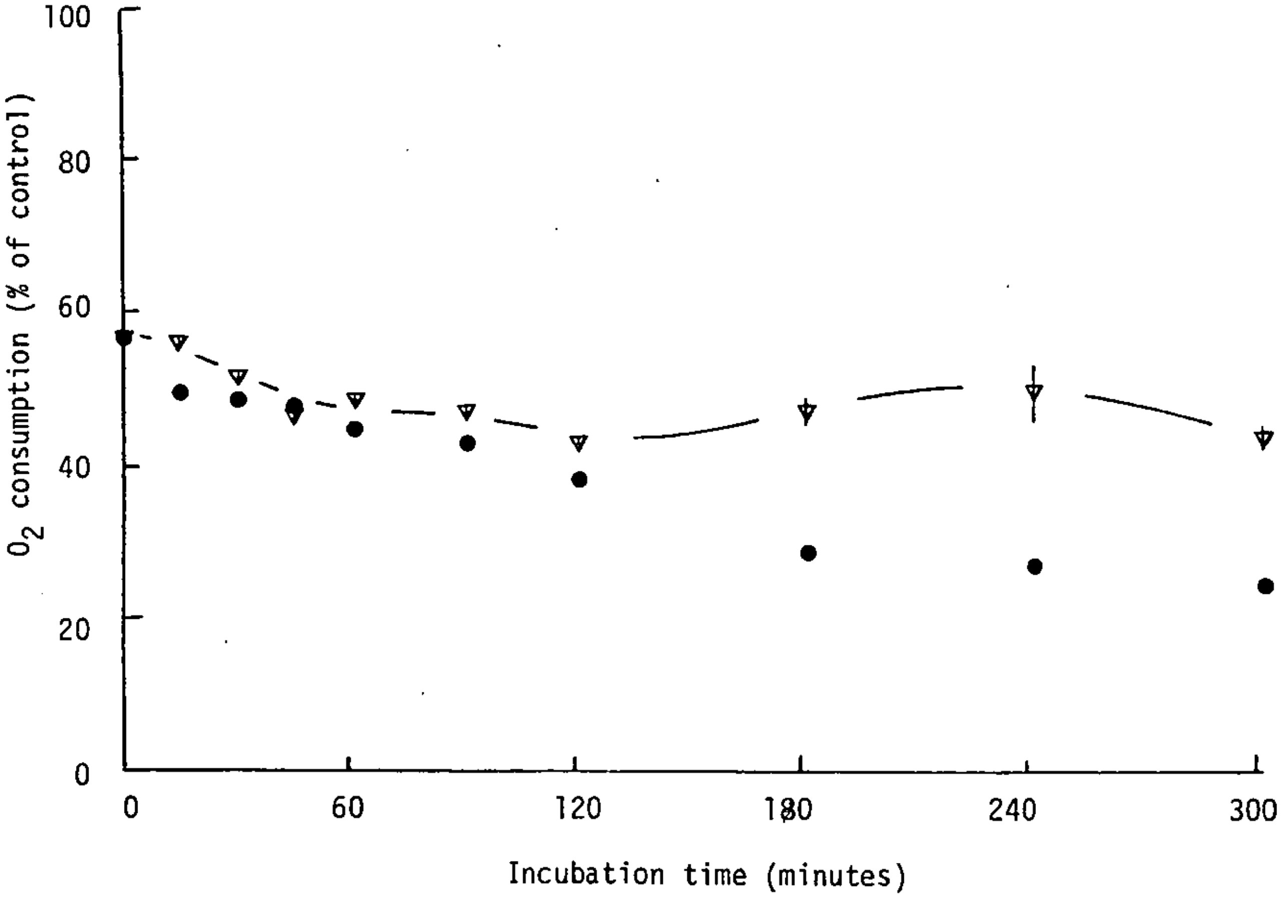

100
80
60
40
20
0
O_2 consumption (% of control)
0
60
120
180
240
300
Incubation time (minutes)

FIGURE 3.61

Assessment of the activity of dazomet against *B.licheniformis* by spectrophotometric measurement of growth at 650 nm

◇-◇	500	$\mu g.ml^{-1}$
□-□	250	"
×-×	100	"
+-+	50	"
▽-▽	10	"
△-△	5	"
○-○	0	"

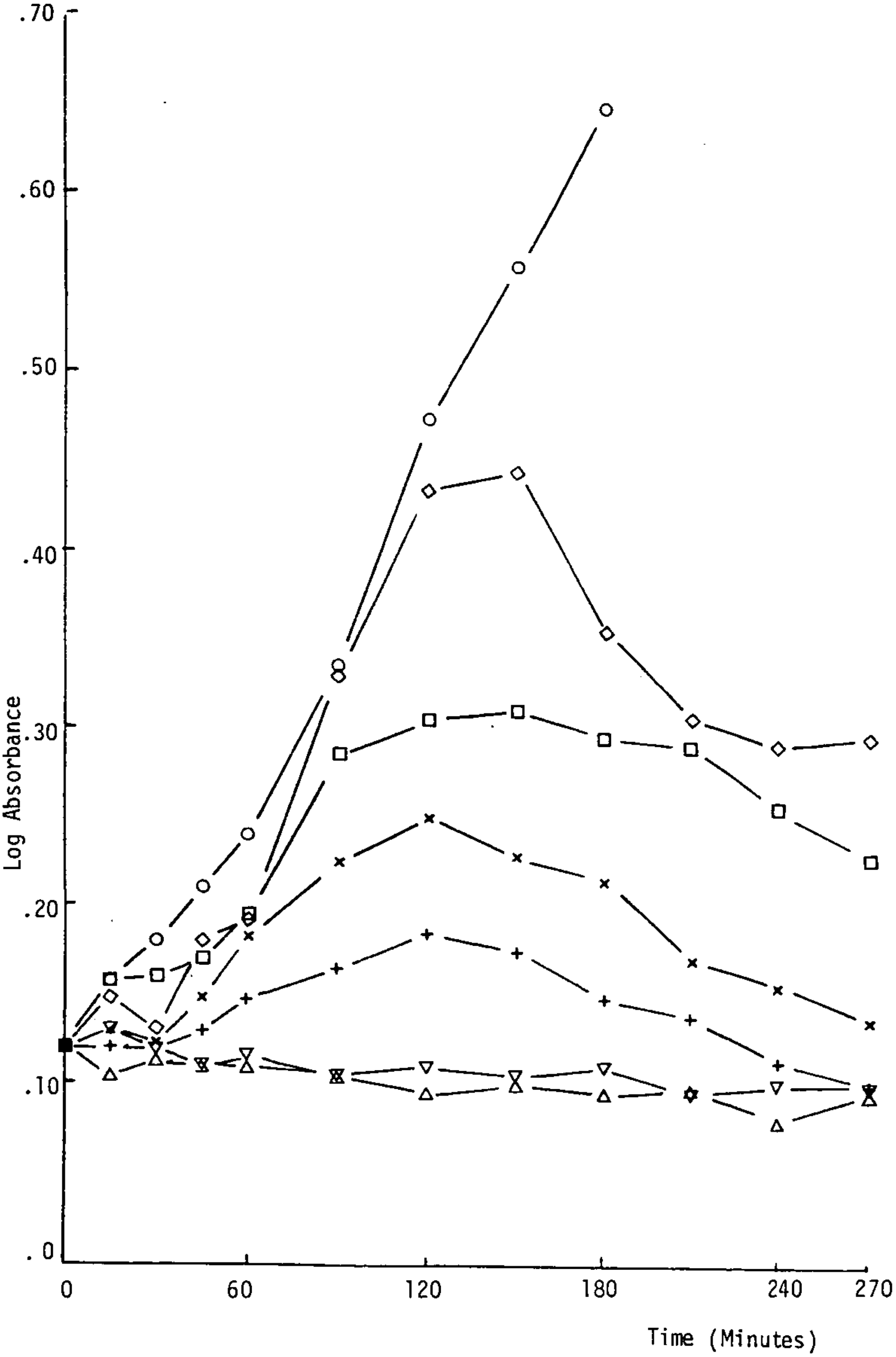

.70
.60
.50
.40
.30
.20
.10
. 0
Log Absorbance
0
60
120
180
240
270
Time (Minutes)

FIGURE 3.62

Assessment of the activity of Na-MDC against *B.licheniformis* by spectrophotometric measurement of growth at 650 nm

◇-◇	500	μg.ml^{-1}
□-□	250	"
×-×	100	"
+-+	50	"
▽-▽	10	"
△-△	5	"
○-○	0	"

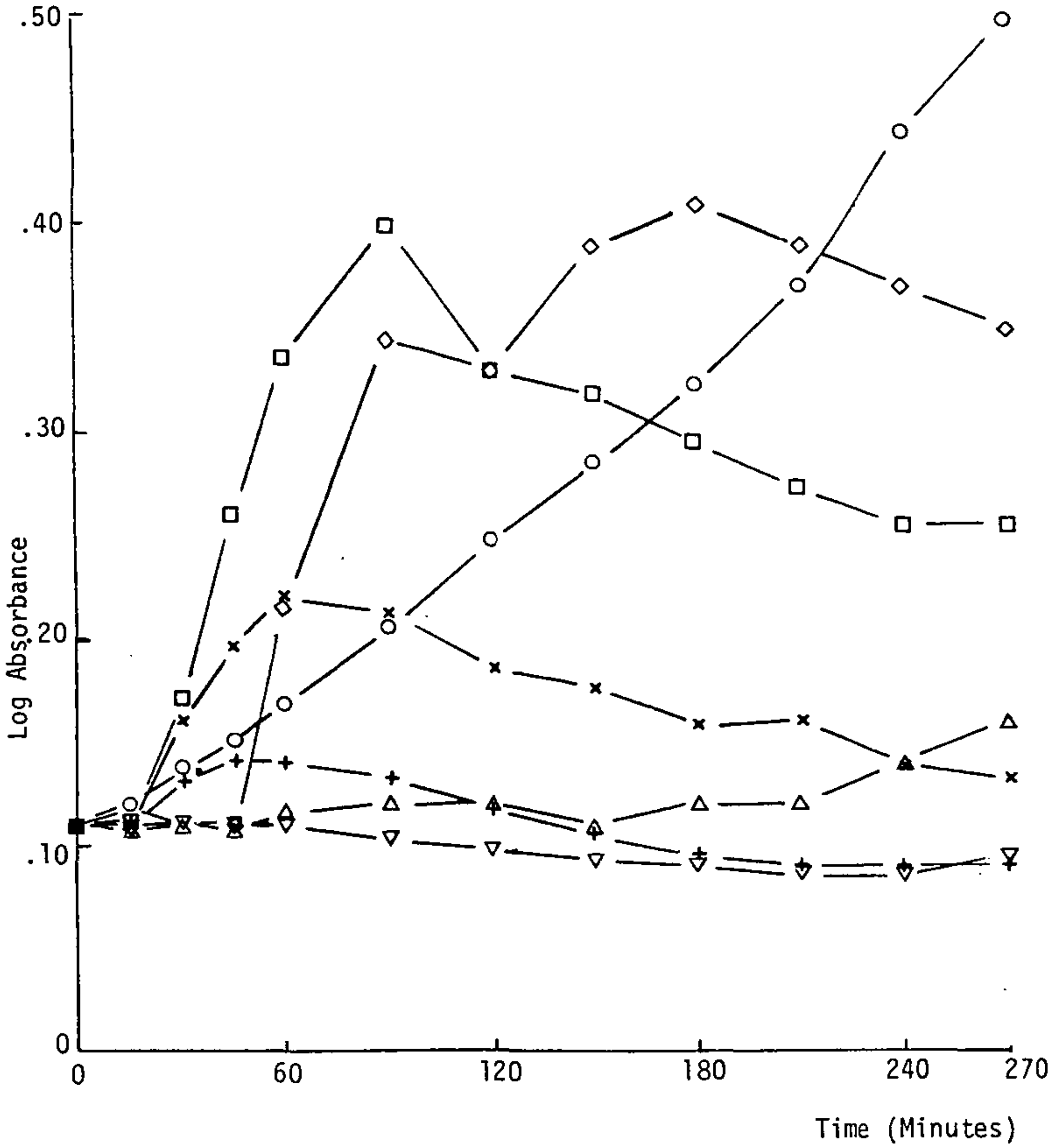

Log Absorbance
.50
.40
.30
.20
.10
0
0
60
120
180
240
270
Time (Minutes)

FIGURE 3.63

Assessment of the activity of MIT against *B.licheniformis* by spectrophotometric measurement of growth at 650 nm

◇-◇	50	$\mu g.ml^{-1}$
□-□	40	"
×-×	30	"
+-+	20	"
▽-▽	10	"
△-△	5	"
○-○	0	"

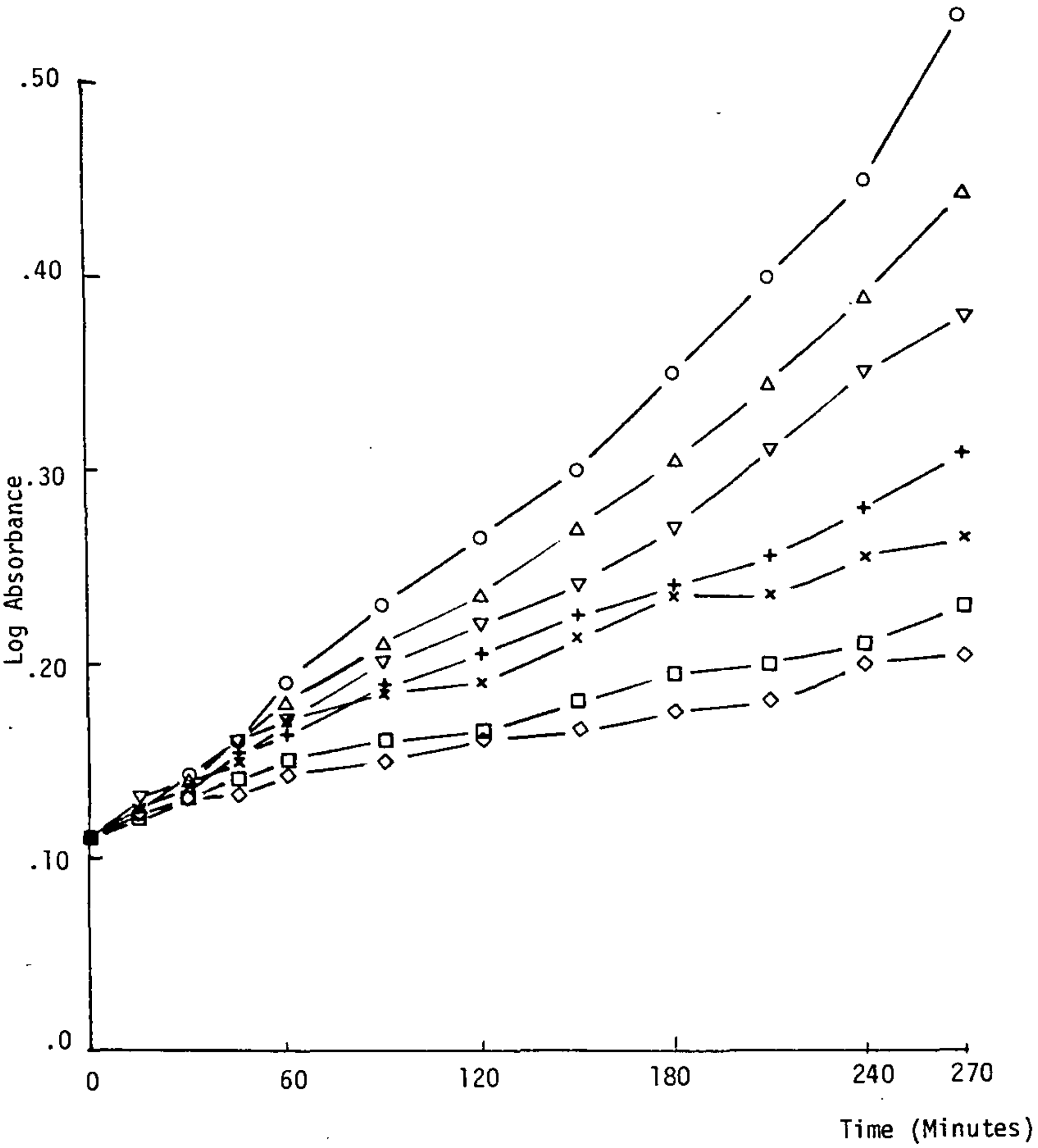

Log Absorbance
.50
.40
.30
.20
.10
.0
0
60
120
180
240
270
Time (Minutes)

FIGURE 3.64

Assessment of the activity of formaldehyde against *B.licheniformis* by spectrophotometric measurement of growth at 650 nm

◇ - ◇	50 $\mu g.ml^{-1}$
× - ×	30 "
+ - +	20 "
▽ - ▽	10 "
△ - △	5 "
○ - ○	0 "

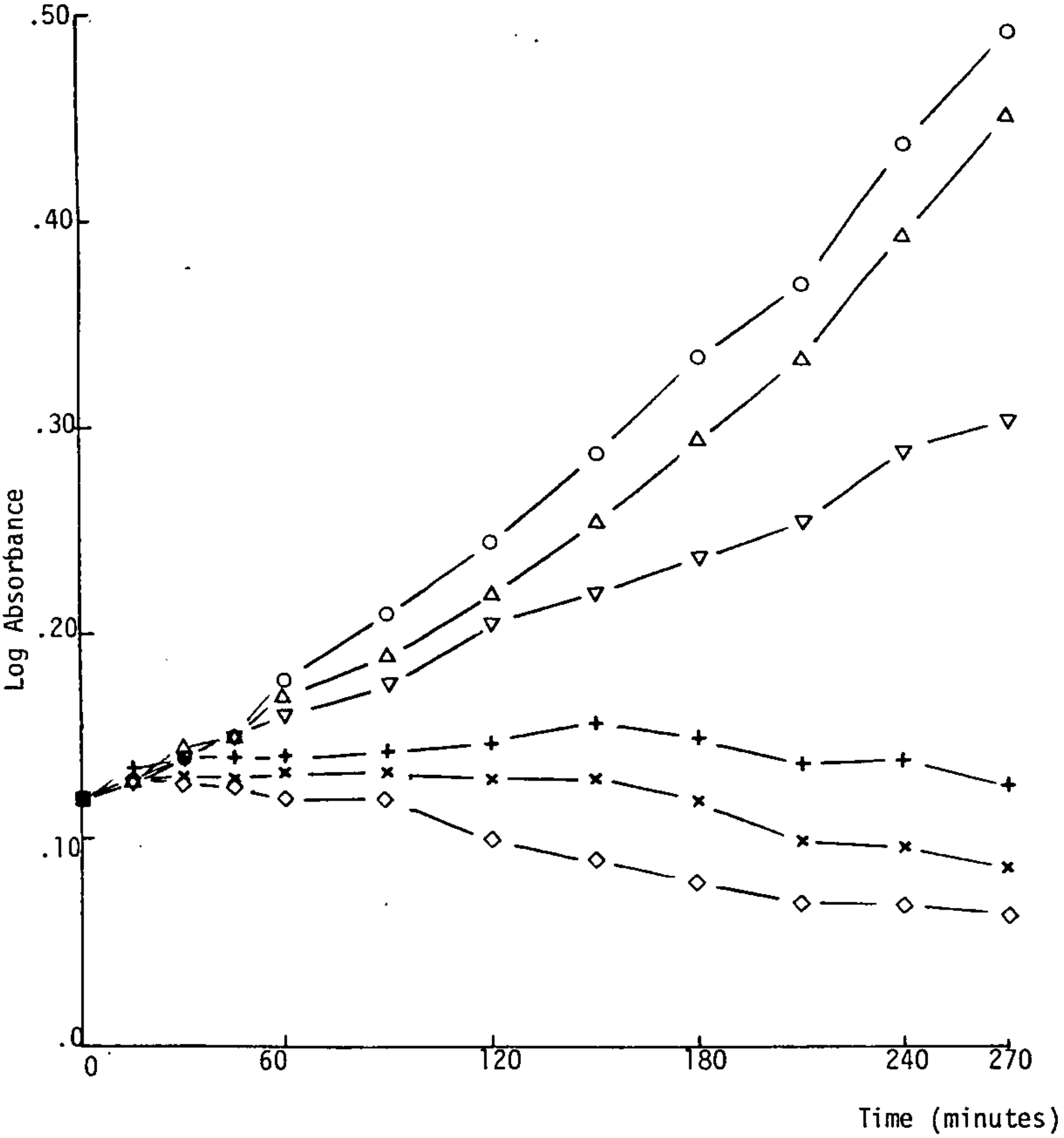

.50
.40
.30
.20
.10
.0
Log Absorbance
0
60
120
180
240
270
Time (minutes)

FIGURE 3.65

Assessment of the activity of dazomet against B.licheniformis in the presence of L-cysteine by spectrophotometric measurement of growth at 650 nm

△ – △ 5 $\mu g.ml^{-1}$

▽ – ▽ 5 " + L-cysteine (0.625 mM)

○ – ○ 0 "

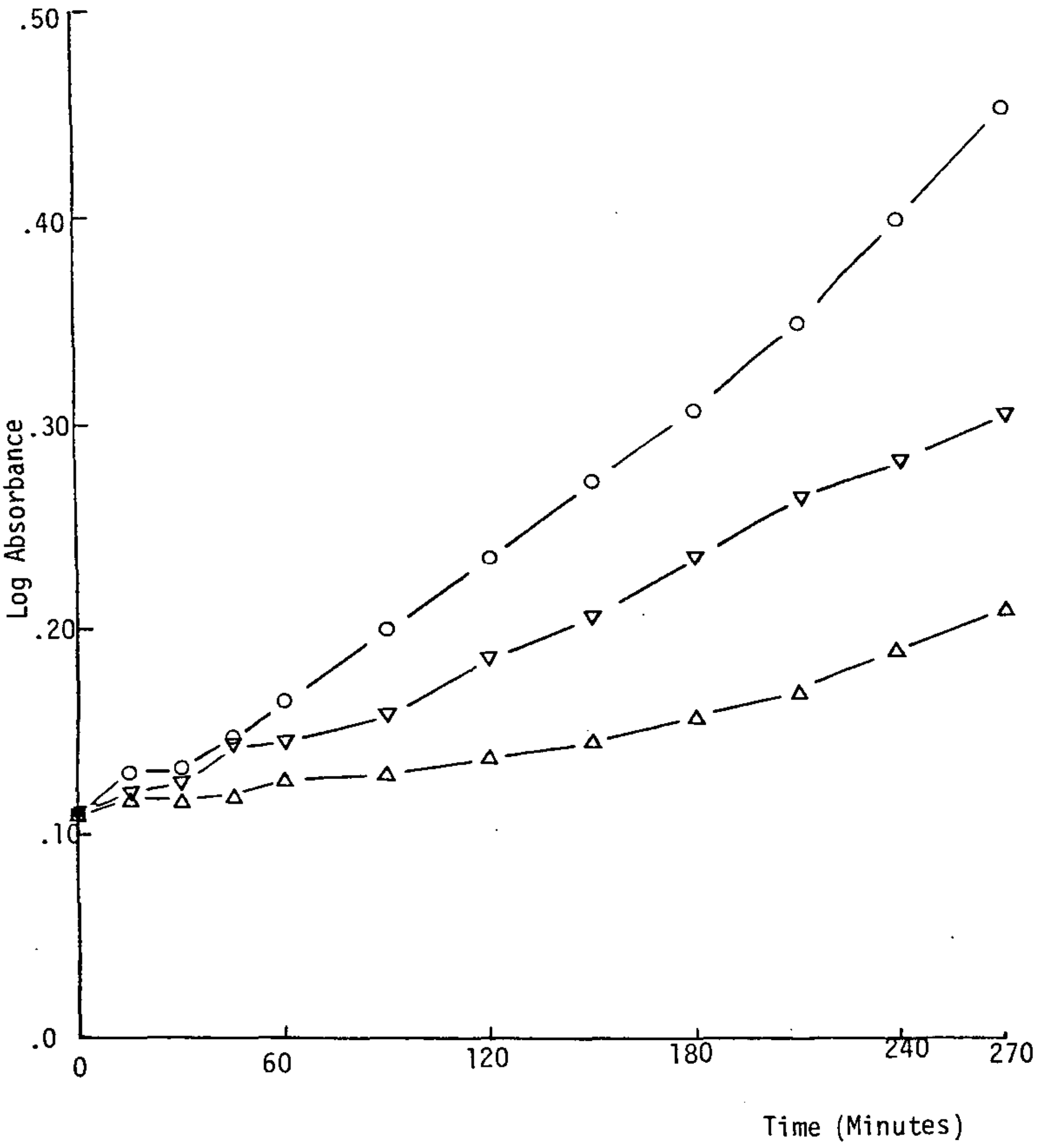

.50
.40
.30
.20
.10
.0
Log Absorbance
0
60
120
180
240
270
Time (Minutes)

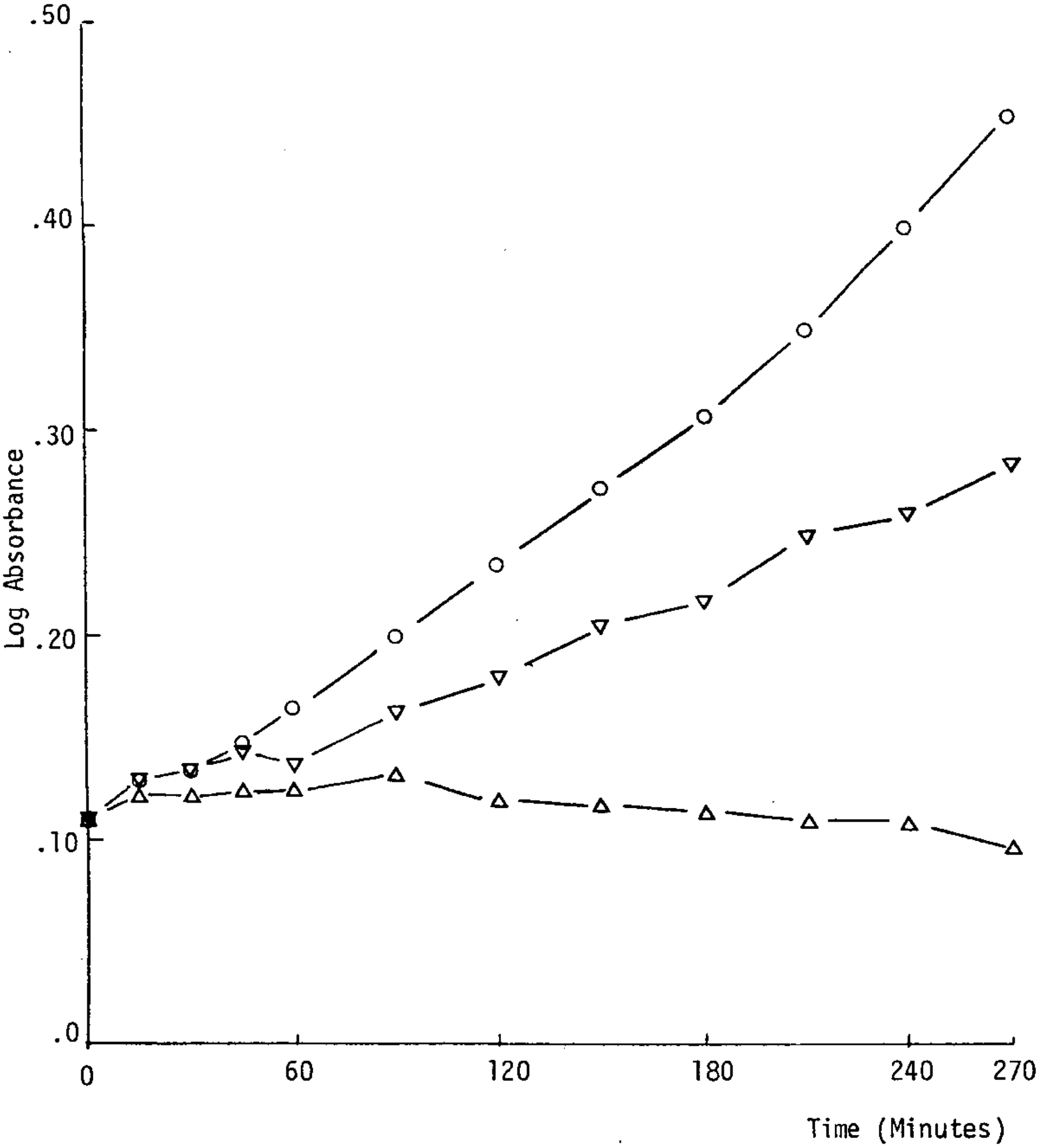
FIGURE 3.66

Assessment of the activity of Na-MDC against B.licheniformis in the presence of L-cysteine by spectrophotometric measurement of growth at 650 nm

△ – △ 10 μg.ml^{-1}

▽ – ▽ 10 " + L-cysteine (0.625 mM)

○ – ○ 0 "



.50
.40
.30
.20
.10
.0
Log Absorbance
0
60
120
180
240
270
Time (Minutes)

FIGURE 3.67

Assessment of the activity of MIT against *B.licheniformis* in the presence of L-cysteine by spectrophotometric measurement of growth at 650 nm

△ — △ 50 $\mu g.ml^{-1}$

▽ — ▽ 50 " + L-cysteine (0.625 mM)

○ — ○ 0 "

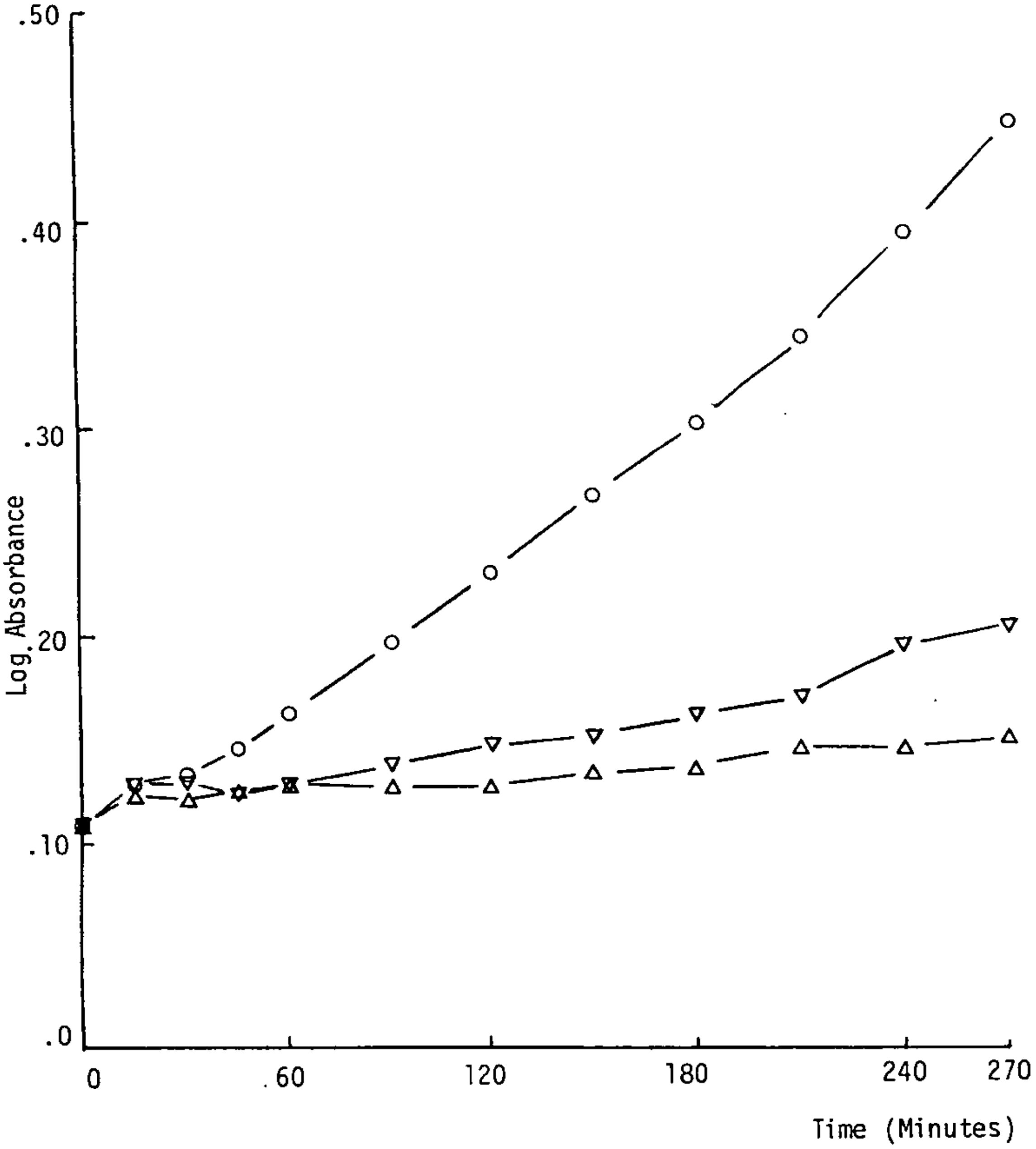

.50
.40
.30
.20
.10
.0
Log. Absorbance
0
60
120
180
240
270
Time (Minutes)

FIGURE 3.68

Assessment of the activity of formaldehyde against *B.licheniformis* in the presence of L-cysteine by spectrophotometric measurement of growth at 650 nm

△ — △ 50 μg.ml^{-1}

▽ — ▽ 50 " + L-cysteine (0.625 mM)

○ — ○ 0 "

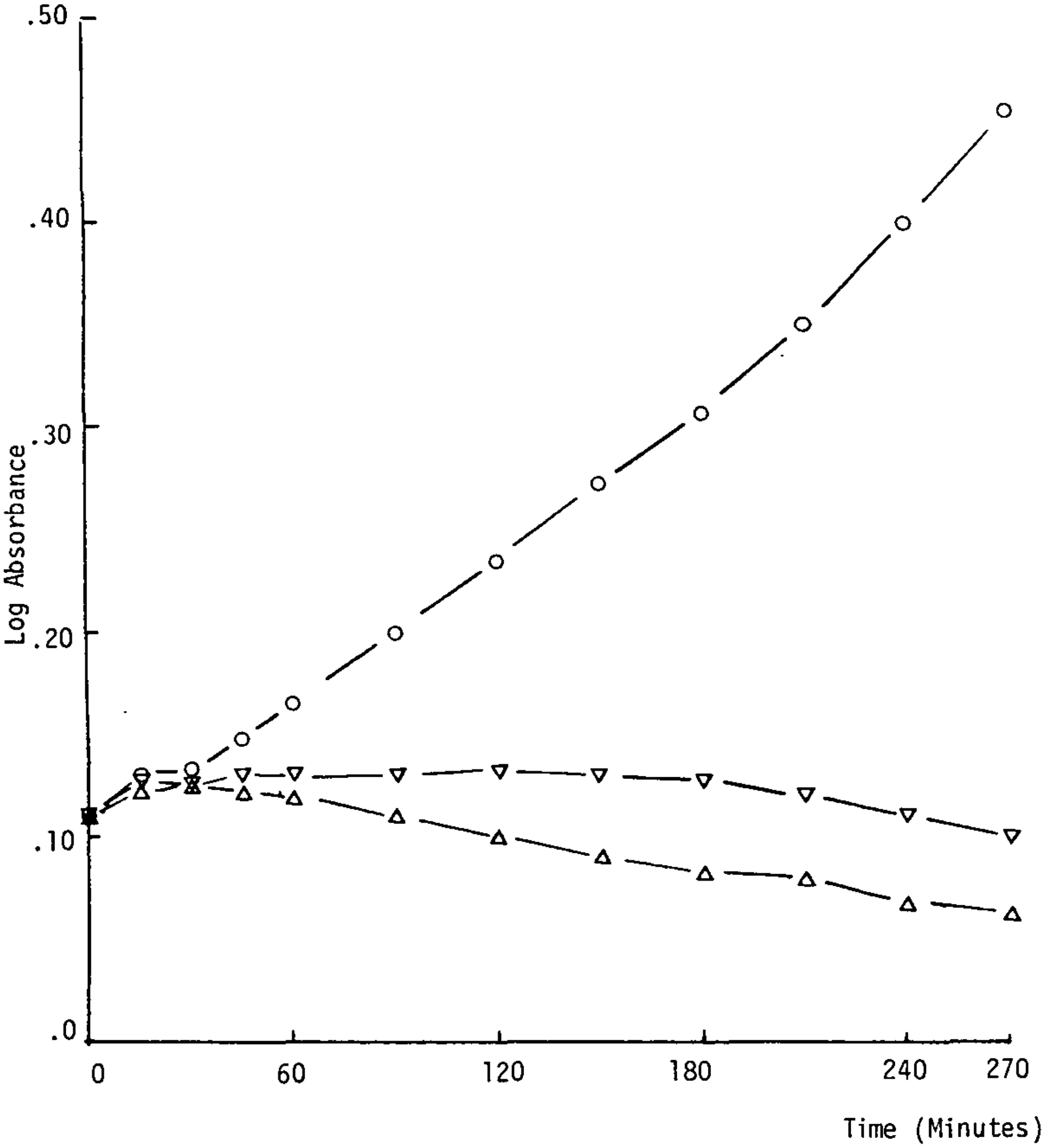

Log Absorbance
.50
.40
.30
.20
.10
.0
0
60
120
180
240
270
Time (Minutes)

FIGURE 3.69

UV spectra of Na-MDC in minimal salts media containing Zn (11), Fe (11), Mn (11) and Mg (11) at 0.09, 0.09, 0.12 and 0.98 mM, respectively

Curves 1-8: 0.04, 0.08, 0.16, 0.40, 0.80, 1.60, 2.78 and 3.97 mM concentrations of Na-MDC, respectively

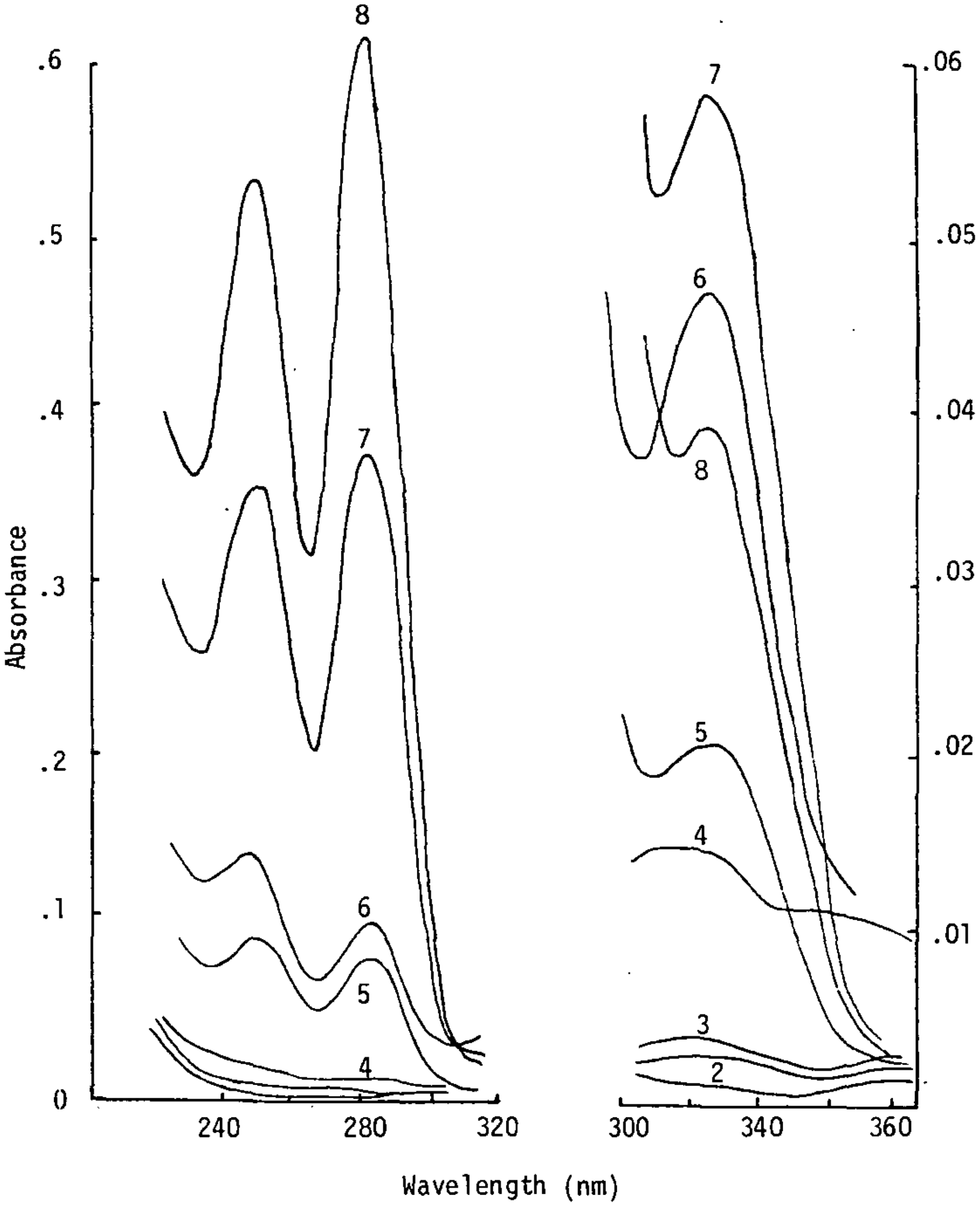
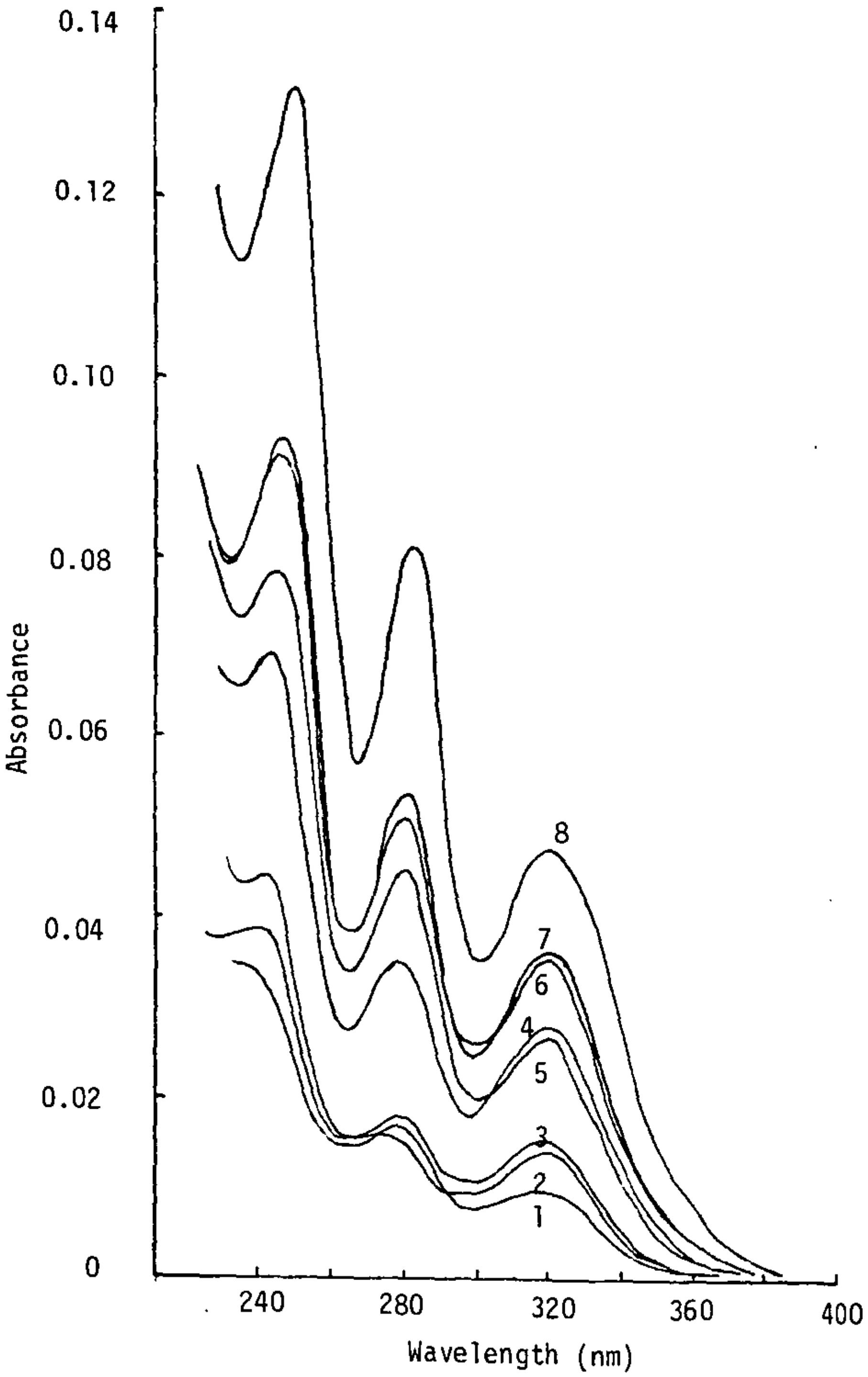

Absorbance
.6
.5
.4
.3
.2
.1
0
.06
.05
.04
.03
.02
.01
240
280
320
300
340
360
Wavelength (nm)
8
7
6
5
4
7
6
8
5
4
3
2

FIGURE 3.70

UV spectra of Na-MDC in minimal salts media containing 0.98 mM Mg (11) ions

Curves 1-8: 1.19, 1.59, 1.99, 2.33, 2.78, 3.18, 3.57 and 3.97 mM concentrations of Na-MDC, respectively


Absorbance
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
240
280
320
360
400
Wavelength (nm)
8
7
6
4
5
3
2
1

TYPICAL TLC PLATES OF DEGRADED DAZOMET AND SOME STANDARDS

Plate 3.1

TLC using calcium oxalate plates

From left to right

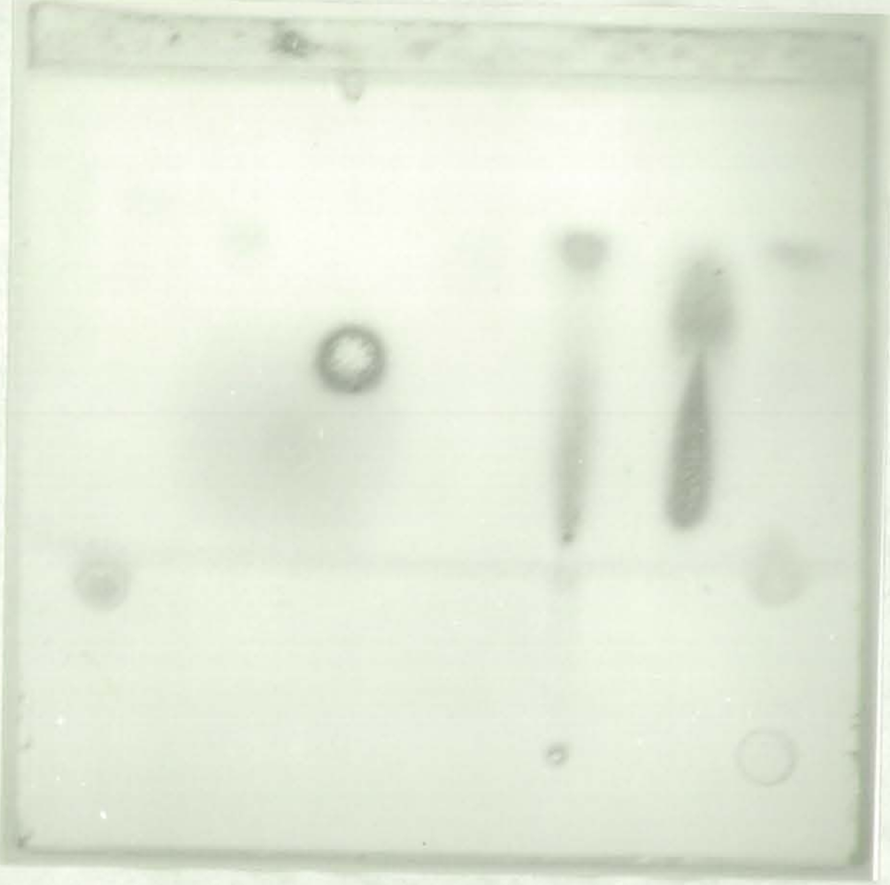
methylamine hydrochloride, DMTD, DMTU, MIT, Na-MDC, dazomet and degraded sample of dazomet (in phosphate buffers for 4 hours at 55°C)

Plate 3.2

TLC using plain silica plates

From left to right

methylamine hydrochloride, DMTU, MIT, Na-MDC, dazomet, degraded dazomet (in phosphate buffers for 4 hours at 55°C, respectively) and sample of dazomet stored in distilled water for 4 months at 45°C.



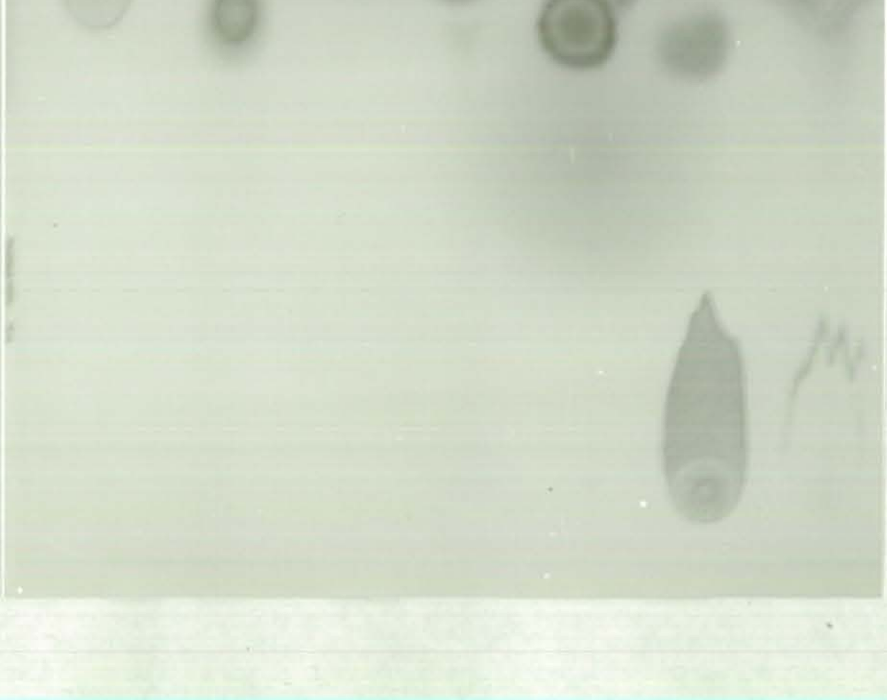


Plate 3.3

Cultures of E.coli grown in minimal salts media at 37^{o}C in the presence of dazomet.

Tubes 1-~~9~~ 10 contain 500, 400, 300, 200, 60, 40, 20, 15, 5 and 0 μg.ml^{-1} dazomet, respectively.

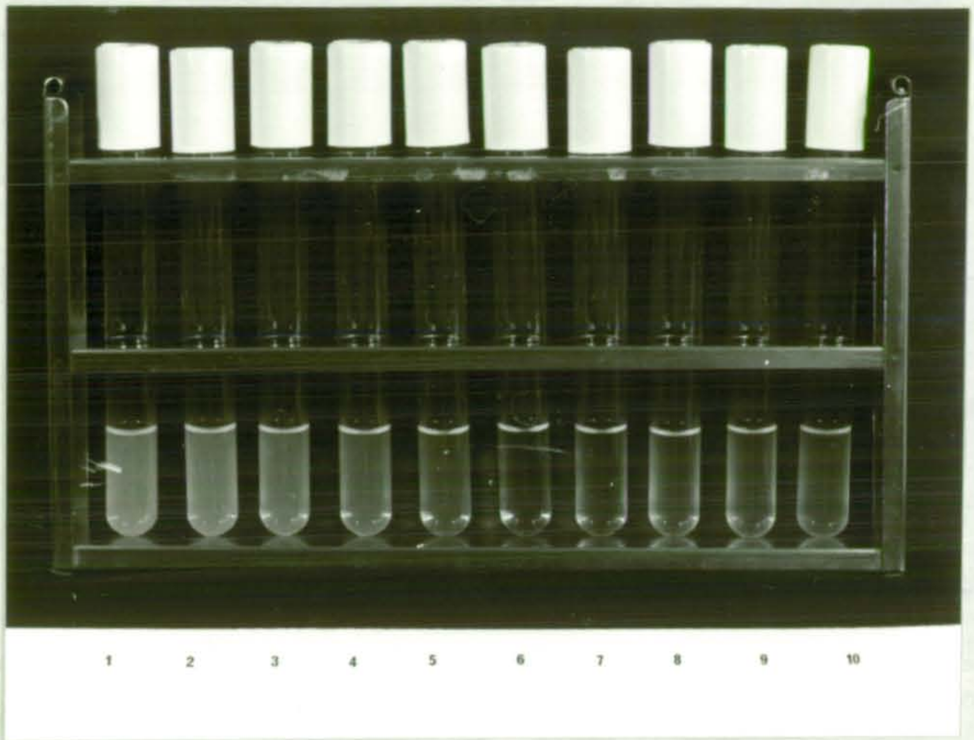
Plate 3.4

Cultures of E.coli grown in minimal salts media at 37^{o}C in the presence of Na-MDC.

Tubes 1-~~9~~ 10 contain 500, 400, 300, 200, 60, 40, 20, 15, 5 and 0 μg.ml^{-1} ~~dazomet~~ Na-MDC, respectively.

E.coli

DAZOMET


1
2
3
4
5
6
7
8
9
10

E.coli

METYLDITHIOCARBAMATE

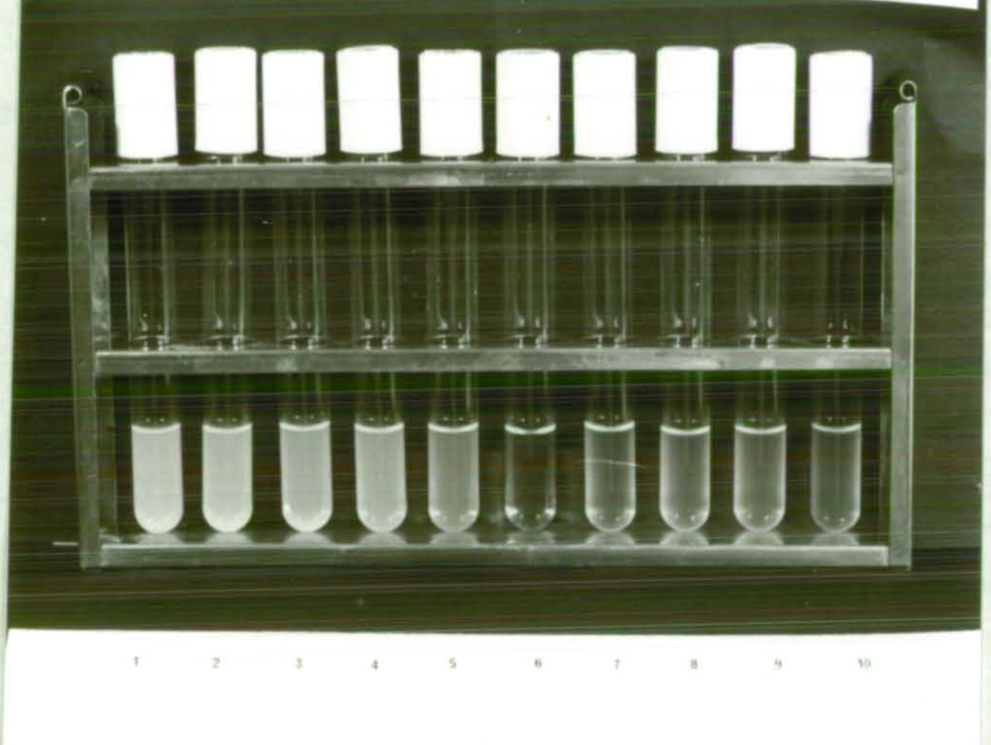

1
2
3
4
5
6
7
8
9
10

Plate 3.5

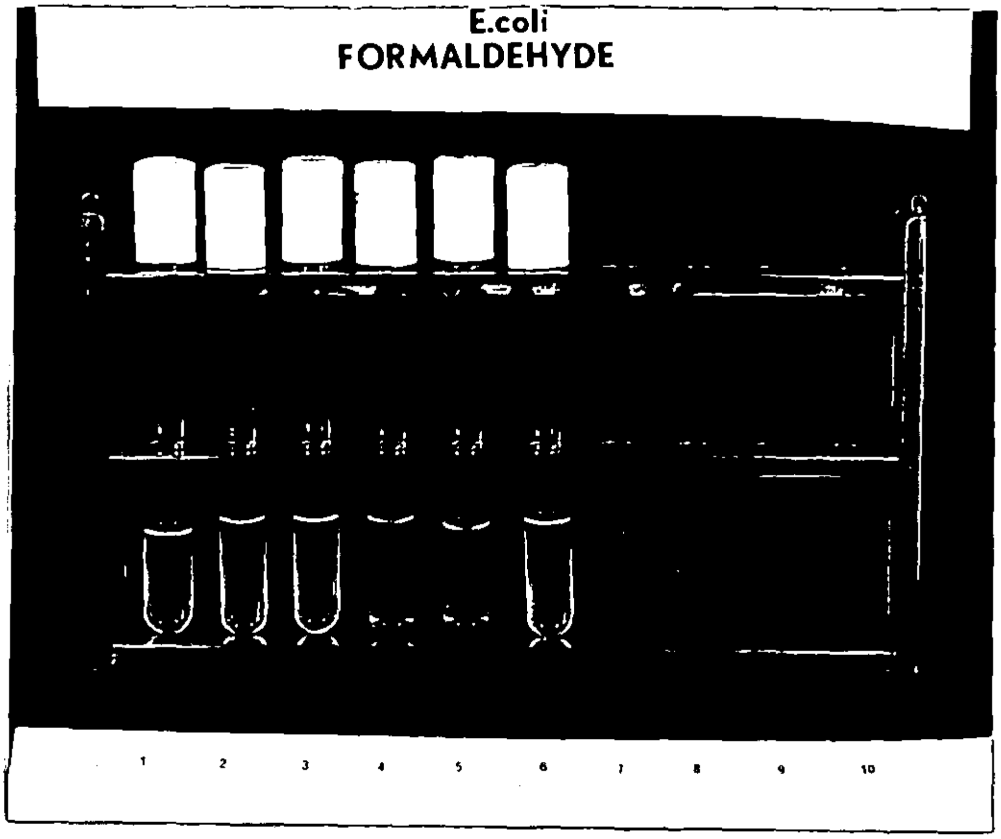
Cultures of E.coli grown in minimal salts media at 37°C in the presence of formaldehyde.

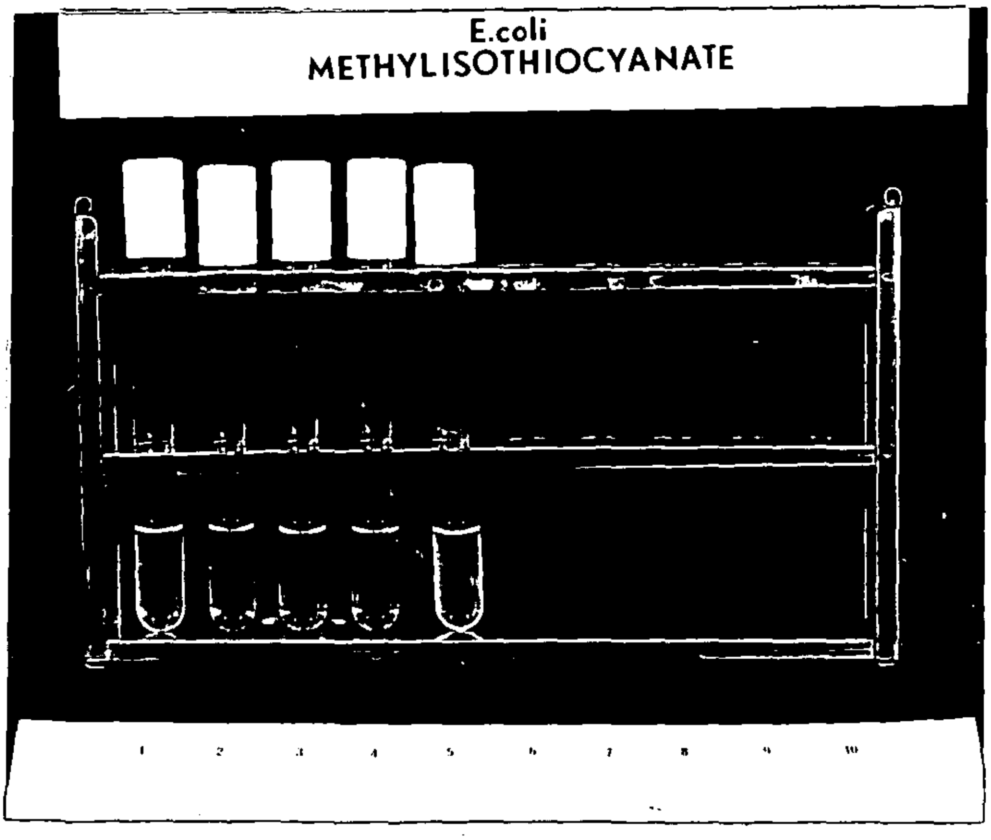
Tubes 1-6 contain 5, 10, 15, 20, 25 and 0 μg ml^{-1} formaldehyde, respectively.

Plate 3.6

Cultures of E.coli grown in minimal salts media at 37°C in the presence of MIT.

Tubes 1-5 contain 5, 10, 15, 20 and 10 μg.ml^{-1} MIT, respectively.


E.coli
FORMALDEHYDE
1
2
3
4
5
6
7
8
9
10


E.coli
METHYLISOTHIOCYANATE
1
2
3
4
5
6
7
8
9
10

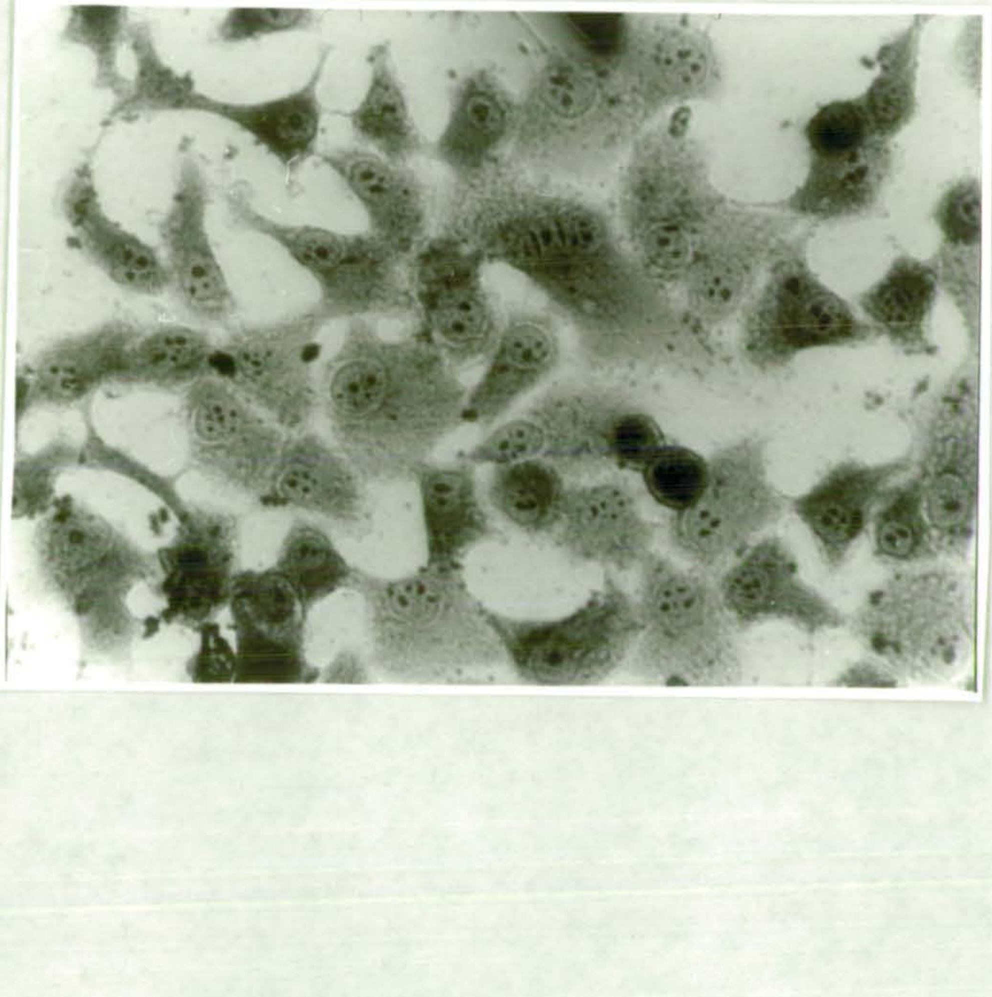
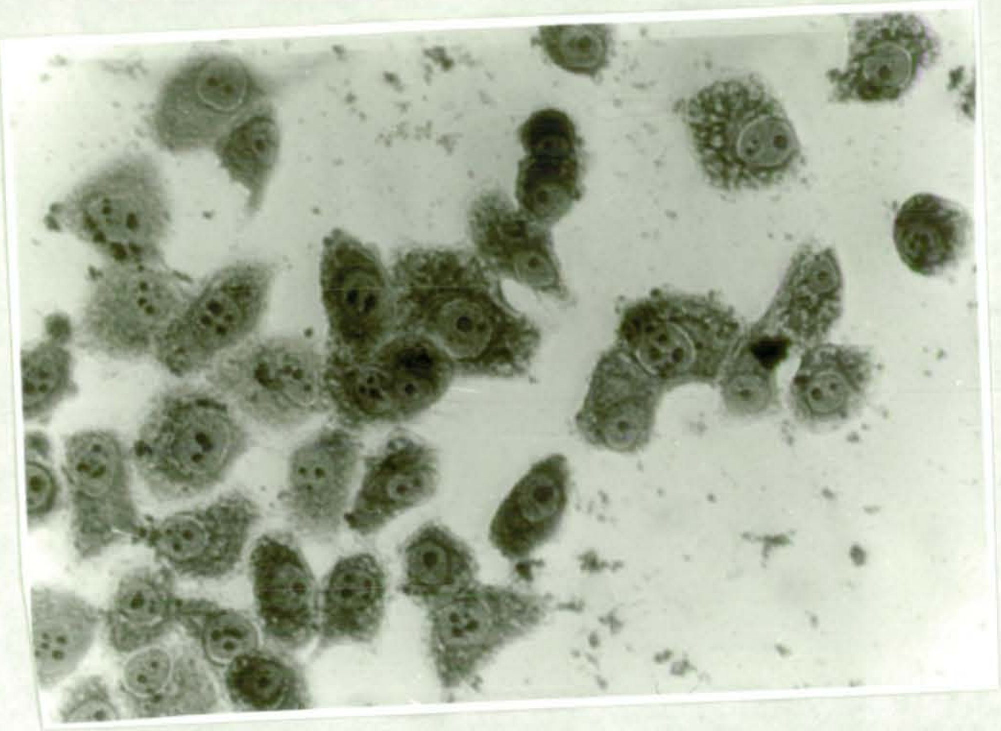


Plate 3.8

Effect of sub-toxic concentration of dazomet (1.6 $\mu g.ml^{-1}$) on Human KB cells cultured in MEM medium at 37°C.

Plate 3.9

Effect of sub-toxic concentration of Na-MDC (8 $\mu g.ml^{-1}$) on Human KB cells cultured in MEM medium at 37°C.



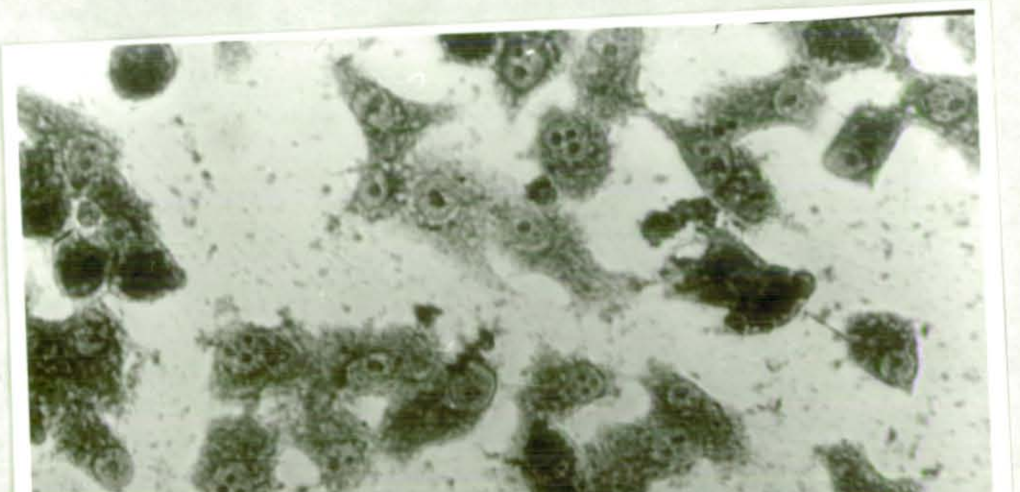
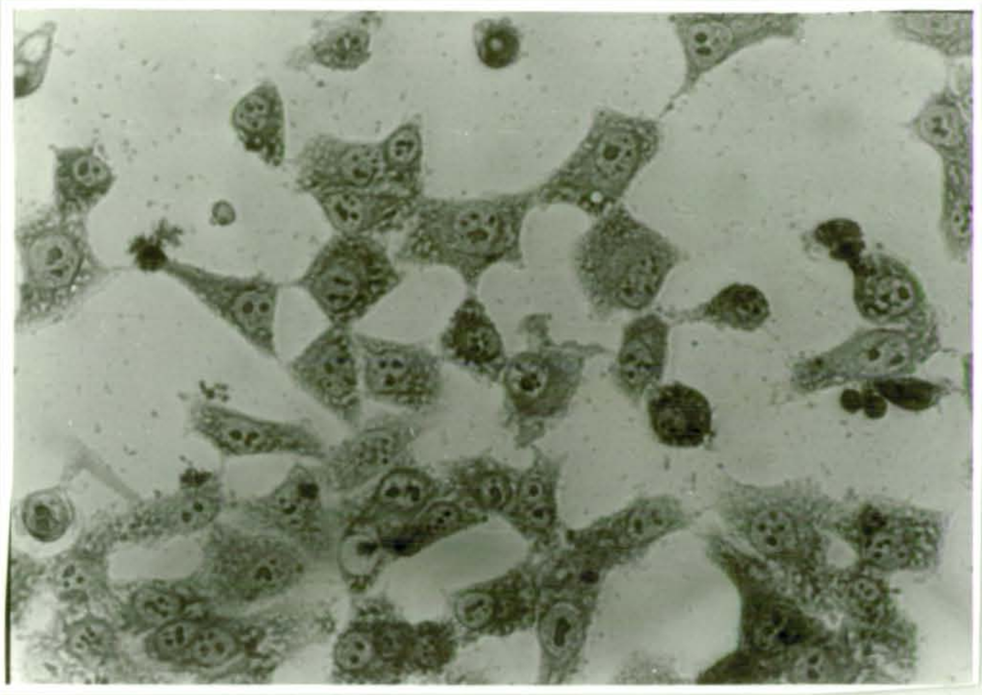


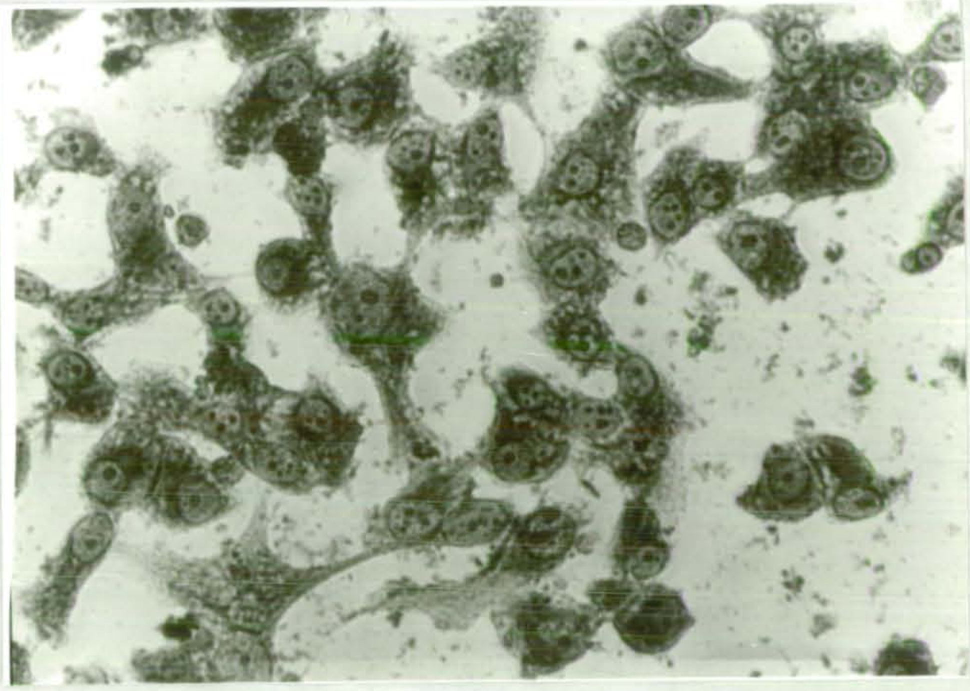
Plate 3.10

Effect of sub-toxic concentration of formaldehyde (0.4 $\mu g.ml^{-1}$) on Human KB cells cultured in MEM medium at $37^{o}C$.

Plate 3.11

Effect of sub-toxic concentration of MIT (2-8 $\mu g.ml^{-1}$) on Human KB cells cultured in MEM medium at $37^{o}C$.





4. KINETICS OF DEGRADATION

4.1 Kinetics of Degradation of Dazomet in Phosphate Buffers

4.1.1 The proposed model

For the kinetic analysis of the experimental data obtained for the degradation of dazomet in phosphate buffers (Figures 3.18-3.21), the simplified reaction network shown in Figure 4.1 was found to hold. This scheme was arrived at after examining other possibilities (see Appendix 6.2) - none of which resulted in a satisfactory representation of the experimental observations.

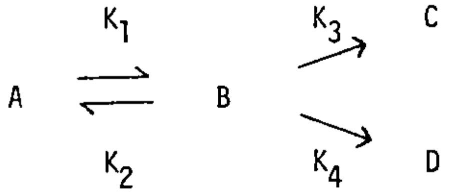


FIGURE 4.1 Simplified reaction network for the degradation of dazomet

where: A is dazomet; B is N-methyldithiocarbamate (MDC);

C is N-methylisothiocyanate (MIT) and D represents all other products not quantified

The objective of the kinetic model presented here is to give an analytical representation of the experimentally obtained concentration versus time profiles for the degradation of dazomet and its main products - namely, MDC and MIT.

As mentioned previously, (3.1.6), concentrations of A, B and C in a reaction mixture at a given time were obtained by HPLC. Concentration of D, however was obtained by a mass balance as follows:

$$C_D = C_{A_0} - C_A - C_B - C_C \qquad (4.1)$$

where: C_{A_0} is initial concentration of A and C_A, C_B, C_C and C_D are concentrations of A, B, C and D, respectively.

A pseudo-first order equilibrium was assumed for the degradation of A to B. This was based on the nature of the degradation profiles (Figures 3.18-3.21). The degradation of dazomet can be seen to proceed in three stages. The first stage, up to about 30 minutes is very rapid and the concentration of dazomet decreases to about 1/3 of the initial concentration. The concentration versus time relationship is approximately a straight line - characteristic of a zero order reaction. The second stage up to about 90 minutes is exponential, and if log concentration versus time is plotted for the region, a straight line can be drawn through the points, characteristic of a first order reaction. The final stage, from 90 to 300 minutes, is a region represented by very little change (less than 0.5 mM). This region resembles the first stage, and a straight line can be drawn through the concentration versus time points, characteristic of zero order reaction. These sequences of events clearly imply establishment of an equilibrium in the system, and since the disappearance of A continues at a rapid rate until large concentrations of B are produced, it is reasonable to assume an equilibrium between A and B.

Degradation of B was treated as two separate reactions of unknown order with respect to the concentrations of the products.

Since C and D appeared in the incubation mixture only after B was formed (Figures 3.18-3.21), they can be assumed to be products arising from B. The rate of formation and the maximum amount of C formed was nearly the same in each case. A straight line through the concentration versus time points for C suggests that its formation is not dependent on the amount of B present in the incubation mixture. However, the amount of D, which was calculated by a mass balance (4.1), was different in each case, and since various possibilities exist as to the nature of secondary reactions, involving both C and various products of D (e.g. carbon disulphide, methylamine, N,N-dimethylthiuram disulphide etc), an unknown order of reaction was assumed with respect to concentration of each.

Thus, rate equations for A, C and D can be written as follows:

$$-\gamma_A = \frac{-d\ C_A}{dt} = K_1\ C_A - K_2\ C_B \qquad (4.2)$$

$$\gamma_C = \frac{d\ C_C}{dt} = K_3\ C_C^{\ n_3} \qquad (4.3)$$

$$\gamma_D = \frac{d\ C_D}{dt} = K_4\ C_D^{\ n_4} \qquad (4.4)$$

where $-\gamma_A$ is the rate of degradation of A

γ_C and γ_D are the rates of formation of C and D, respectively.

K_1, K_2, K_3 and K_4 are the rate constants and n_3, n_4 are the orders of reactions

By integrating equations (4.2), (4.3) and (4.4) with appropriate boundary conditions, the following concentration versus time profiles were obtained (see Appendix 6.3)

$$C_A = C_{A_e} + (C_{A_o} - C_{A_e}) \exp(-Kt) \qquad (4.5)$$

where: - $K = K_1 + K_2$ (rate constants for the forward and reverse reactions) and C_{A_e} = equilibrium concentration of A, taken as the final value of C_A.

$$C_C = [(1 - n_3)K_3]^{\frac{1}{1-n_3}} t^{\frac{1}{1-n_3}} \qquad (4.6)$$

$$C_D = [(1-n_4)K_4]^{\frac{1}{1-n_4}} t^{\frac{1}{1-n_4}} \qquad (4.7)$$

For B, the concentration versus time profiles were obtained from mass balance (4.1).

4.1.2 Evaluation of K_1 to K_4 (rate constants) and n_3 and n_4 (order of reactions for C and D respectively)

Evaluation of K_1 and K_2

Equation (4.5) can be written as:

$$\ln \frac{C_A - C_{A_e}}{C_{A_o} - C_{A_e}} = - Kt \tag{4.8}$$

The best value of K was obtained using a non-linear optimization routine G02CBF (NAG Library). The initial estimate of K required for this routine was obtained as follows:

By plotting $\ln \frac{C_A - C_{A_e}}{C_{A_o} - C_{A_e}}$ versus t, a straight line was obtained through the origin (Figure 4.2). The slope of this line gave the required estimate of K.

From the best value of K obtained using the NAG routine G02CBF, K_1 and K_2 were calculated using the following relationships (Appendix 6.3):

$$K_1 = \frac{-K\ (C_{A_o} - C_{A_e})}{C_{A_o}} \tag{4.9}$$

$$K_2 = -(K + K_1) = -K \frac{C_{A_e}}{C_{A_o}} \tag{4.10}$$

Evaluation of K_3 and n_3

Equation (4.6) can be written as:

$$C_C = at^b \tag{4.11}$$

where:
$$a = [(1 - n_3)K_3]^{\frac{1}{1-n_3}} \tag{4.12}$$

$$b = \frac{1}{1-n_3} \tag{4.13}$$

Constants a and b were obtained using a non-linear optimization routine E04FDF (NAG library routine). Initial estimates of a and b required in the routine E04FDF were obtained as follows:

From equation (4.11), we have

$$\log C_C = \log a + b \log t \tag{4.14}$$

Thus, by plotting log C_C versus log t and using only two selected points, a straight line was obtained. The slope of this line gave the value of b and the intercept was used to get the value of a. The individual values of these estimates are shown in the computer program (Appendix 6.4).

Using the optimum values of a and b obtained from the routine E04FDF, K_3 and n_3 were calculated from equations (4.12) and (4.13).

Evaluation of K_4 and n_4

K_4 and n_4 were evaluated using exactly the same procedure described above for the evaluation of K_3 and n_3.

4.1.3 Results from the kinetic model

A computer program was developed to evaluate the rate constants (K_1, K_2, K_3, K_4) and the orders of reactions (n_3, n_4) using the methods outlined in the previous sections (see Appendix 6.4). The values of the constants K_1, K_2, K_3, K_4, n_3 and n_4 were obtained for different pH conditions and are shown in Table 4.1. For each of the reaction components, namely, dazomet, MDC and MIT represented as A, B and C respectively, the standard deviations of the experimental data as well as the standard deviations between the predicted values and the mean of the experimental values are also shown in Table 4.1.

It can be seen that the standard deviations of the experimental data and the standard deviations of the predicted data using the kinetic model are very similar. The standard deviations of the predicted points for D (representing all other products not quantified) are also in close agreement with the values obtained from the mass balance.

The orders of reactions provided for C and D are fractional and in some cases negative as shown in Table 4.1.

The predicted concentration versus time profiles for A, B C and D at pH 5, 6, 7 and 7.5 are shown in Figures 3.18-3.21,

TABLE 4.1

Evaluated rate constants and orders of reaction for the degradation of dazomet

Component	A				B		C				D		
pH	K_1 $\times 10^6, s^{-1}$	K_2 $\times 10^6, s^{-1}$	SD OF[1] EXP DATA	SD OF[2] PRED DATA	SD OF[1] EXP DATA	SD OF[2] PRED DATA	n_3	K_3 $\times 10^6, s^{-1}$	SD OF[1] EXP DATA	SD OF[2] PRED DATA	n_4	K_4 $\times 10^6, s^{-1}$	SD OF[3] PRED DATA
5	737.8	58.7	.0335	.0954	.2302	.1938	-0.173	27.2	.0384	.0141	0.005	223.6	.1641
6	607.8	68.2	.0437	.0841	.0917	.0924	-0.198	23.0	.0293	.0133	0.484	198.6	.0435
7	533.3	57.4	.0612	.0910	.0729	.1010	0.169	23.0	.0534	.0369	.0516	186.5	0.0333
7.5	613.3	54.6	.0421	.0999	.1098	.1229	-0.145	21.2	.0191	.0189	-0.946	218.9	0.1888

A, B, C and D = dazomet, N-methyldithiocarbamate, N-methylisothiocyanate and all other products not quantified, respectively

K_1, K_2, K_3 and K_4 are rate constants

n_3 and n_4 are orders of reactions

SD = standard deviation (defined in Appendix 6.1)

1 = standard deviation of points determined experimentally

2 = standard deviation of predicted points from the mean of experimentally determined points

3 = standard deviation of predicted points from the mean of calculated values (by mass balance) of D

respectively. The figures show excellent agreement between the predicted curves and the experimental data.

4.1.4 Influence of various factors on the degradation of dazomet

The influence of various factors on the rate constants K_1 and K_2 for the degradation of dazomet are summarised in Tables 4.2 (effect of oxygen and nitrogen rich environments at various pH's), 4.5 (effect of temperature) and 4.4 (effects of some products of degradation in the incubation mixture).

Under normal conditions of degradation (in phosphate buffer at 55°C), the influence of pH on the rate constants was very little (Table 4.1). However, small differences could be observed and the value of the rate constant K_1 (forward reaction of the equilibrium) was the highest at pH 5.0, decreasing slightly with increasing pH (pH 6 and 7), but showing slight increase at pH 7.5. However, for the corresponding values of K_2 (reverse reaction of the equilibrium), the effect was reversed. Thus, the value of K_2 was the lowest at pH 5.0, increasing slightly with increasing pH (pH 6 and 7), but showing a slight decrease at pH 7.5.

Under the conditions of oxygen and nitrogen rich environments (Table 4.2), the effect of pH on the rate constants K_1 and K_2 is the same as under normal conditions(Table 4.1). However, the values of K_1 are slightly higher than those under normal conditions, with values of K_1 for oxygen rich environment being slightly greater than under nitrogen rich environments. The values of K_2 for oxygen rich environments are lower than those under normal conditions, while those for nitrogen rich environments are slightly higher. Table 4.2 also shows the influence of dark environments on the rate constants, K_1 and K_2. Compared to the degradation under normal conditions (Table 4.1), there is no significant effect on K_1. However, the value of K_2 is slightly less.

The influence of temperature on the rate constants is shown in Table 4.3. The effect of pH on the rate constants K_1 and K_2 is the same as under normal conditions of degradation considered previously (Table 4.1). However, the value of K_1 is lower at 25°C compared with that of 37°C, while the value of K_2 is higher at lower temperatures, and both the rate constants are significantly smaller than at 55°C. The variation of rates with temperature was tested using Arrhenius equation:

$$K = A \exp^{-E/RT} \tag{4.15}$$

where: K = rate constant
T = temperature (Kelvin)
R = gas constant
E = activation energy
A = frequency factor

The equation may be written in the following simplified form:

$$\ln K = \ln A - E/RT \tag{4.16}$$

Hence, a plot of ln K versus $\frac{1}{T}$ gave a straight line (Figure 4.7). The value of E was evaluated from the slope of the graph, and was found to be 21.28 Kcal mol^{-1}; and by substituting this value in (4.16), the value of A was found to be 1.08×10^{11} s^{-1}.

The influence of some products of degradation on the rate constants K_1 and K_2 is shown in Table 4.4. Na-MDC, MIT, methylamine and carbondisulphide, all have little or no effect on the value of K_1 compared to degradation under normal conditions (Table 4.1). However, the value of K_2 is increased by nearly two times in the presence of Na-MDC, and a little less in the presence of MIT, but is not affected much in the presence of either methylamine or carbon disulphide. The predicted points using the kinetic model show excellent agreement with the experimental data for the degradation of dazomet in the presence of Na-MDC, MIT, methylamine and carbon disulphide, (Figures 4.3-4.6 respectively). Figures 4.3-4.6 also show the effect of these products on the formation of MDC. Na-MDC, methylamine and carbon disulphide have very little effect or may cause a slight decrease in the total amount of MDC formed. In the presence of MIT however (Figure 4.4), slightly higher amounts of MDC are formed.

TABLE 4.2

Evaluated rate constants for the degradation of dazomet under oxygen or nitrogen rich environments

pH	CONDITION[1]	K_1 x 10^6, s^{-1}	K_2 x 10^6, s^{-1}	SD OF[2] EXP DATA	SD OF[3] PRED DATA
5	Oxygen	805.1	48.7	0.0473	0.0902
	Nitrogen	1053.6	72.9	0.0490	0.0460
6	Oxygen	762.6	77.1	0.0626	0.1213
	Nitrogen	461.0	174.5	0.0594	0.4254
7	Oxygen	795.2	54.4	0.1013	0.1357
	Nitrogen	567.8	103.4	0.0619	0.2301
	Dark	539.0	46.0	0.0571	0.0609
7.5	Oxygen	947.6	7.5	0.0503	0.1402
	Nitrogen	719.2	96.0	0.0517	0.0832

K_1 and K_2 are rate constants.

SD = standard deviation (defined in Appendix 6.1)

1 = dazomet degraded at 55°C under oxygen or nitrogen rich environments or in dark

2 = standard deviation of points determined experimentally

3 = standard deviation of predicted points from mean of experimentally determined points

TABLE 4.3

Evaluated rate constants for the degradation of dazomet at different temperatures

pH	Temperature[1] °C	K_1 x 10^6, s^{-1}	K_2 x 10^6, s^{-1}	SD OF[2] EXP DATA	SD OF[3] PRED DATA
5	25	22.5	5.9	0.0544	0.1271
	37	36.4	1.8	0.0597	0.1654
	55	737.8	58.7	0.0335	0.0954
6	25	20.2	7.1	0.0535	0.0828
	37	31.1	3.9	0.0431	0.1504
	55	607.8	68.2	0.0437	0.0841
7.5	25	22.2	6.5	0.0522	0.0866
	37	36.9	4.7	0.0604	0.1429
	55	613.3	54.6	0.0421	0.0999

K_1 and K_2 are rate constants.

SD = standard deviation (defined in Appendix 6.1)

1 = degradation temperature of dazomet

2 = standard deviation of points determined experimentally

3 = standard deviation of predicted points from mean of experimentally determined points

TABLE 4.4

Evaluated rate constants for dazomet in the presence of some products of degradation

Rate constant x 10^6, s^{-1} / Degradation product [1]	K_1	K_2
Na-MDC	558.5	102.1
MIT	542.3	67.5
Methylamine	654.2	50.9
Carbon disulphide	599.9	50.1

[1] dazomet degraded in pH 7 phosphate buffer at 55°C in presence of some degradation products

4.2 Kinetics of Degradation of Dazomet in Minimal Salts Media and Nutrient Broth

The degradation of dazomet in minimal salts media and nutrient broth at different concentrations and under incubation conditions required for the growth of bacteria (B.licheniformis and P.aeruginosa NCTC 6749) and yeast (S.cerevisiae) was discussed previously in Section 3.1.9 (Figures 3.23-3.38) with respect to the amount of MDC and MIT produced under each condition. For dazomet, the figures show that under each condition the degradation is exponential and either reaches zero concentration or tends towards it at the end of the incubation period. The differences between the various curves are minimal, hence kinetic analysis of the results was attempted in order to study the overall trend.

The reactions in minimal salts media and nutrient broth were performed under conditions of aeration and at concentrations of 500, 100 and 50 $\mu g.ml^{-1}$ (3.086, 0.617 and 0.309 mM, respectively), were in contrast to degradation in phosphate buffers (Section 3.1.8). The latter was studied in sealed containers at 1000 $\mu g.ml^{-1}$ (6.173 mM). Under these conditions (Figures 3.18-3.21) an equilibrium was observed and a pseudo-first order kinetic treatment (equation (4.2)), gives the best representation of the experimentally obtained data. However, for the degradation of dazomet in minimal salts media and nutrient broth (Figures 3.23-3.38) an equilibrium was not established and hence equation (4.2) can be written as follows:

$$-\gamma_A = -\frac{d\,C_A}{dt} = K\,C_A \qquad (4.17)$$

where: $-\gamma_A$ is the rate of degradation of dazomet and C_A is the concentration of dazomet at time t.

Therefore, the reaction in this case can be said to follow first order kinetics.

By integrating equation (4.17), we obtain the following concentration versus time profile:

$$C_A = C_{A_o}\,e^{-Kt} \qquad (4.18)$$

This equation can also be written as:

$$\ln\frac{C_{A_o}}{C_A} = Kt \qquad (4.19)$$

where: C_{A_o} is the initial concentration of dazomet.

∴ by plotting ln (C_{A_o}/C_A) vs. t a straight line was obtained with slope = K.

The values of half life (τ) for the degradation of dazomet under various conditions were calculated by taking the integrated form of the rate equation (4.19) and setting t equals τ and C_A to $C_{A_o}/2$.

Thus:

$$\tau = \frac{1}{K} \ln \frac{C_{A_o}}{C_{A_o}/2} = \frac{1}{K} \ln 2 \qquad (4.20)$$

The values of rate constants (K) and half lives (τ) for the degradation of dazomet under various conditions are summarised in Table 4.5. The table shows excellent correlation coefficients (of the order of 0.99) for predicting a straight line through log concentration versus time plots for the first order degradation of dazomet under these conditions.

The values of K and τ were evaluated for the degradation of dazomet at different concentrations in minimal salts media (at 30°and 37°C) and in nutrient broth (30°C) and under similar conditions in the presence of microorganisms (Table 4.5).

For the degradation of dazomet in minimal salts media at 37°C, the rate constants do not change to any significant extent in the presence of either B.licheniformis or P.aeruginosa compared with the controls, at each of the three concentrations studied. However, under each condition, the value of K decreases with an increase in the concentration of dazomet. The difference being greater for a two-fold increase in concentration from 50 to 100 $\mu g.ml^{-1}$, and significantly less for a five-fold increase from 100 to 500 $\mu g.ml^{-1}$. Similar effect was observed also with S.cerevisiae in both minimal salts media and nutrient broth at 30°C. However, the values of the rate constants are less for both the controls and in the presence of microorganisms compared with 37°C. The influence of concentration on the rate constant under different conditions is also reflected in the evaluated values of τ.

TABLE 4.5

Evaluated rate constants and half-lives for the degradation of dazomet in minimal salts media and nutrient broth

Condition of degradation	Concentration ($\mu g.ml^{-1}$)$^+$	50 (.31)	100 (.62)	500 (3.09)
In minimal salts media at 37°C:				
Control	(K	200.6	137.7	129.5
	(CR	.9957	.9951	.9838
	(τ	57.6	83.9	89.2
In presence of *B.licheniformis*	(K	209.3	171.8	137.4
	(CR	.9943	.9972	.9951
	(τ	55.2	67.2	84.1
In presence of *P.aeruginosa* NCTC 6749	(K		161.1	122.7
	(CR	(-)	.9926	.9963
	(τ	(-)	71.7	94.1
In minimal salts media at 30°C:				
Control	(K		77.0	50.7
	(CR	(-)	.9994	.9980
	(τ	(-)	150.0	227.9
In presence of *S.cerevisiae*	(K		71.6	54.2
	(CR	(-)	.9988	.9975
	(τ	(-)	161.3	213.3
In nutrient broth at 30°C:				
Control	(K		54.2	42.3
	(CR	(-)	.9863	.9982
	(τ	(-)	213.2	272.9
In presence of *S.cerevisiae*	(K		61.4	42.2
	(CR	(-)	.9799	.9976
	(τ	(-)	188.1	273.9

K = rate constant (x 10^6, s^{-1}) for first order degradation of dazomet

τ = half life (minutes) for the decomposition of dazomet

(-) not studied

CR correlation coefficients for predicting a straight line through log concentration versus time plots for the first order degradation of dazomet (Appendix 6.1)

+ figures in brackets are concentrations in mM

FIGURE 4.2

Estimation of the initial value of K required for the determination of pseudo-first order rate constants K_1 and K_2

C_{A_o} = initial concentration of dazomet (mM)

C_{A_e} = equilibrium concentration of dazomet (mM)

C_A = concentration of dazomet at time t (mM)

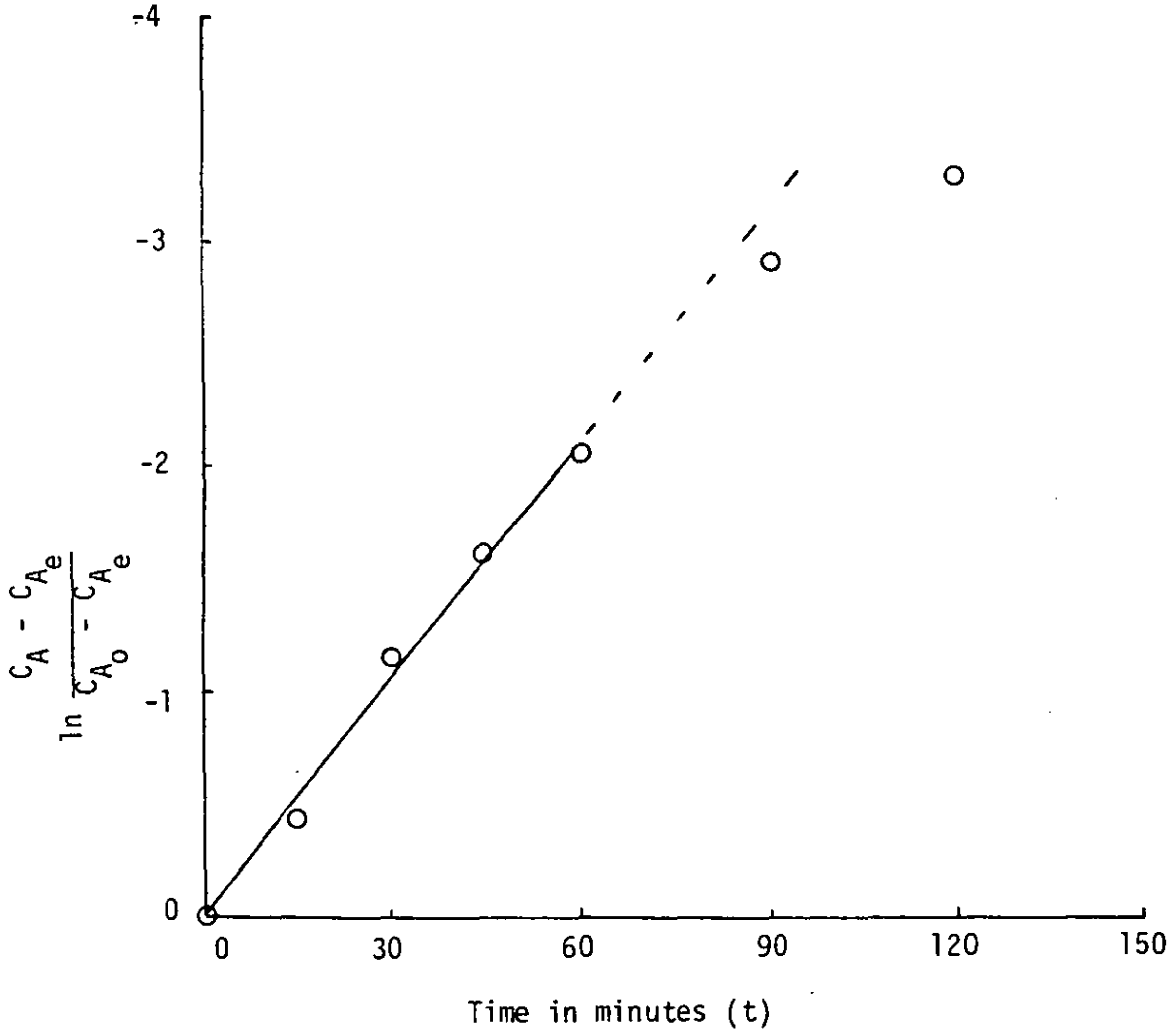

-4
-3
-2
-1
0
$\ln \frac{C_A - C_{A_e}}{C_{A_o} - C_{A_e}}$
0
30
60
90
120
150
Time in minutes (t)

FIGURE 4.3

Degradation study of dazomet in pH 7 phosphate buffer at 55°C in the presence of Na-MDC

○ dazomet

□ MDC

— solid line represents theoretical profile using the kinetic model

FIGURE 4.4

Degradation study of dazomet in pH 7 phosphate buffer at 55°C in the presence of MIT

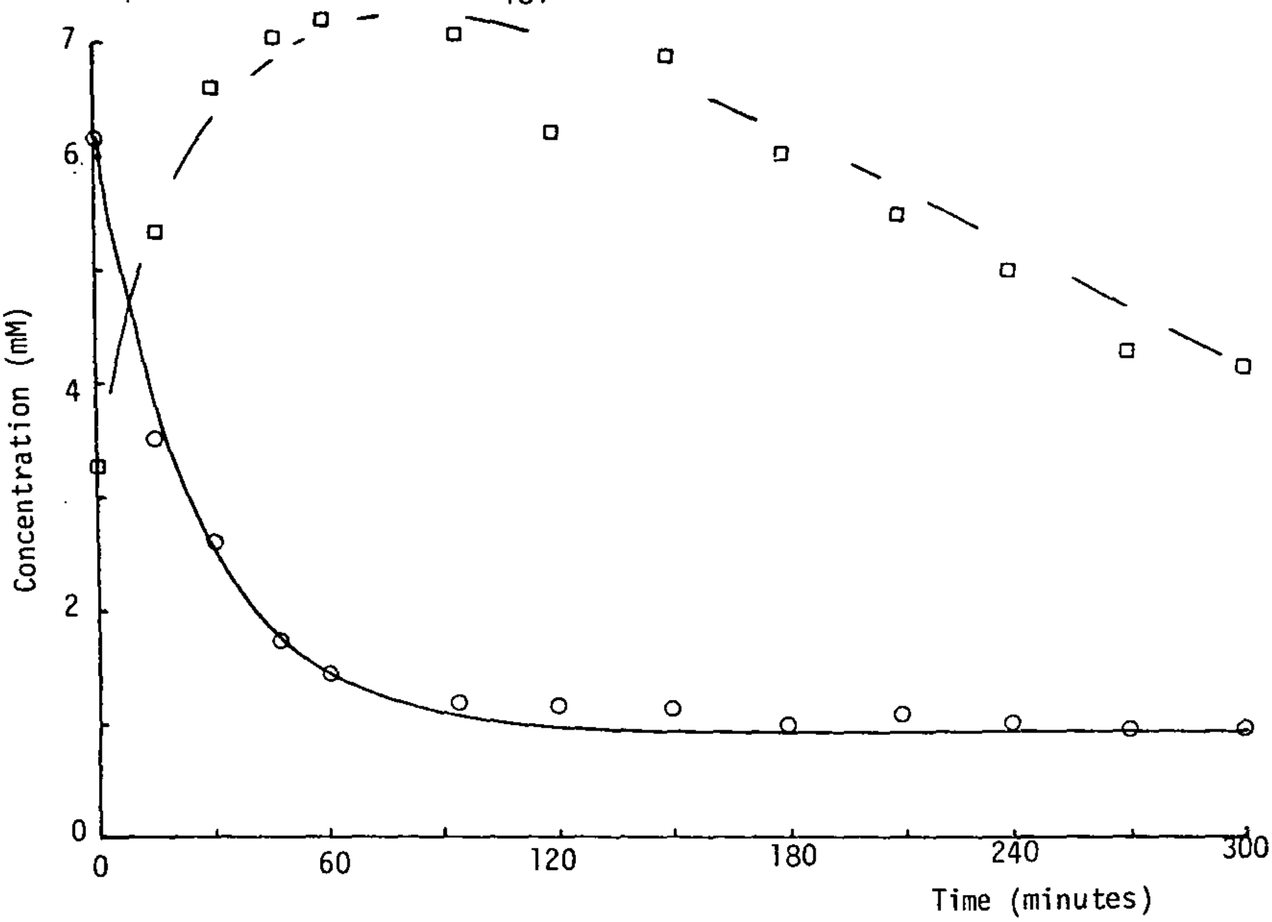
○ dazomet

□ MDC

△ MIT

◇ methylamine

— solid line represents the theoretical profile using the kinetic model


7
6
4
2
0
Concentration (mM)
0
60
120
180
240
300
Time (minutes)

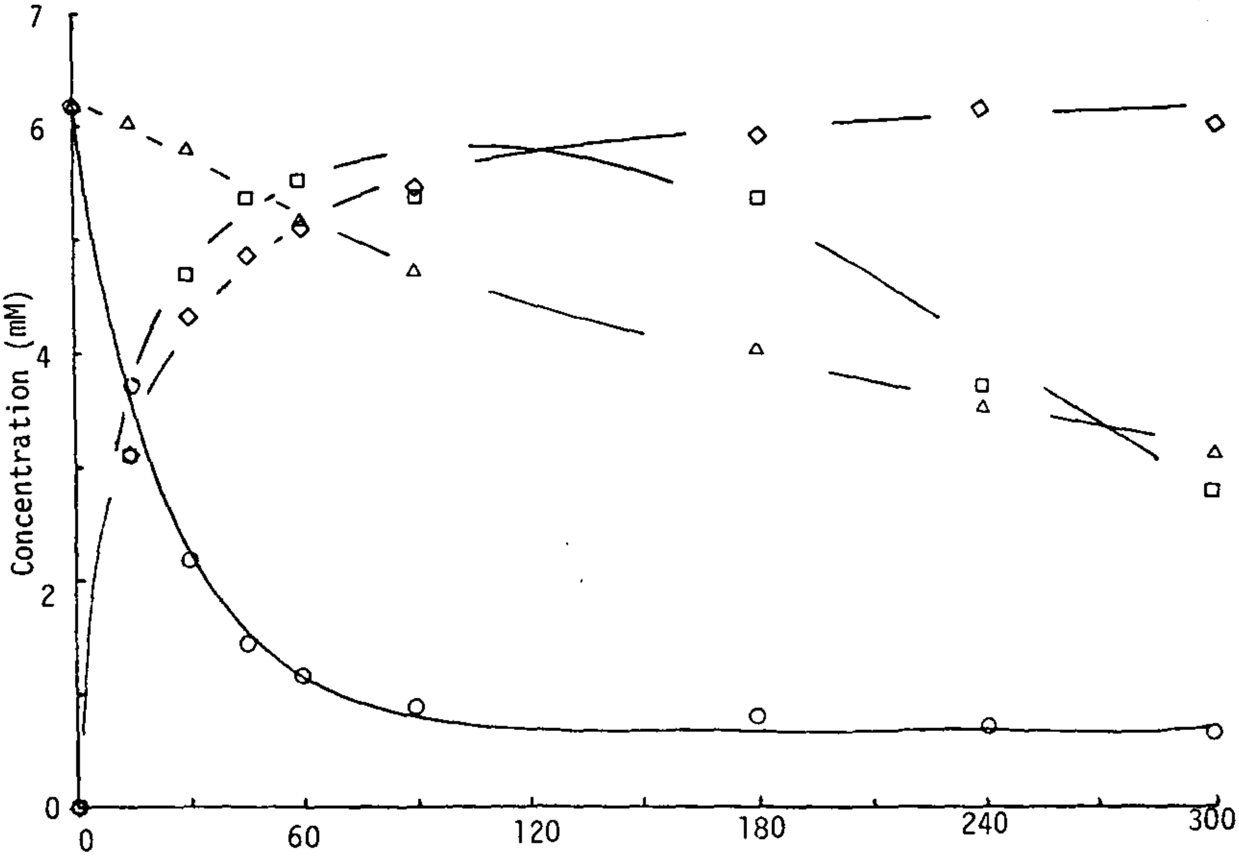

7
6
4
2
0
Concentration (mM)
0
60
120
180
240
300
Time (Minutes)

FIGURE 4.5

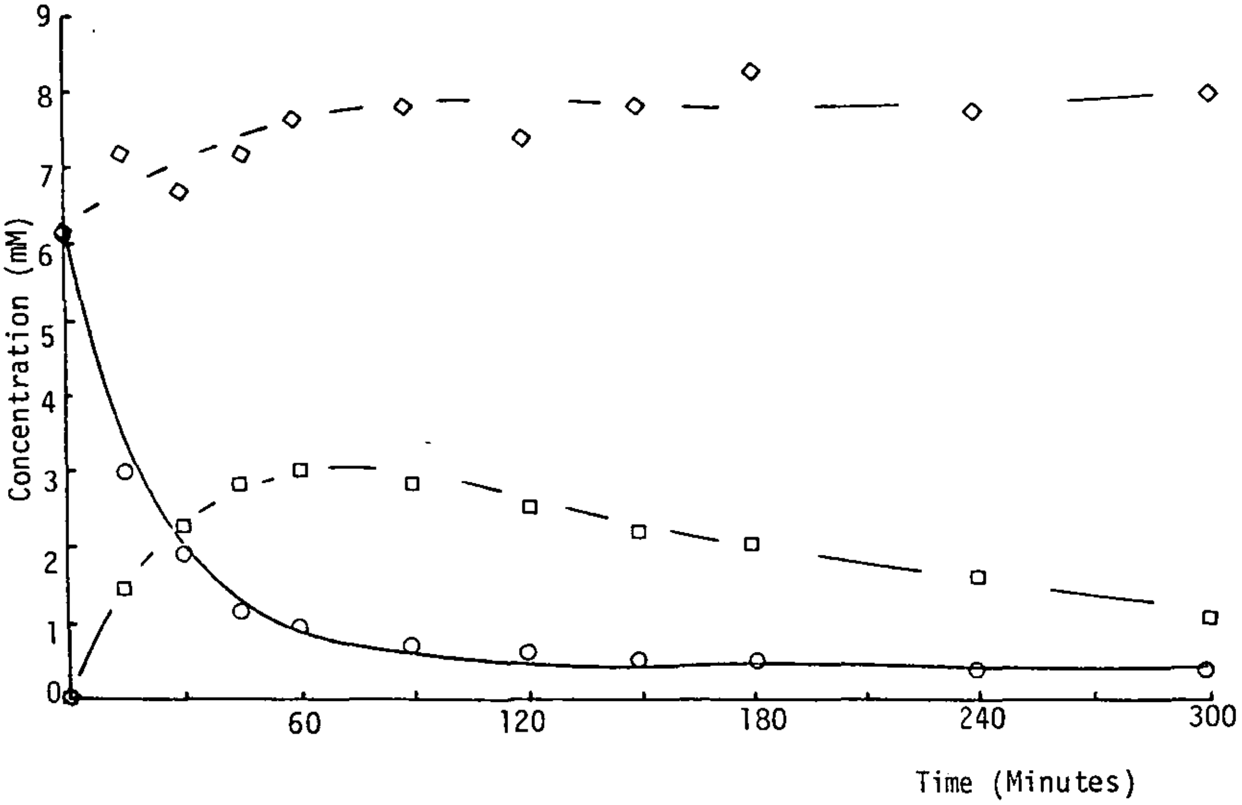
Degradation study of dazomet in pH 7 phosphate buffer at 55°C in the presence of methylamine

○	dazomet
□	MDC
◇	methylamine
——	solid line represents the theoretical profile using the kinetic model

FIGURE 4.6

Degradation study of dazomet in pH 7 phosphate buffer at 55°C in the presence of carbon disulphide

○	dazomet
□	MDC
——	solid line represents the theoretical profile using the kinetic model


Concentration (mM)
0
1
2
3
4
5
6
7
8
9
60
120
180
240
300
Time (Minutes)

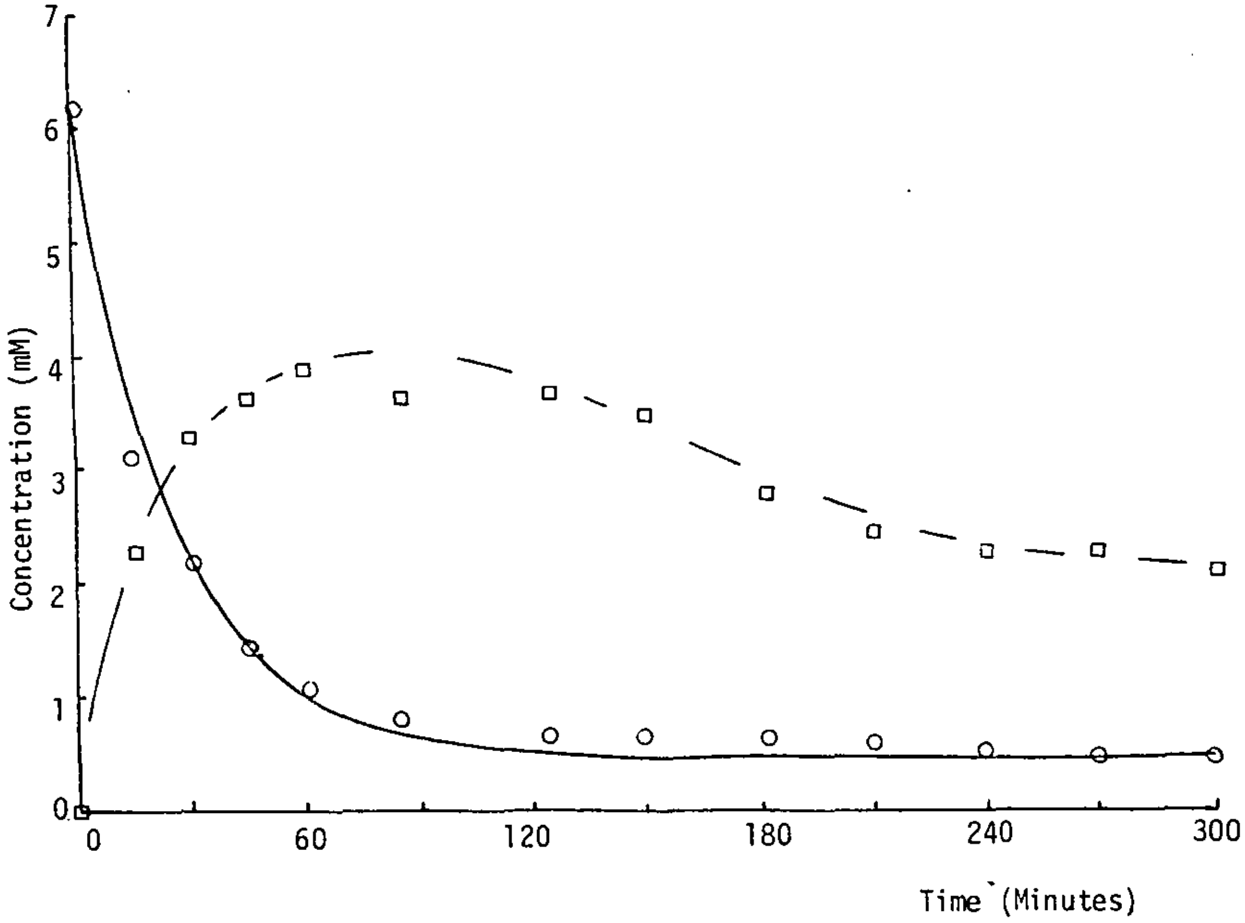

Concentration (mM)
0
1
2
3
4
5
6
7
0
60
120
180
240
300
Time (Minutes)

FIGURE 4.7

Arrhenius plot for the degradation of dazomet

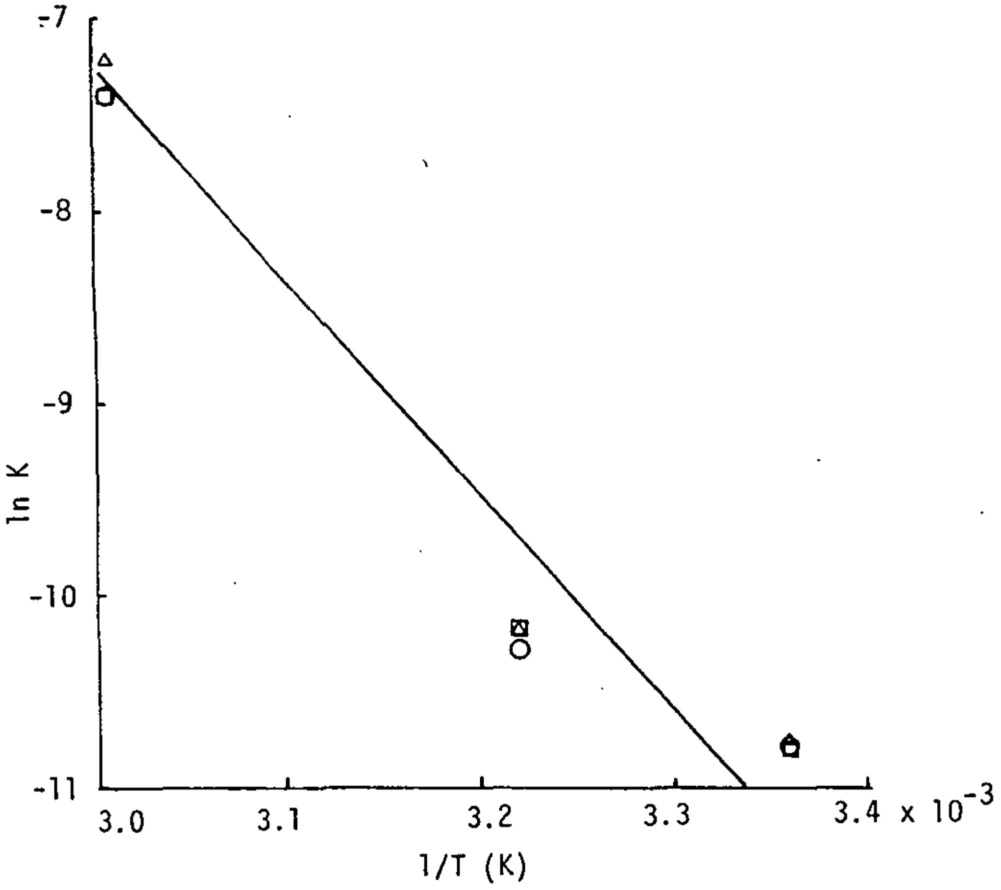
△ — △ pH 5.0

○ — ○ pH 6.0

□ — □ pH 7.0

$E = 21.28 \text{ K cal mol}^{-1}$

$A = 1.08 \times 10^{11} \text{ s}^{-1}$


-7
-8
-9
-10
-11
ln K
3.0
3.1
3.2
3.3
3.4 x 10^-3
1/T (K)

5. DISCUSSION

Analysis of Dazomet and Dithiocarbamates by HPLC

Analysis of a degraded sample of dazomet is complicated by the presence of polar, non-polar and neutral components in the mixture (Plates 3.1-3.2 and Table 3.1). HPLC using a reverse phase column (Figure 3.13) could separate dazomet and N-methylisothiocyanate (MIT), however, an unresolved peak also appeared at the solvent front, suggesting very polar nature of the eluent. TLC studies (Plates 3.1-3.2 and Table 3.1) indicated that the compound was probably N-methyldithiocarbamate (MDC). Reduction of the pH of the eluting solvent (up to pH 4) to suppress ionisation had no effect on the retention of the compound. Lower pH was not tried due to unstable nature of dithiocarbamates below pH 4 (Bode, 1954). Ion-pair reagents tetrabutylammonium hydoxide and cetrimide were also tried but appeared to facilitate the degradation of MDC.

As transition-metal salts of a number of dithiocarbamates have been successfully separated using liquid chromatography (Schwedt, 1977, 1978 and 1979), the use of transition-metal ions as ion-pair reagents in the eluting solvent was investigated. Both cobalt (11) and nickel (11) metal ions (Table 3.3) gave good separation and peak efficiencies without markedly affecting the retention time of other components of degradation (Table 3.2). Cobalt (11) was selected since it has a lower retention time. Using cobalt (11) as the pairing reagent, dazomet, MDC, MIT and N,N'-dimethylthiuram disulphide (DMTD) could all be determined in the same run (Figure 3.14). The peaks were readily detected without background interference from the metal ions in the solvent. The efficiency of the column was unchanged compared with the unpaired eluents, suggesting that complex formation was rapid. The capacity factors of the eluents remained unchanged at different concentrations of the metal-ions, hence on-column complex formation was the mode of chromatography. In addition, the relative order of elution of other components in the degradation mixture remained unaltered (Table 3.2), suggesting that they are not affected by the presence of cobalt (11) ions.

The calibration graphs for the determination of dazomet, MDC and MIT shows a linear detector response in the concentration ranges studied (Figures 3.15-3.17, respectively). The calibration graph for MDC (Figure 3.16) shows a negative intercept, suggesting a small but constant amount of decomposition.

The potential application of the on-column pairing technique using transition-metal ions was further investigated for the analysis of various dithiocarbamates and thiuram disulphide which are commonly used as fungicides, rubber vulcanisers and in pharmaceutical products (Table 3.3). In these studies, the transition-metal ions in the eluting solvent form a neutral non-aqueous soluble metal-dithiocarbamate complex, enabling them to be resolved from the solvent front and to be eluted under similar conditions to the corresponding disulphides (Table 3.3). As each of these complexes has different capacity factor, it should be possible, by selection of metal-ions, to adjust the retention of the complex so that it can be resolved from other components of a mixture.

A number of important fungicides are based on ethylenebisdithiocarbamic acid (Table 1.1), including zineb, maneb and mancozeb, the metal complexes of which are polymeric and attempts to examine the sodium salt using cobalt (11) as a reagent were unsuccessful. Many dithiocarbamates form stable silver (1) salts (Raizman and Thompson, 1972) but a trial using silver nitrate appeared to result in the decomposition of the ethylenebisdithiocarbamate (3.1.7). Similarly complexes of iron (111), mercury (11), copper (11) and lead (11) ions, resulted in decomposition.

Preliminary studies were carried out to examine the use of other transition metal ions in the analysis of dithiocarbamates and thiuram disulphides. It was found that cobalt (11) complex eluted as a cobalt (111) complex, confirming a report by Gaetani, Laureri and Mangia (1979). Problems were encountered in analysis if more than one of the dithiocarbamates and/or thiuram disulphide was present because of mixed complex formation and alkyl interchanges. This area has been considered further and reported (see Appendix 6.5).

Hence, the use of nickel or cobalt ions as complexation reagents is a useful technique for the determination of individual dithiocarbamates, though the interpretation of the results can be complicated if dithiocarbamates and/or thiuram disulphides with different amino function are present in the sample.

Degradation Studies on Dazomet

A freshly prepared solution of dazomet in distilled water showed two absorbance peaks at 250 and 277 nm (Figure 3.1). The dithiocarbamate grouping, as in esters of dithiocarbamic acids, or in tetramethylthiuram monosulphide, show strong UV bands in the regions 275-290 nm and 245-260 nm (Thorn and Ludwig, 1962). However, a corresponding solution of dazomet in carbon tetrachloride showed only a single peak at 290 nm (Figure 3.1). The very much lower absorbancy in water indicates that the ring structure has been split and the spectrum of the aqueous solution is very similar to spectra of MDC in distilled water (Figure 3.2) and is characteristic of dithiocarbamate derivatives (Goksoyr, 1964). UV studies of dazomet in distilled water (Figures 3.3-3.7) showed that the solutions were very unstable and the degradation was most rapid at high temperatures (e.g. 70^{o}C; Figure 3.7). The relative ratios of the height of the peaks were also different, indicating that the solution contains a mixture of UV-absorbing compounds. van der Kerk (1956) and Goksoyr (1964) who compared the fungicidal activity of dazomet and Na-MDC, concluded (without furnishing chemical evidence) that dazomet hydrolyses in aqueous solution to form formaldehyde and N-methyldithiocarbamate ester (MDC-ester). The ester is formed by the splitting of the ring between the two nitrogens at positions 3 and 5, which decomposes further to MDC and MIT (Figure 1.2).

However, the single UV peak shown by dazomet at the longer wavelength in carbon tetrachloride is similar to the spectral effect shown by mercaptobenzthiazole (Figure 5.1).

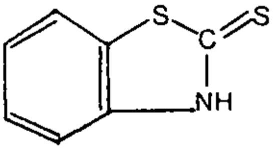


FIGURE 5.1 Mercaptobenzothiazole

Zahradnik (1959) observed that mercaptabenzothiazole shows strong absorption at 332 nm and a weak absorption band at 265 nm, which was explained on the basis of -S-C(S)-group giving the ring a pseudoaromatic character. Hence, the incorporation of the sulphur atom in the dazomet ring structure (Figure 5.2) appears to effect the loss of the characteristic dithiocarbamate spectra. Thus, the spectra of dazomet in aqueous solution (Figure 3.1) suggests that the ring structure splits at position 1 between the methylene group and sulphur atom as shown in Figure 5.2.

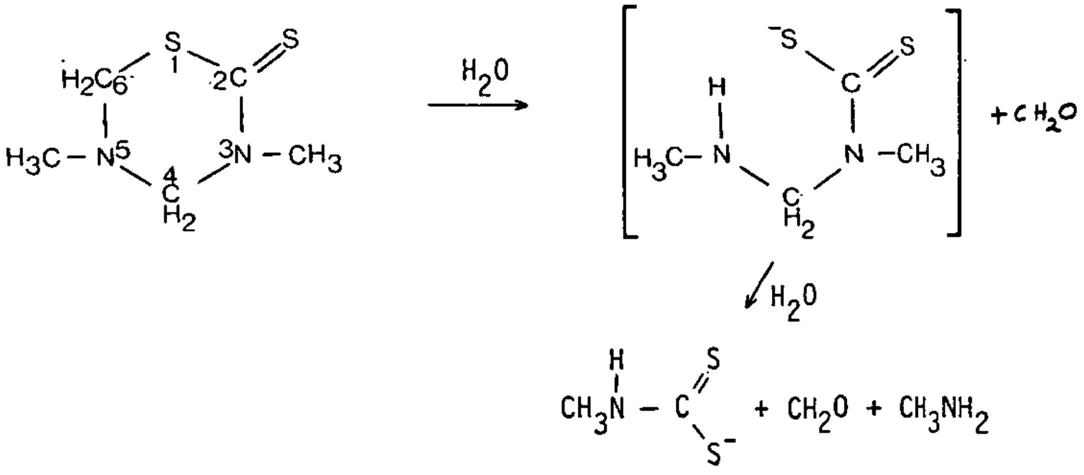


FIGURE 5.2 Proposed degradation of dazomet in aqueous solution

Evidence in support of this suggestion comes from the antibacterial and antifungal activities of dazomet and Na-MDC. Solutions of freshly prepared dazomet and Na-MDC both show similar inhibitory activity on oxygen consumption by B.licheniformis (Figures 3.39-3.42) and S.cerevisiae (Figures 3.48-3.49). The nature and extent of inhibition as shown by the inhibition curves was similar at the various concentrations studied. This suggests that the active group responsible for the inhibitory activity of both these compounds could be similar. The ability of the dithiocarbamate moiety to form metal chelates is well known (Thorn and Ludwig, 1962; Lukens, 1971) and will be considered in more detail later with respect to the biological activity of dazomet and Na-MDC. If however, the dazomet ring splits between positions 3 and 5 as suggested by van der Kerk (1956), then an alternate metal chelate may be formed between the two nitrogen atoms at positions 3 and 5 of the dazomet ring (Figure 1.2). Such a coordinate metal chelate is of the type formed by histidine (Figure 5.3)

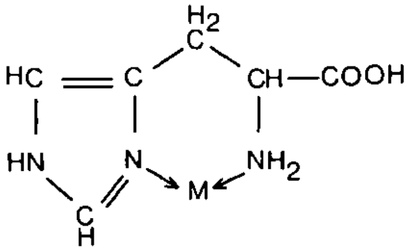


FIGURE 5.3 Chelate of histidine with metal (Lukens, 1971)

However, it has been shown that histidine has a much lower complex strength compared with complexes where the dithiocarbamate group is actively involved (Kaars Sijpesteijn and Janssen, 1957 and 1958). Hence, it is expected that a ring chelate involving MDC-ester will show quite a different spectrum of biological activity due to complex strength and steric effects compared with Na-MDC, where the dithiocarbamate group is involved in the formation of a metal chelate.

The similarity in the biological activity of dazomet and

MDC (Figures 3.39-3.42 and 3.48-3.49) also suggests another possibility that dazomet acts as MDC in aqueous solutions. However, the nature of the UV spectra, as mentioned ealier, (Figure 3.1), suggests a mixture of compounds in the solution. Furthermore, degradation studies of dazomet in phosphate buffers by HPLC and fluorimetry (Figures 3.18-3.21) show that both MDC and methylamine are produced in increasing concentration with time. The quantitative release of formaldehyde from dazomet solutions should also indicate that dazomet is present as a split ring form (Figure 5.2) and not as MDC. The presence of formaldehyde in fresh solutions was detected by a spot test (3.1.4). However, this does not give any positive indication of whether the formaldehyde is from position 6 of the ring as proposed (Figure 5.2), or from position 4, as suggested by van der Kerk (1956; Figure 1.2), or both. Studies using Nash's reagent (Figures 3.10-3.11) show that a total of two moles of formaldehyde are released per mole of dazomet. However, due to high temperatures required for the development of the coloured reaction, the method does not provide any answers about the initial steps involved in the degradation of dazomet.

There also exists a possibility that in a reaction medium containing zinc and other metal ions, dazomet ring splits catalytically to release MDC. This possibility was first suggested for copper and other heavy metals by Mulder (1873), but, studies on the degradation of dazomet in minimal salts media (containing Zn (11), Fe (11), Mn (11) and Mg (11) ions) in the presence and absence of microorganisms (Figures 3.23-3.38) discounts this possibility, since individual peaks due to dazomet and MDC were observed at various time intervals, as with degradation studies in phosphate buffers (Figure 3.14).

Hence, it is reasonable to assume that in aqueous solutions dazomet is present as an open ring form (Figure 5.2), and will therefore be referred to as dazomet (I) in subsequent discussions.

Further studies on the degradation of dazomet in aqueous solutions could not be made using the UV technique, because as mentioned earlier, the nature of the spectra (Figure 3.1) suggests the presence of a mixture of UV absorbing compounds. An alternative technique using paper chromatography (Figures 3.8-3.9) resolved only dazomet (I) and was not investigated further because of the length of time taken for the analysis and limitations of the detection procedure.

Hence, degradation studies using HPLC on-column pairing (discussed previously) was the preferred technique, since dazomet (I) as well as the major decomposition products namely MDC, MIT and DMTD could all be analysed in the same run (Figure 3.14). The degradation profiles obtained for dazomet (I) at a narrow pH range of phosphate buffers (5, 6, 7 and 7.5) show very similar decay curves (Figures 3.18-3.21). The three stages of degradation starting with a very rapid stage followed by an exponential stage and finally an equilibrium stage could not be explained in terms of simple kinetic treatment of first-order, second order, or of multiple orders (see Chapter 4, and Appendix 6.2).

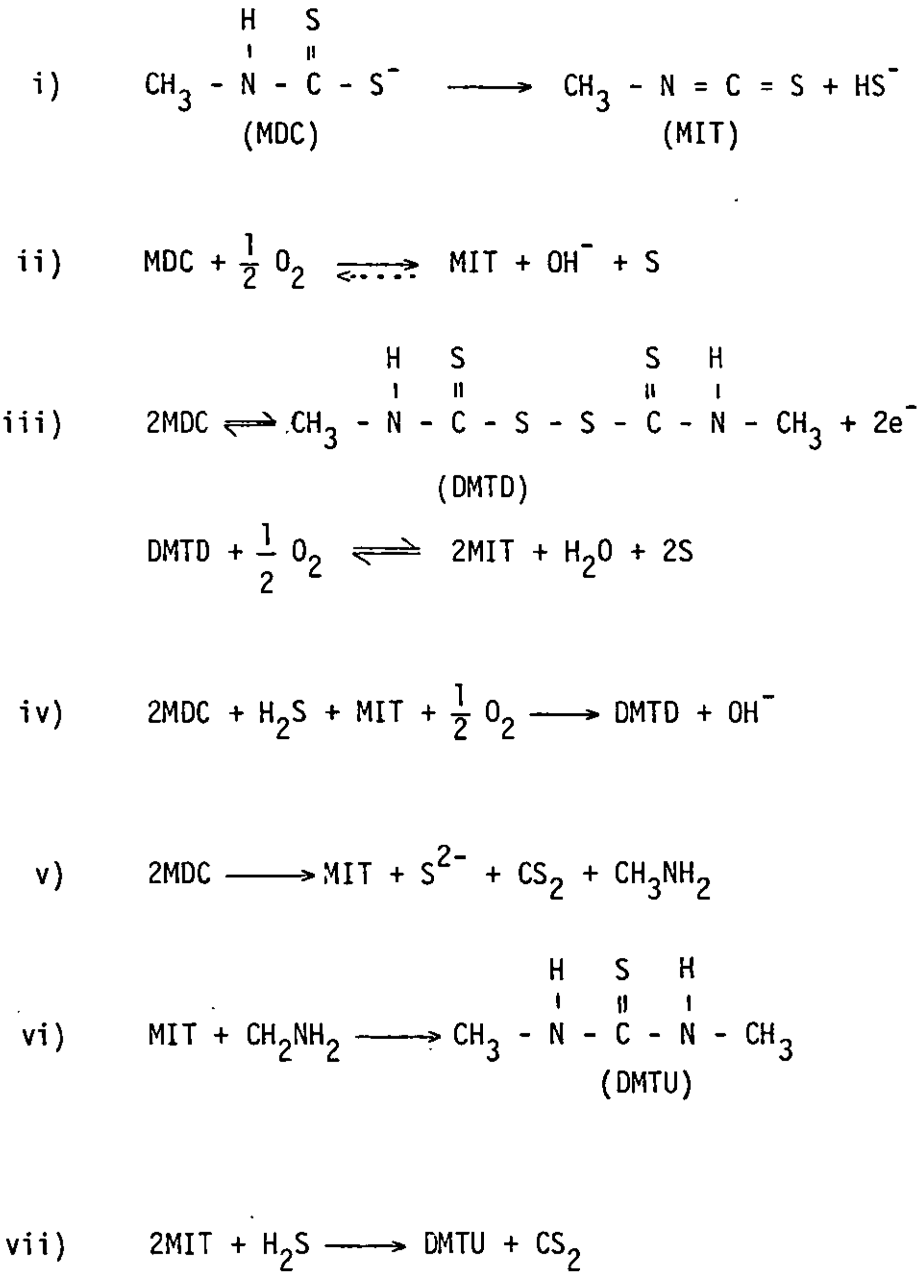
A kinetic model assuming a pseudo-first order equilibrium between dazomet (I) and MDC is proposed (Figure 4.1). Further degradation which results in the formation of MIT and D (all other products not quantified), were assumed to be of unknown orders with respect to the concentrations of each; since they may react with MDC in several possible side reactions (Figure 5.4). A computer program (Appendix 6.4) was developed to evaluate the rate constants and orders of reaction based on methods outlined in Chapter 4. The results obtained using the proposed reaction network (Figure 4.1) shows excellent agreement between the experimental points and the predicted curves (Figures 3.18-3.21 and Table 4.1).

The formation of MIT from MDC (Figures 3.18-3.21) was time dependent and was not affected by the concentration of the latter, suggesting a zero order kinetics. Evaluation of the order of reaction using the kinetic model confirms this (Table 4.1). Since several possible side reactions occur in aqueous solutions involving MDC and other products of degradation (Figure 5.4), all unquantified products were treated together (D). The evaluated orders of reaction for these products suggests that the reactions are either changing in characteristic all the time or that the formation of the products is dependent upon time (Table 4.1).

Thus the proposed kinetic model for the degradation of dazomet (I) assumes that the many reactions in aqueous solution involve the primary degradation product MDC. Hence, the other products of degradation which may influence the stability of MDC are also expected to affect the stability of dazomet (I) indirectly.

Degradation of dazomet (I) in the presence of MDC (Table 4.4), while not having much effect on the forward reaction to form MDC, causes nearly two fold increase in the rate of reverse reaction. A similar effect was observed in the presence of a nitrogen rich environment (Table 4.2), which would result in greater stabilisation of MDC, if it is assumed (see later) that its decomposition is primarily an oxidative process. Further evidence is provided by degradation in the presence of an oxygen rich environment (Table 4.2) which shows that the rate of reverse reaction is greatly reduced with the highest effect being observed at higher pH.

The other products of degradation, namely carbon disulphide and methylamine do not affect the rate of reverse reaction (Table 4.4). This is also reflected in the amounts of MDC formed in the presence of these products (Figures 4.4 and 4.5, respectively), which shows very little change or a slight decrease in the maximum amounts formed. MIT may have a small effect however, since the rate of reverse reaction (Table 4.4) and the maximum amount of MDC produced (Figure 4.3) were higher. The formation of MIT is depen-



i) $CH_3 - \overset{H}{\overset{|}{N}} - \overset{S}{\overset{\|}{C}} - S^- \longrightarrow CH_3 - N = C = S + HS^-$

(MDC) (MIT)

ii) $MDC + \frac{1}{2} O_2 \rightleftharpoons MIT + OH^- + S$

iii) $2MDC \rightleftharpoons CH_3 - \overset{H}{\overset{|}{N}} - \overset{S}{\overset{\|}{C}} - S - S - \overset{S}{\overset{\|}{C}} - \overset{H}{\overset{|}{N}} - CH_3 + 2e^-$

(DMTD)

$DMTD + \frac{1}{2} O_2 \rightleftharpoons 2MIT + H_2O + 2S$

iv) $2MDC + H_2S + MIT + \frac{1}{2} O_2 \longrightarrow DMTD + OH^-$

v) $2MDC \longrightarrow MIT + S^{2-} + CS_2 + CH_3NH_2$

vi) $MIT + CH_2NH_2 \longrightarrow CH_3 - \overset{H}{\overset{|}{N}} - \overset{S}{\overset{\|}{C}} - \overset{H}{\overset{|}{N}} - CH_3$

(DMTU)

vii) $2MIT + H_2S \longrightarrow DMTU + CS_2$

FIGURE 5.4 Chemical reactions associated with the decomposition products of dazomet (I) in aqueous solutions

dent upon the oxygen content of the solutions. When dazomet (I) was degraded in an oxygen rich environment, the amount of MIT formed was nearly four times the concentration obtained under a nitrogen rich environment (Table 3.4). The oxygen content of the solutions also affected the formation of sulphur. Solutions of dazomet (I) in either distilled water or phosphate buffer when incubated for long periods, showed the presence of sulphur (Figure 3.22). However, no sulphur was detected when dazomet (I) was degraded in a nitrogen rich environment (3.1.8). This indicates that in a limited oxygen environment with sufficiently high concentrations of MIT and sulphur, MDC may be reformed in an equilibrium reaction (Figure 5.4(ii)).

The effect of pH on the degradation of dazomet (I) under these conditions showed that, as with normal conditions (Table 4.1), the rate of degradation of dazomet (I) under either oxygen or nitrogen rich environments (Table 4.2) was high at pH 5 and was affected very little or only decreased slightly at pH 6 and 7, increasing again at pH 7.5. The increase in the rate of degradation at pH 7.5 was more pronounced under an oxygen rich environment, while at pH 5.0 the increase was more pronounced under a nitrogen rich environment.

These studies suggest that the decomposition of the primary degradation product MDC, at least in basic solution, is primarily an oxidation reaction Figure 5.4(ii)). Further evidence is provided by faster increase in pH of dazomet (I) solution in distilled water when air was passed through it compared with solutions just left exposed to air (3.1.8).

The effect of oxygen on the rate of decomposition of Na-MDC in aqueous solutions was first observed by Turner and Corden (1963). However, no details were given on the possible oxidation mechanism. It has been suggested that at pH 9.5, studied by Turner and Corden (1963), the formation of MIT by oxidation of MDC (Figure 5.4(ii)), involves an intermediate equilibrium reaction (Figure 5.4(iii)) which results in the formation of DMTD (Joris, Aspilla and Chakarbarti, 1970b). The presence of such an equilibrium suggests that MIT will be formed only after the appearance of DMTD. Also the amounts of MIT produced will be much greater. Present studies show that DMTD may be formed from MDC early in the reaction, and in large quantities, only at pH greater than 7. Hence at pH 7.5 (Figure 3.21), MDC appears to degrade readily and the maximum amount detected is much less compared with lower pH's (Figures 3.18-3.20). However, this does not result in increased formation of either MIT or methylamine (see later), suggesting that the oxidation reaction leading to the formation of DMTD (Figure 5.4(iii)) from MDC must be important. DMTD may also be produced from MDC in an alternate mechanism involving MIT and hydrogen sulphide (Figure 5.4(iv)). However, MIT forms quite late in the reaction (Figure 3.21) and hydrogen sulphide was not detected in any of the studies (3.1.8). Hence, the oxidation reaction involving hydrogen sulphide is not considered to be significant under these conditions. Further oxidation of DMTD, according to Joris, et al (1970b), should result in an increased production of MIT. However, the total amount and the rate of MIT produced was similar at all pH's studied (Figures 3.18-3.21). This suggests that further oxidation of DMTD is not favoured. However, DMTD is not very stable (Thorn and Ludwig, 1962) and degrades readily to form MDC (Table 3.2). This is also reflected in greater stabilisation of MDC with longer incubation periods at pH 7.5 (Figure 3.21), compared with lower pH's (Figures 3.18-3.20). Hence, DMTD may be formed from MDC in an oxidation reaction at pH 7.5; however, further oxidation leading to the formation of MIT may result directly from MDC (Figure 5.4(ii)) and the intermediate equilibrium involving DMTD (Figure 5.4(iii)), as suggested by Joris, et al (1970b), may not be significant under these conditions.

The reactions of dazomet (1) at pH < 7.5, which were considered previously, are also dependent on the oxygen content of the solution (Table 3.4), and are thus considered to be primarily oxidation reactions (Figure 5.4(ii)). DMTD was detected at all pH's, but it appears only after long incubation periods, suggesting that reaction (iii) (Figure 5.4) is not significant. Also, the formation of DMTD in significantly large quantities would result in greater stabilisation of MDC as in the case of pH 7.5 (Figure 3.21), however, at pH < 7.5, MDC degrades much faster (Figures 3.18-3.20).

Previous studies (Turner and Corden, 1963; Joris, Aspilla and Chakrabarti, 1970b) have shown that when MDC is degraded in acid conditions, hydrogen sulphide is formed (Figure 5.4(ii)). However, in the present studies at pH 5 and above, no hydrogen sulphide was detected (3.1.8). Its absence may be partly due to formation of DMTD in an oxidation reaction (Figure 5.4(iv)) or due to the formation of N,N'-dimethylthiurea (DMTU) in a reaction involving MIT (Figure 5.4(vii)). DMTU was identified in the degraded sample of dazomet (Plates 3.1-3.2 and Table 3.1). However, MIT and methylamine are both present in a degraded sample of dazomet (I), and may also react together (Figure 5.4(vi)) to form DMTU (Moore and Crossley, 1955). Hence, the formation of DMTU may result from any of these reactions.

At pH 5.0, an additional mechanism of degradation may be involved, since the rate of degradation of dazomet (I) was high even in a nitrogen rich environment, (Table 4.2). More than one mole of methylamine was released per mole of dazomet (I) degrading (an effect which was not observed at higher pH), suggesting that the extra methylamine arises from the splitting of the MDC molecule itself (Figure 5.4(v)).

The observed shift in mode of decomposition of the primary degradation product MDC, is similar to the change in oxidative to acid decomposition that occurs with disodium ethylenebisdithiocarbamate or nabam (Table 1.1), as the pH is lowered (Ludwig and Thorn, 1962). The evaluated orders of reactions for the formation of all the products which were not quantified, also confirms this change in the mode of decomposition (Table 4.1).

So far the reactions of MDC in aqueous solutions have been considered and indicate that the stabilities of MDC and dazomet (I) are interdependent at various pH's studied. However, studies on the effect of temperature (Table 4.3) on the rate of dazomet (I) degradation, shows an increase of nearly twenty times for temperature increase from 37° to 55°C, while for an increase in temperature from 25° to 37°C the increase in the rate was of the order of two times. These increases in the rates due to temperature are in accordance with the Arrhenius law (Figure 4.7). Hence, temperature is the critical factor responsible for the stability of dazomet (I) in aqueous solution, while pH has very little direct effect, but may influence the stability indirectly through reactions involving MDC.

In the degradation studies on dazomet (I) in phosphate buffers described previously, only one concentration (6.2 mM) was studied. To study the effect of microorganisms on the degradation of dazomet (I) in minimal salts media, lower concentrations (3.1, 0.6 and 0.3 mM) as well as aerated conditions, were used. Under these conditions the decay curves for dazomet (I) show characteristic exponential form and tend towards completion (Figures 3.23-3.38). Hence, concentration must be the critical factor since the degradation of dazomet (I) under oxygen rich environments, which was discussed previously (Table 4.2), showed good pseudo-first order fitting. The decay curves at lower concentrations were found to fit first order kinetics. The evaluated rate constants and half lives (Table 4.5) show that under each concentration studied, the presence of microorganisms has either no effect or may cause a very slight increase in the rate of degradation. The calculated rate constants at these concentrations also show that the stability of dazomet (I) increases with higher concentrations in the solution (Table 4.5), and, as with degradation in phosphate buffers (Table 4.3 and Figure 4.7), the stability of dazomet (I) is greatly reduced at higher temperatures, such that for an increase in temperature from 30^{0} to 37^{0}C, the corresponding increase in rate of degradation was nearly two times.

The conclusions derived from the study of dazomet in aqueous solutions may be useful for efficient use of the compound in field applications as a fumigant. MIT is regarded as the active constituent in its use as a soil fumigant (Helling, Kearney and Alexander, 1977; Kaufman, 1977; Cremlyn, 1979). However, several other products, both volatile and non-volatile, have been reported to be formed in the soil which may affect the activity as a fumigant. This is expected considering the complex nature of soils, which will introduce such factors as temperature, pH, moisture content, as well as soil microorganisms and adsorption of compounds on soil particles.

There does not appear to be microbial involvement in the degradation of dazomet (Munnecke and Martin, 1964), thus confirming the present findings (Table 4.5).

It has been suggested that methylaminomethyl dithiocarbamate forms in aqueous solutions (Vacek and Flegr, 1964) as well as in soil (Kaufman, 1977; Worthing, 1979). However, in aqueous solutions such a compound is expected to exist as free dithiocarbamate and methylamine. HPLC studies on the degradation of dazomet confirm this, since both MDC and methylamine are produced in increasing concentration with time (Figures 3.18-3.21). In soils however, such a compound could be formed, but due to volatile nature of methylamine, the concentrations produced are not expected to be significant; besides the fact that the compound is unstable and has been suggested to degrade easily to form methylamine, hydrogen sulphide and MIT (Kaufman, 1977). As discussed previously, methylamine and hydrogen sulphide are considered to.be important reactions only below pH 5 (Figure 5.4). Also the formation of these products will be favoured only in low oxygen and high acidity environments.

Another product which has been isolated is a triazine, 1,3,5-trimethylhexahydro-s-triazine-2-thione (Drescher and Otto, 1968). The product which has been isolated from sandy soil, in which dazomet degrades slowly, is a condensation product involving DMTU, formaldehyde and methylamine. However, this reaction is unlikely in soils which contain higher moisture content; since addition of clay to sandy soils (thereby increasing the moisture content) results in increased production of MIT (Munnecke, Martin and Moore, 1967). The decreased amount of MIT produced in sandy soils may be due to its reaction with methylamine to form DMTU (Figure 5.4(vi)). Hence, in the presence of sufficient amounts of DMTU, the reaction which leads to the formation of the triazine may be important.

In soils treated with dazomet, detectable amounts of hydrogen sulphide have also been reported, but not at high enough concentrations considered to be toxic (Munnecke, Domsch and Martin, 1962). The bioassay technique used by these authors, which involved passing of air through columns of soil and assaying the fungitoxicity of the evolved gas, suggests that under these conditions of aeration, oxidation reaction leading to the formation of MIT and sulphur (Figure 5.4(ii)) will be important, as discussed previously. Hence, the little hydrogen sulphide produced may be due to the reduction of sulphur in the soil by microorganisms (Lukens, 1971). Thus, the oxidation reaction which forms MIT and sulphur (Figure 5.4(ii)) should result in an increase in soil pH. This was observed in soils treated with dazomet (Munnecke, Martin and Moore, 1967).

It has been suggested that methylamine, hydrogen sulphide and formaldehyde may react together to form additional products, which in turn degrade to form carbon dioxide, ammonia, sulphur dioxide and water (Kaufman, 1977). However, fluorimetry studies failed to show any presence of ammonia. Similarly, formation of sulphur dioxide is not considered significant since it was not detected. Any side reactions which result in the formation of sulphur dioxide should cause a reduction in pH when produced in significant quantities; but in the present studies an increase in pH was always observed in aqueous solutions of dazomet. Similarly, treatment of soil with dazomet also results in an increase in pH (Munnecke, Martin and Moore, 1967), suggesting that these reactions are not significant. Further evidence is shown by observations that fungitoxicity of vapours released from dazomet can be accounted for in terms of the total MIT produced (Munnecke, Domsch and Eckert, 1962). It has also been suggested that MIT may react with water to form carbon dioxide, hydrogen sulphide and methylamine (Kaufman, 1977). The reactions which result in the formation of hydrogen sulphide and methylamine from MDC have already been considered previously (Figure 5.4) and are not considered to be significant above pH 5. Hence, the suggested reaction of MIT with water leading to the formation of hydrogen sulphide and methylamine are not likely to occur; they may be important below pH 5, and may involve MDC directly and not MIT.

The effect of temperature, pH and soil moisture content on the degradation of dazomet in soil has been studied previously (Munnecke and Martin, 1962; Munnecke, Martin and Moore, 1967). These studies confirm the present findings that all these factors are important in the overall stability of dazomet. However, they do not consider the initial factors which are responsible for the splitting of the molecule itself. Also other factors such as concentration and aeration have not been considered.

Present findings show that degradation of dazomet in aqueous solution is mainly temperature dependent and pH has very little effect. In soil however, moisture content may be the limiting factor but the critical factor resulting in the maximum breakdown is expected to be high soil temperatures. Further degradation resulting in the formation of MIT from MDC is dependent on pH as well as oxygen content. Hence, at more basic soil pH and increased aeration, the amount of MIT produced will be highest. Oxygen content of the soil will also be influenced by soil moisture content, since under saturated soil conditions less MIT may be produced due to decreased aeration. Dazomet is more stable at high concentrations; and this coupled with saturated soil conditions, will lead to greater stability and hence slower decomposition rates.

Biological Studies on Dazomet, MDC, MIT and Formaldehyde

The stability of dazomet in aqueous solutions has already been considered. These studies suggest that dazomet is present as an open ring structure (dazomet (I)), which further decomposes to MDC with a total release of two moles of formaldehyde (Figure 5.2). Subsequent reactions of MDC to form MIT and other products of degradation were also studied (Figure 5.4). Since most biological studies involving dazomet (I) were carried out in minimal salts media at pH 6.4 and under aeration, oxidation reactions (Figure 5.4(ii),(iii)), which result in the formation of MIT and DMTD must be considered important in the overall toxicity of the compound.

The MIC of DMTD against various fungi was found to be twice that of MDC (Klopping and van der Kerk, 1951), and due to its monomeric nature was suggested to decompose in solution to form two molecules of MDC. Present studies also indicate that pure samples of DMTD are unstable and decompose readily to form MDC (Table 3.2). Hence the biological activity of DMTD was not investigated. Also carbon disulphide, the other potential toxicant was not studied because as discussed previously, the reaction which leads to its formation (Figure 5.4(v)), is favoured below pH 5. Similarly sulphur which is produced by the oxidation of MDC (Figure 5.4(ii)) can show toxic action. However, its toxicity depends on participation in cellular oxidation-reduction reactions to produce hydrogen sulphide (H_2S) (Lukens, 1971), which may also be produced from MDC in acid conditions (Figure 5.4(i)). But, no H_2S was detected in the present studies (3.1.8). This may be due to either the limits of the detection technique, or as discussed previously, due to the formation of DMTU (Table 3.1) by reaction with MIT (Figure 5.4(vii)). DMTU may also be formed by the reaction between MIT and methylamine (Figure 5.4(vi)).

Dazomet (I), formaldehyde, MDC and MIT, the main components of the degradation mixture, were thus studied for their biological activity. The other degradation products such as DMTU are non-toxic, while H_2S and carbon disulphide are toxic only at very high concentrations (Clarke and Shepherd, 1965; Ludwig and Thorn, 1962), and are hence not considered important in the biological activity of dazomet (I).

A comparison of the activity of dazomet (I), Na-MDC, formaldehyde and MIT in terms of minimum inhibitory concentrations (MIC's) (Table 3.5), shows that both dazomet (I) and Na-MDC are equally toxic and show similar spectrum of activity. They are both relatively more toxic against Gram-positive organisms compared with Gram-negative organisms. The biological activity of dazomet has been ascribed to its decomposition products in solution (Goksoyr, 1964). Studies on the stability of dazomet (previously considered) show that it is unstable, particularly at low concentrations. Hence, at concentrations such as those used for MIC determinations, the degradation reaction is expected to go to completion within few hours of incubation (Table 4.5). This should result in the formation of one mole of MDC and two moles equivalent of formaldehyde (Figure 5.2). Further decomposition of MDC to MIT under these conditions would be favoured by oxidation reaction (Figure 5.4(ii)). Thus, the concentrations of formaldehyde and/or MIT produced at MIC value of both dazomet and MDC should account for their biological activity. The concentration of formaldehyde which is expected to be produced when dazomet (I) degrades completely, is toxic enough to explain its activity against Gram-negative organisms, but not against the Gram-positive organisms and fungi. Whereas the expected concentrations of MIT produced are toxic enough to explain the activity of both dazomet and Na-MDC against Gram-negative organisms and fungi, but not Gram-positive organisms. Hence, the activity of dazomet (I), at least against Gram-positive organisms cannot be explained in terms of the expected degradation products.

This suggests one possibility that both dazomet and MDC may act by a similar mode of action. The higher activity of the compounds compared with their degradation products may be due to the ability of both compounds to form metal complexes (see later) which may penetrate the cells easily and exert their action by degrading to form MIT and formaldehyde which may be toxic intracellularly. The antibacterial activity of Taurolin, for example, may be due to the release of formaldehyde intracellularly (Myers and Allwood, et al, 1980). However, in the case of dazomet (I) and MDC the proposed 1:1 metal-dazomet (I)/MDC complexes may combine with cellular reactive sites and form the stable 1:2 type of complexes which are toxic (see later), in which case the activity of the compounds will not depend on further degradation. This is confirmed by the observation that fresh solutions of dazomet (I) and MDC show maximum inhibition of oxygen consumption against B.licheniformis (Figures 3.39-3.42) and S.cerevisiae (Figures 3.48-3.49) at the same concentrations as the MIC values (Table 3.5). Cell growth was also inhibited at these concentrations immediately after incubation (Figures 3.61-3.62), suggesting that the activity does not depend on the degradation products. Formaldehyde which could be released from dazomet (I) molecules (Figure 5.2) immediately after being taken up by the cell, also has no significant effect on the overall inhibitory effect. This is reflected in similar spectrum of activity and toxicity due to both dazomet (I) and MDC in terms of MIC values (Table 3.5), oxygen consumption inhibition (Figures 3.39-3.42 and Figures 3.48-3.49) and growth inhibition studies (Figures 3.61-3.62).

Both dazomet (I) and MDC show similar reactions in minimal salts media. They form precipitates at concentrations above MIC's in the presence of all microorganisms tested (Plates 3.3-3.4). This indicates that both compounds react with some constituent of the media at high concentrations. The effect was only observed some time after incubation as is shown by growth inhibition studies (Figures 3.61-3.63). It was not due to either formaldehyde or MIT which would be formed in the media after long incubation times. Both formaldehyde (Plate 3.5 and Figure 3.64) and MIT (Plate 3.6 and Figure 3.63) show no such effect in presence of cells even at high concentrations and after long incubation periods.

The precipitate in the solutions may be formed due to highly insoluble nature of sulphur (Lukens, 1971), which may be produced in an oxidation reaction involving MDC (Figure 5.4(ii)). However, it is unlikely that at concentrations just above MIC values, the amount of sulphur produced will be sufficient enough to account for the observed precipitation. This is confirmed by the observation that for Gram-negative organisms, for example, which have slightly higher MIC values compared with other organisms, the precipitation was still observed at concentrations just above MIC values (Table 3.5).

An alternative possibility for the presence of the precipitate may be due to the formation of insoluble metal complexes by the reaction of dithiocarbamates with various heavy metal ions (Goksoyr, 1955). Spectral studies of Na-MDC in minimal salts media containing zinc (11), ferrous (11), manganese (11) and magnesium (11) ions required for optimum growth of microorganisms (Figure 3.69) indicates such a possibility. However, the formation of complexes by any chelating agent depends on complex strengths and solubilities.

Mellor and Maley (1948) showed that for a number of chelating agents, essentially the same order of stability is obtained for metals: $Pd > Cu^{2+} > Ni > Co > Zn > Cd > Fe^{2+} > Mn > Mg$. This observation is in good agreement with the series given by Malatesta (1941) and Eckert (1957) for metal dithiocarbamate complex strengths and solubilities. Hence in the minimal salts media containing various metal ions, the formation of the complexes would be: first the formation of 1:1 zinc-MDC at lower concentrations of Na-MDC, followed by 1:2 zinc-MDC complex; secondly the formation of 1:1 ferrous-MDC complex at lower concentrations of the excess MDC, followed by 1:2 complex; and similarly for manganese (11) and magnesium (11) ions, respectively. The spectral studies (Figure 3.69) show that in the presence of Na-MDC, a peak appears at 324 nm and increases in intensity at higher concentrations. However, when the concentration of Na-MDC exceeds twice the concentration of Zn (11) ions, the intensity of the peak increases to a much higher level. This suggests a switch-over from the 1:1 zinc-MDC complex to the 1:2 complex. Similarly, the formation of other metal complexes is expected when the excess MDC in the solution, after complexation exceeds the concentrations of each of the metal ions Fe (11), Mn (11) and Mg (11) by a factor of two. At very high concentrations of MDC the peak intensity decreases, possibly due to insolubility of metal complexes. Preliminary investigations (Figure 3.70) indicate that this effect may be due to Mg (11) ions. In the presence of Mg (11) ions alone in the media (Figure 3.70), the enhancement of the 324 nm peak is observed when the Na-MDC concentration exceeds Mg (11) ions by a factor of two. At a little higher concentration the intensity of the peak decreases slightly, indicating that the effect may be due to the insoluble 1:2 magnesium-MDC complex.

To summarise therefore, both dazomet (I) and MDC show similar reactions in aqueous media containing full complement of metal ions required for the growth of microorganisms (Plates 3.3-3.4 and Figures 3.61-3.62). UV studies (Figures 3.69-3.70) suggests this to be due to the formation of an insoluble 1:2 metal-MDC/dazomet complex. This confirms the suggestion that the dithiocarbamate group is important in the open ring form of dazomet in aqueous solutions (Figure 5.2).

The antimicrobial activity of dazomet (I) and Na-MDC in terms of MIC values (Table 3.5) suggests their activity to be similar. However, their activities against human KB cells (Table 3.6) show that the toxic concentrations in the presence of dazomet (I) are considerably less than Na-MDC. While toxicity of both formaldehyde and MIT is comparable to that of dazomet (I). Under the conditions of incubation, it is expected that all or most of dazomet (I) will degrade (Table 4.5) to release two moles equivalent of formaldehyde and one mole of MDC (Figure 5.2). However, the concentration of the latter in the media is not sufficient enough to explain the toxicity of dazomet (I), but the concentration of formaldehyde produced is toxic enough on its own to account for the activity of dazomet (I). This suggests that MDC is stable under the conditions of incubation and does not decompose further to produce the toxic MIT (Figure 5.2). Since these studies were conducted under carbon dioxide rich environment and at relatively basic pH, MDC is stabilised and the expected decomposition does not occur.

Although Na-MDC was found to be relatively less toxic compared with dazomet (I) and MIT, all three compounds are toxic towards KB cells at sub-inhibitory concentrations (Plates 3.9, 3.8 and 3.11, respectively). This was shown by morphological changes from normal squamous (Plate 3.7) to rounded forms, as well as a reduction in the number of living cells. This suggests that the compounds may act on the components of the cell membranes either by forming metal chelates, as in the case of dazomet (I) and MDC; or by direct action on the sulphydral and amino groups of the cell, as in the case of MIT (see later). However, formaldehyde showed a slight stimulatory effect on the cells at sub-inhibitory concentrations (Plate 3.10). Although potentially toxic at high concentrations, formaldehyde is also a normal metabolite of biological systems and there are biotransformation mechanisms available in man which can metabolise formaldehyde to formic acid and thence to carbon dioxide and water (Neely, 1964). Hence, at low concentration the stimulatory effect on KB cells (Plate 3.10) may be due to its participation in some cellular metabolic activity.

The relative activities of dazomet (I), MDC, MIT and formaldehyde as studied by MIC determinations against bacteria and fungi (Table 3.5) and activities against KB cells (Table 3.6), are good indicators of the overall toxicities of the compounds, especially when longer incubation times are considered as in these studies. However, they do not provide any answers about the toxicities of the specific molecules themselves without much interference from other products of degradation.

The relative activities of undegraded dazomet (I) and Na-MDC as well as that of formaldehyde and MIT was compared by their by their inhibitory activities on oxygen consumption. An oxygen electrode was used as a model system for respiration of D-glucose or sodium succinate by B.licheniformis and S.cerevisiae (Figures 3.39-3.51). This method has an advantage due to its rapidity (within minutes) of assay. While other commonly used techniques in the studies of dithiocarbamates, and which require longer incubation periods, such as spore germination tests (Dimond, 1941; van der Kerk and Kloping, 1952; Goksoyr, 1955), growth responses (Kaars Sijpesteijn, 1952 and 1954), substrate utilisation (Sisler and Cox, 1955; Goksoyr, 1955 and 1964) and radiorespirometric technique (Domsch and Corden, 1973b), leave considerable doubt as to whether the compounds acted in their original form throughout the duration of the experiments.

The respiration of D-glucose by B.licheniformis in the presence of dazomet (I) and Na-MDC (Figures 3.39 and 3.41) shows an unusual phenomenon which was first demonstrated by Dimond et al (1941) for spore germination tests with tetramethylthiuram disulphide. The response is characterised by a first zone of inhibition, followed by a reduction of percent inhibition with increasing dose of the inhibitor, and at further increase of concentration - by a second zone of very little change or a slightly increased inhibition. This effect was also demonstrated for oxygen consumption inhibition using Warburg apparatus

(Figures 3.52-3.53). The concentrations of dazomet (I) and Na-MDC which cause maximum inhibition corresponds to the formation of the 1:1 zinc-dazomet (I)/MDC complex, which was discussed previously (Plates 3.3-3.4 and Figure 3.69). Higher concentrations of the compound which probably results in the formation of the insoluble 1:2 zinc-dazomet (I)/MDC complex, corresponds to a reduction in inhibitory activity, and suggests that the cells are less susceptible to the 1:2 complex.

The reduced activity of dazomet (I) and MDC at concentrations which form 1:2 metal-dazomet (I)/MDC complex is similar to the phenomenon of 'concentration quenching' observed for 8-hydroxyquinoline (Albert, 1979) and Phanquone (Husseni and Stretton, 1980). The mode of action of these compounds against Gram-positive organisms, for example, is thought to be due to the formation of toxic 1:1 or 1:2 complex, while in the presence of excess compounds, a 1:3 non-toxic complex is formed which is harmless because of saturation.

The zone of reduced activity also corresponds to the formation of Fe (11) and Mn (11) ions in the media, however, since their formation is not reflected in additional zones of inhibition at the respective concentration, both these metal ion complexes are considered to be less effective inhibitors of glucose respiration under these conditions. At higher concentrations of the compounds which would probably correspond to the formation of 1:1 magnesium-dazomet (I)/MDC complexes, little change or slight increase in inhibition is observed, suggesting that Mg (11) ions are also less effective compared with zinc (11) ions. Since only one peak of inhibition was observed corresponding to the formation of 1:1 zinc-dazomet (I)/MDC complex, it is possible that the toxicity at low concentration could be due to chelation of essential metals rather than the complex itself participating in producing the toxicity (Horsfall, 1956). However, in the absence of zinc (11) ions, very high concentrations of Na-MDC are required to show any inhibitory activity on Fusarium oxysporum f.sp lycopersici (Domsch and Corden, 1973a), suggesting that zinc (11) ions act synergestically at low concentrations of Na-MDC.

The effect of both dazomet (I) and Na-MDC on glucose respiration by S.cerevisiae (Figures 3.48-3.49) also shows maximum inhibition at concentrations which correspond to the formation of 1:1 zinc-dazomet (I)/MDC complex. However, with increasing concentration which would result in the formation of the insoluble 1:2 zinc-dazomet (I)/MDC complex, the reduction in activity is more pronounced than in the presence of B.licheniformis (Figures 3.39 and 3.41). At slightly higher concentrations of the chelating agent which probably corresponds to the formation of 1:1 ferric-dazomet (I)/MDC complex, an enhanced inhibitory effect is observed compared with B.licheniformis (Figures 3.39 and 3.41). Further increase in concentration of either dazomet (I) or Na-MDC results in little change or slight increase in inhibition, corresponding to molar ratios which would be required for the formation of manganese-dazomet (I)/MDC complex. At higher concentrations still, which probably corresponds to the formation of 1:1 magnesium-dazomet (I)/MDC complex, slightly further increase in inhibitory activity is observed. At concentrations which may form 1:2 magnesium-dazomet (I)/MDC complex little change or slight decrease in inhibitory activity is observed. Hence, the activity of both dazomet (I) and MDC in minimal salts media can be accounted for in terms of the metal ions present.

The effect of dazomet (I) and Na-MDC on succinate respiration by B.licheniformis (Figures 3.40 and 3.42) also shows maximum inhibition at equimolar concentrations which lead to the formation of 1:1 zinc-dazomet (I)/MDC complex. Higher concentration results in a decrease in activity, suggesting that the insoluble 1:2 zinc-dazomet (I)/MDC complex is ineffective. At concentrations which correspond to the molar ratios required to form 1:1 and 1:2 ferric dazomet (I)/MDC complexes, further decrease in inhibitory activity is observed, suggesting that Fe (11) ion complexes are probably ineffective. With further increase in concentration which would probably result in the formation of Mn (11) and Mg (11) ion complexes, respectively, MDC (Figure 3.42) shows further reduction in inhibitory activity followed by little change, while dazomet (I) (Figure 3.40) showed slight increase in inhibition. This difference in activity at high concentrations between dazomet (I) and MDC may be due to structural differences (Figure 5.2). A long chain metal chelate of dazomet (I) may be more lipid soluble, and hence easily accessible to the site of action (Albert, 1979). However, further investigation is required.

Thus the inhibitory activity of both dazomet (I) and Na-MDC on glucose and succinate respiration is shown to be maximum when 1:1 zinc-dazomet (I)/MDC complex is likely to be formed in the medium (Figures 3.39-3.42). Studies with other metal dithiocarbamates (Lukens, 1971; Corbett, 1974) suggest that the 1:1 metal-dithiocarbamates, act by interacting with vital thiol-groups in the cell. Since the oxidation of glucose and succinate involves thiol containing pyruvate and succinic dehydrogenases, the mechanism of action of 1:1 metal-dazomet (I)/MDC complex may be explained in terms of the formation of 1:2 type of complex with the vital groups on the enzymes leading to a blockage of biosynthetic steps. An expected consequence of any blocked biosynthetic steps would be accumulation of metabolites occurring prior to the blocked steps of synthesis. From studies with N,N-dimethyldithiocarbamate (DMDC), it has been shown that small amounts of pyruvic acid accumulates at concentrations which result in the first zone of inhibition (Goksoyr, 1955; Kaars Sijpestein and van derk Kerk, 1957). Hence, for pyruvate dehydrogenase complex, for example, the 1:1 zinc-dazomet (I)/MDC complex may combine with the dithiol of lipoic acid, a component of the dehydrogenase system, to form a mixed 1:2 metal-dithiocarbamate +

dithiol complex. This reaction is very similar to the 'biochemical lesion' site of Arsenic, which has an affinity for dithiol group of lipoic acid (Doull et al, 1981). A characteristic of this type of interaction is that their inhibitory activity is counteracted by dithiols such as dimercaptopropanol (BAL) and L-cysteine (Stocken and Thompson, 1946).

The inhibitory activity of dazomet (I) and Na-MDC on glucose oxidation (Figures 3.54 and 3.56) and growth of B.licheniformis (Figures 3.65-3.66) was reduced in the presence of L-cysteine, suggesting that thiols are probably the important sites of action of these compounds. However, the antagonistic effect due to L-cysteine on oxygen consumption by B.licheniformis was much less when the cells were pretreated with D-glucose and Na-MDC was the inhibitor (Figure 3.55), compared with dazomet (I) (Figure 3.54). This effect cannot be explained in terms of substrate acting as an antagonist, since the inhibitory activity of Na-MDC is not reduced by the addition of D-glucose (Figure 3.56). This suggests a possibility that some intermediate of the substrate metabolism may be acting as a competitor for the toxic 1:1 metal-MDC complex. It has been shown that pyruvic acid and other α-keto acids may antagonise the inhibitory activity of DMDC on the growth of Aspergillus niger in the first zone of inhibition which corresponds to the formation of the toxic 1:1 complex (Kaars Sijpesteijn and van der Kerk, 1957). Hence, pretreatment of cells with substrate (Figure 3.55) may result in the formation of such intermediates which would protect the enzymes by competing for the toxic 1:1 complex. The higher activity of dazomet (I) under the same conditions (Figure 3.54) may be due to structural differences (Figure 5.2), as discussed previously. A long chain metal chelate of dazomet (I) may be more lipid soluble, and thus easily accessible to sites inside the cell.

The inhibitory activity of MIT on both glucose oxidation (Figure 3.57) and growth of B.licheniformis (Figure 3.67) also shows a reduction in activity in the presence of L-cysteine. This suggests that the target sites for the inhibitory activity may be of a similar type to that of dazomet (I) and MDC. Although the target sites may be the same, MIT reacts with these sites by a mechanism that is different. MIT reacts with thiols to form methyldithiocarbamic esters (Lukens, 1971). The formation of esters was demonstrated in the presence of S.cerevisiae (Goksoyr, 1964). However, the effect of MIT on glucose or succinate oxidation by B.licheniformis (Figures 3.43-3.44) or S.cerevisiae (Figure 3.51) suggests that the nature of the inhibitory effect is very different compared with dazomet (I) and MDC (Figures 3.39-3.42 and Figures 3.48-3.49). At very low concentrations of MIT little effect or a slight stimulation of respiration is observed, while at higher concentrations, inhibition is observed. The observed response is similar to the effect of the classical uncoupler 2,4-dinitrophenol (DNP) (Figure 3.45). Isothiocyanates belong to a class of uncouplers which uncouple respiration at low concentrations and inhibit respiration at high concentrations (Drobnica et al, 1974). The uncoupling effect may be exerted by a non-specific interaction with cell membranes (Miko and Chance, 1974).

The target site of action of formaldehyde is different from that of dazomet(I), MDC or MIT. This is shown by their respective activities against microorganisms in the presence of L-cysteine. The inhibitory activity of formaldehyde on both glucose oxidation (Figure 3.58) and growth of B.licheniformis (Figure 3.68) is affected very little in the presence of L-cysteine; dazomet, MDC and MIT all show significant reduction in activities, (Figures 3.54, 3.56 and 3.57; and Figures 3.65-3.67). However, the activity of formaldehyde on succinate oxidation (Figure 3.47) shows a similar effect to that of MIT (Figures 3.43-3.44), with a slight stimulation of respiration at low concentrations and an inhibition at higher concentrations. This effect was not observed for glucose oxidation (Figure 3.46) which is inhibited to a greater extent. These observations cannot be easily explained due to non-specific action of formaldehyde on proteins (Sykes, 1965; Albert, 1979). However, binding of formaldehyde at low concentrations to essential enzymes of oxidative phosphorylation may result in configurational changes whereby coupling activity is lost and hence stimulation of respiration (Figure 3.47). Another possibility which cannot be ruled out is that, due to configurational changes in the membrane, more substrate may be able to reach the active sites on the enzyme and hence result in stimulation of respiration. The higher inhibitory activity of formaldehyde on glucose oxidation compared with succinate oxidation may be due to higher specificity for enzyme(s) involved in oxidation of the former. However further investigation is required to explain these effects.

The biological studies considered so far show that dazomet (I) as well as its main decomposition products MDC, MIT and formaldehyde all have inhibitory action of their own on microorganisms. Further evidence for the activity due to dazomet (I) is shown by studies involving the assay of the inhibitory activity of predegraded solutions of dazomet (I) on oxygen consumption by B.licheniformis (Figures 3.59-3.60). The results show that the toxicity of the solutions varies according to the initial concentration of the toxicant and the length of preincubation time. This suggests that the composition of the degradation mixture is important in the overall toxicity of the solutions. Comparison of these values with that of estimated inhibitions at respective times due to the remaining dazomet (I) in the solution (Table 3.7) shows good agreement between the points, especially after shorter periods of incubation (Figures 3.59-3.60). However, the differences are greater at higher incubation times. The observed differences in activity may be explained in terms of the reactions of dazomet (I) and MDC in minimal salts media containing full complement of metal ions. This reaction has already been described previously in terms of the toxic 1:1 metal-dazomet (I)/MDC complex. However, at low concentrations of dazomet (I) and MDC considered here (Table 3.7), the contribution of other metal ions besides zinc (11) ions is not considered important in the overall toxicity of the compounds. Hence, with increasing age of dazomet (I) solutions, the concentrations of dazomet (I) and/or MDC should be less than or equal to the molar concentration of zinc (11) ions required to form the toxic 1:1 zinc-dazomet (I)/MDC complexes. This effect is clearly shown at both concentrations of dazomet (I) studied (Table 3.7; Figures 3.59-3.60). With longer preincubation times, the composition of the degradation mixture also changes such that higher concentrations of MDC are produced (Figures 3.26 and 3.29). This is also reflected in greater deviation from estimated inhibition due to dazomet (I) alone (Figures 3.59-3.60). Thus, the corresponding decrease in inhibitory activity of the solutions at this point may be due to the formation of the non toxic 1:2 zinc-dazomet (I)/MDC complex. However, in a mixture containing two dithiocarbamates such as dazomet (I) and MDC, mixed 1:2 complexes of the type dazomet (I) + MDC may be formed (see Appendix 6.5). Thus the decrease in the toxicity of the preincubated solutions may be due to the formation of such complexes. The reduced inhibitory activity after long preincubation times (Figures 3.59-3.60) could also be due to MIT (Figures 3.26 and 3.29) which causes a slight stimulation of respiration at low concentrations (Figure 3.43). However, it is unlikely that at the very low concentrations of dazomet (I) studied here (Table 3.7) either MIT (Figure 3.43) or formaldehyde (Figure 3.46), the other component of degradation, have any effect on oxygen consumption.

Thus, dazomet is expected to degrade (depending on concentration, temperature, and soil moisture content) to dazomet (I) followed by MDC. Further degradation of MDC will result in the formation of MIT and various other products (depending on pH, oxygen content, etc). Hence, in the presence of heavy metal ions, the initial reactions which result in the formation of dazomet (I) and MDC, must be considered to be important in assessing its effectiveness as an antimicrobial agent.

6. APPENDIX

6. APPENDIX

6.1 Statistical Analysis of Results and Definitions

The following standard formulae were used:

Standard deviation:

$$S = \sqrt{\frac{1}{N-1} \sum_{i=1}^{N} (x_i - \overline{X})^2}$$

Pearson product-moment correlation coefficient:

$$r = \frac{\sum_{i=1}^{N} (x_i - \overline{x})(y_i - \overline{y})}{\sqrt{\sum_{i=1}^{N} (x_i - \overline{x})^2 \sum_{i=1}^{N} (y_i - \overline{y})^2}}$$

Column capacity ratio:

$$K' = \frac{t_r - t_o}{t_o}$$

6.2 Attempted Kinetic Networks for the Degradation of Dazomet

When dazomet degrades in solution several products may be formed including MDC, MIT, DMTD, sulphur, hydrogen sulphide, methylamine and DMTU. However, only dazomet (A), MDC (B) and MIT (C) were measured. Since several possible side reactions may take place involving the other products of degradation (Figure 5.4), they were treated together (D). The following networks were tried for the degradation of dazomet (A) to B, C and D:

i) $A \rightarrow B + C + D$

ii)
$$\begin{tikzcd} A \arrow[r] & B \arrow[rr] \arrow[dr] & & C \arrow[dl] \\ & & D & \end{tikzcd}$$

iii)
$$\begin{tikzcd} A \arrow[r] & B \arrow[r] \arrow[dr] & C \\ & & D \end{tikzcd}$$

iv) $A \rightarrow B + D \rightarrow C$

v) $A \rightarrow B + C \rightarrow D$

vi)
$$\begin{tikzcd} A \arrow[r, shift left] & B \arrow[l, shift left] \arrow[r] \arrow[dr] & C \\ & & D \end{tikzcd}$$

6.3 Integration of the Rate Equations

The rate of degradation of dazomet may be represented by the following equation:

$$-\gamma_A = \frac{d\,C_A}{dt} = -K_1C_A + K_2C_B \tag{6.1}$$

where: C_A is the concentration of dazomet at time t

C_B is the concentration of N-methyldithiocarbamate

If C_{A_o} = initial concentration of dazomet i.e. $C_A = C_{A_o}$ at t =0; then C_B at time t is given by:

$$C_B = C_{A_o} - C_A \tag{6.2}$$

$$\therefore \quad \frac{d\,C_A}{dt} = -K_1C_A + K_2\,(C_{A_o} - C_A) \tag{6.3}$$

At equilibrium, $C_A = C_{A_e}$, and $\frac{d\,C_A}{dt} = 0$

$$\therefore \quad 0 = -K_1\,C_{A_e} + K_2\,(C_{A_o} - C_{A_e}) \tag{6.4}$$

$$\therefore \quad K_2 = \frac{K_1\,C_{A_e}}{C_{A_o} - C_{A_e}} \tag{6.5}$$

$$\therefore \quad \frac{d\,C_A}{dt} = -K_1C_A + \frac{K_1\,C_{A_e}}{C_{A_o} - C_{A_e}}\,(C_{A_o} - C_A) \tag{6.6}$$

Simplifying, and using limits of integration:

$$\int_{C_{A_o}}^{C_A} \frac{d\,C_A}{C_A - C_{A_e}} = K \int_o^t dt \qquad (6.7)$$

where:

$$K = \frac{-K_1\,C_{A_o}}{C_{A_o} - C_{A_e}} \qquad (6.8)$$

Integrating equation (6.7) with specified limits,

$$\ln \frac{C_A - C_{A_e}}{C_{A_o} - C_{A_e}} = -Kt \qquad (6.9)$$

or

$$\underline{C_A = C_{A_e} + (C_{A_o} - C_{A_e}) \exp(-Kt)} \qquad (6.10)$$

Rate of formation of N-methylisothiocyanate is represented by the following equation:

$$\gamma_c = \frac{d\,C_c}{dt} = K_3\,C_c^{n_3} \qquad (6.11)$$

$$\therefore \quad \int_{o}^{C_c} \frac{d\,C_c}{C_c^{\,n_3}} = K_3 \int_{o}^{t} dt \qquad (6.12)$$

$$\therefore \quad C_c^{\,1-n_3} = (1 - n_3)\,K_3 t \qquad (6.13)$$

or

$$\underline{C_c = [(1 - n_3)\,K_3]^{\frac{1}{1-n_3}}\,t^{\frac{1}{1-n_3}}} \qquad (6.14)$$

Rate of formation of D (all other products not quantified) is represented by the following equation:

$$\gamma_D = \frac{d\,C_D}{dt} = K_4\,C_D^{\,n_4} \qquad (6.15)$$

$$\therefore \quad \int_{o}^{C_D} \frac{d\,C_D}{C_D^{\,n_4}} = K_4 \int_{o}^{t} DT \qquad (6.16)$$

$$\therefore \quad C_D^{\,1-n_4} = (1 - n_4)\,K_4 t \qquad (6.17)$$

or

$$\underline{C_D = [(1 - n_4)\,K_4]^{\frac{1}{1-n_4}}\,t^{\frac{1}{1-n_4}}} \qquad (6.18)$$

########/####

6.4 Computer Program for Fitting a Kinetic Model to the Degradation Profile of Dazomet Using Experimentally Obtained Data

```
      MASTER FITTING
C**** THIS PROGRAM FITS A KINETIC MODEL TO THE DEGRADATION PROFILE    ****
C**** OF DAZOMET USING EXPERIMENTALLY OBTAINED DATA.                   ****
C****                                                                 ****
C**** THE VALUES OF THE CONSTANTS K1, K2, K3, AND K4 ARE EVALUATED     ****
C**** FOR THE FOLLOWING DEGRADATION SCHEME OF DAZOMET (SIMPLIFIED):    ****
C****                                                                 ****
C****                   K1    K3                                      ****
C****                A  →  B  →  C                                    ****
C****                   ←     ↘                                       ****
C****                   K2    K4 D                                    ****
C****                                                                 ****
C**** WHERE : A IS DAZOMET ,                                           ****
C****         B IS N-METHYLDITHIOCARBAMATE ,                           ****
C****         C IS N-METHYLISOTHIOCYANATE ,                            ****
C**** AND,    D IS ALL OTHER PRODUCTS ,                                ****
C****           WHICH INCUDES :                                       ****
C****           N,N'-DIMETHYLTHIURAM DISULPHIDE, METHYLTHIOUREA ETC.****
C****                                                                 ****
C**** K1 AND K2 ARE EVALUATED ASSUMING A PSEUDO-FIRST ORDER KINETIC    ****
C**** RELATIONSHIP.                                                    ****
C**** K3 AND K4 ARE EVALUATED ASSUMING A RELATIONSHIP OF THE FORM :    ****
C****                                                                 ****
C**** CONCENTRATION = (CONSTANT 1) * (TIME ** CONSTANT 2) ,            ****
C****                                                                 ****
C**** FOR THE C AND D DATA .                                           ****
C****                                                                 ****
C****                                                                 ****
      INTEGER IT(11),IW(2)
      REAL T(11),A(11),B(11),C(11),D(11),AD(11),BD(11),CD(11)
      REAL ADP(11),ADN(11),BDP(11),BDN(11),CDN(11),CDP(11)
      REAL AL(10),AP(11),BP(11),CP(11),DP(11)
      REAL AERROR(11),BERROR(11),CERROR(11),DERROR(11)
      REAL XK(6),CC(6)
      REAL RESULT(20)
      REAL X(2),W(110)
      COMMON/BL1/CC,XK/BL2/NPTS,CCB,XKB/BL3/IVALUE/BL4/T,C,D
      DO 110 J=1,4
C**** LOOP 4 TIMES TO OPERATE ON 4 SETS OF DATA                        ****
      READ(1,1)N
C**** N IS THE NUMBER OF DATA                                          ****
      READ(1,2)(IT(I),A(I),AD(I),B(I),BD(I),C(I),CD(I),D(I),I=1,N)
      WRITE(2,3)
      DO 101 I=1,N
      WRITE(2,4)IT(I),A(I),AD(I),B(I),BD(I),C(I),CD(I),D(I)
  101 CONTINUE
C**** READ AND WRITE THE EXPERIMENTAL DATA                             ****
C**** A,B,C,D AND AD,BD,CD ARE THE CONCENTRATIONS AND THEIR            ****
C**** RESPECTIVE DEVIATIONS AT TIME IT                                 ****
      DO 102 I=1,N
      T(I)=FLOAT(IT(I))
C**** CONVERTS INTEGER TIME VALUES TO REAL                             ****
      ADP(I)=A(I)+AD(I)
      ADN(I)=A(I)-AD(I)
      BDP(I)=B(I)+BD(I)
      BDN(I)=B(I)-BD(I)
      CDP(I)=C(I)+CD(I)
      CDN(I)=C(I)-CD(I)
C**** ADD AND SUBTRACT DEVIATIONS FROM RESPECTIVE CONCENTRATIONS       ****
```

```
C****                                                                      ****
C**** THE KINETIC MODEL USED FOR A IS OF THE FORM :                        ****
C****                                                                      ****
C****                A = AE + (AI - AE) * EXP(K * T)                       ****
C****                                                                      ****
C****                WHERE , K = -(K1 + K2)                                ****
C****                                                                      ****
C**** THUS ,          LOG NATURAL ((A-AE)/(AI-AE)) = K * T                 ****
C****                                                                      ****
C**** SO A PLOT OF T VERSUS LOG NATURAL ((A-AE)/(AI-AE)) ,                 ****
C**** WILL GIVE A STRAIGHT LINE THROUGH THE ORIGIN WITH SLOPE K            ****
C**** K IS OBTAINED BY LINEAR REGRESSION ANALYSIS                          ****
C**** K IS NOW CALCULATED USING THE NAG ROUTINE G02CBF                     ****
C**** K1 AND K2 ARE CALCULATED FROM K                                      ****
      AE=A(N)
C**** THE FINAL VALUE OF A IS SET AS THE EQUILIBRIUM VALUE                 ****
      AI=A(1)
C**** THE FIRST VALUE OF A IS SET AS THE INITIAL VALUE                     ****
      ADIFF=AI-AE
      DO 103 I=1,9
      AL(I)=ALOG((A(I)-AE)/ADIFF)
  103 CONTINUE
C**** AL IS THE NATURAL LOGARITHM OF (A-AE)/(AI-AE)                        ****
      K=1
      DO 104 L=4,9
      CALL G02CBF(L,T,AL,RESULT,0)
      XK(K)=RESULT(6)
C**** RESULT(6) IS THE REGRESSION COEFFICIENT                              ****
      CC(K)=RESULT(5)
C**** RESULT(5) IS THE CORRELATION COEFFICIENT                             ****
      K=K+1
  104 CONTINUE
C**** LINEAR REGRESSION IS CARRIED OUT USING 4 TO 9 POINTS                 ****
C**** THE REGRESSION COEFFICIENTS AND THE CORRELATION COEFFICIENT          ****
C**** AT EACH PASS IS STORED IN ARRAYS XK AND CC RESPECTIVELY              ****
      CALL CCMAX
C**** THIS RETURNS THE VALUE OF THE BEST CORRELATION COEFFICIENT ,         ****
C**** CCB , THE RESPECTIVE REGRESSION COEFFICIENT , XKB , AND ,            ****
C**** NPTS, THE NUMBER OF POINTS USED TO OBTAIN IT                         ****
      XK1=(-XKB*ADIFF)/AI
      XK2=XK1*AE/ADIFF
C**** XK1 AND XK2 ARE THE VALUES OF K1 AND K2                              ****
      DO 105 I=1,N
      AP(I)=AE+ADIFF*EXP(XKB*T(I))
C**** AP IS THE PREDICTED VALUE OF A                                       ****
      AERROR(I)=ABS(A(I)-AP(I))
C**** AERROR IS THE DIFFERENCE BETWEEN THE EXPERIMETAL AND PREDICTED****
C**** VALUE OF A                                                           ****
  105 CONTINUE
      CALL SDEV(AD,N,ADMEAN,SDAD)
C**** ADMEAN IS THE MEAN VALUE OF AD                                       ****
C**** SDAD IS THE S.D. OF AD                                               ****
      CALL SDEV(AERROR,N,EAMEAN,SDAERR)
C**** EAMEAN IS THE MEAN VALUE OF AERROR                                   ****
C**** SDAERR IS THE S.D. OF AERROR                                         ****
      WRITE(2,5)
      WRITE(2,6)CCB,NPTS,XKB,XK1,XK2
      WRITE(2,7)
      WRITE(2,8)(IT(I),A(I),AP(I),AERROR(I),I=1,N)
      CALL REGPLOT(NPTS,XKB,T,AL)
C**** THIS ROUTINE PLOTS THE STRAIGHT LINE OBTAINED USING LINEAR      ****
C**** REGRESSION                                                      ****
C**** WE NOW CALCULATE K3 USING THE NAG ROUTINE E04FDF                ****
C**** THIS ROUTINE EVALUATES THE CONSTANTS CK1 AND CK2 IN THE         ****
C**** FUNCTION  C = CK1 * TIME ** CK2 BY MINIMIZING THE SUM OF        ****
C**** SQUARES , WHERE X(1) = CK1  AND  X(2) = CK2                     ****
      IVALUE=0
C**** WHEN IVALUE=0 THE SUBROUTINE LSFUN1 CALCULATES RESIDUALS FOR C****
C*****INITIAL ESTIMATES OF X(1) AND X(2) ARE FOUND BY FITTING A
C****** STRAIGHT LINE ALOG(C)=ALOG(X(1))+X(2)*ALOG(T) BETWEEN 6 TH AND
C****** THE LAST POINT. SLOPE OF THE LINE IS X(2) AND INTERCEPT GIVES X(1
      DELC=ALOG(C(10))-ALOG(C(6))
      DELT=ALOG(T(10))-ALOG(T(6))
      X(2)=DELC/DELT
      X(1)=-X(2)*ALOG(T(6))+ALOG(C(6))
      X(1)=EXP(X(1))
      WRITE(2,225)X(1),X(2)
  225 FORMAT(1H ,'ESTIMATES FOR C ARE   X(1)=',F9.6,5X,'X(2)=',F9.6)
C**** THESE ARE ESTIMATED REQUIRED TO BE INPUT E04FDF                 ****
      JJ=0
      IFAIL=1
      CALL E04FDF(N,2,X,FSUMSQ,IW,2,W,110,IFAIL)
      IF(IFAIL.NE.0)GO TO 110
      CN=1.0-1.0/X(2)
      CK3=X(2)*X(1)**(1.0/X(2))
C**** CN IS THE ORDER OF REACTION                                     ****
C**** CK3 IS THE CONSTANT K3                                          ****
      DO 106 I=1,N
      CP(I)=X(1)*T(I)**X(2)
C**** CP IS THE PREDICTED VALUE OF C                                  ****
      CERROR(I)=ABS(C(I)-CP(I))
C**** CERROR IS THE DIFFERENCE BETWEEN THE EXPERIMENTAL AND THE       ****
C**** PREDICTED VALUE OF C                                            ****
  106 CONTINUE
      CALL SDEV(CD,N,CDMEAN,SDCD)
C**** CDMEAN IS THE MEAN VALUE OF CD                                  ****
C**** SDCD IS THE S.D. OF CD
      CALL SDEV(CERROR,N,ECMEAN,SDCERR)
C**** ECMEAN IS THE MEAN VALUE OF CERROR                              ****
C**** SDCERR IS THE S.D. OF CERROR                                    ****
      WRITE(2,11)
      WRITE(2,12)CN,CK3
      WRITE(2,13)
      WRITE(2,8)(IT(I),C(I),CP(I),CERROR(I),I=1,N)
      WRITE(2,9)CDMEAN,SDCD,ECMEAN,SDCERR
C**** WE NOW CALCULATE K4 USING THE NAG ROUTINE E04FDF                ****
C**** THIS ROUTINE EVALUATES THE CONSTANTS DK1 AND DK2 IN THE         ****
C**** FUNCTION  D= DK1 * TIME ** DK2  BY MINIMIZING THE SUM OF        ****
C**** SQUARES , WHERE  X(1) = DK1  AND  X(2) = DK2                    ****
      GO TO 201
  200 WRITE(2,30)IFAIL
      JJ=IFAIL
  201 IFAIL=1
      IVALUE=1
C**** WHEN IVALUE=1 , THE SUBROUTINE LSFUN1 CALCULATES RESIDUALS      ****
C**** FOR D                                                           ****
C*****INITIAL ESTIMATES OF X(1) AND X(2) ARE FOUND BY FITTING A
C****** STRAIGHT LINE ALOG(C)=ALOG(X(1))+X(2)*ALOG(T) BETWEEN 6 TH AND
```

```
      DELD=ALOG(D(10))-ALOG(D(6))
      X(2)=DELD/DELT
      X(1)=-X(2)*ALOG(T(6))+ALOG(D(6))
      X(1)=EXP(X(1))
      WRITE(2,230)X(1),X(2)
  230 FORMAT(1H ,'ESTIMATES FOR D ARE   X(1)=',F9.6,5X,'X(2)=',F9.6)
C**** THESE ARE ESTIMATES REQUIRED TO BE INPUT BY E04FDF                  ****
      CALL E04FDF(N,2,X,FSUMSQ,IW,2,W,110,IFAIL)
      DN=1.0-1.0/X(2)
      DK4=X(2)*X(1)**(1.0/X(2))
C**** DN IS THE ORDER OF REACTION                                         ****
C**** DK4 IS THE CONSTANT K4                                              ****
      DO 107 I=1,N
      DP(I)=X(1)*T(I)**X(2)
C**** DP IS THE PREDICTED VALUE OF D                                      ****
      DERROR(I)=ABS(D(I)-DP(I))
C**** DERROR IS THE DIFFERENCE BETWEEN THE 'EXPERIMENTAL' AND THE         ****
C**** PREDICTED VALUE OF D                                                ****
  107 CONTINUE
      CALL SDEV(DERROR,N,EDMEAN,SDDERR)
C**** EDMEAN IS THE MEAN VALUE OF DERROR                                  ****
C**** SDDERR IS THE S.D. OF DERROR                                        ****
      WRITE(2,14)
      WRITE(2,15)DN,DK4
      WRITE(2,16)
      WRITE(2,8)(IT(I),D(I),DP(I),DERROR(I),I=1,N)
      WRITE(2,17)EDMEAN,SDDERR
      IF(JJ.NE.0)GO TO 110
      GO TO 300
  202 WRITE(2,31)IFAIL
      GO TO 110
C**** USING THE MASS BALANCE EQUATION , B IS NOW PREDICTED USING          ****
C**** THE CALCULATED VALUES OF A , C , AND  D                             ****
C****                                                                     ****
C****                      B = AI - A - C - D                             ****
C****                                                                     ****
  300 DO 108 I=1,N
      BP(I)=AI-AP(I)-CP(I)-DP(I)
C**** BP IS THE PREDICTED VALUE OF B                                      ****
      BERROR(I)=ABS(B(I)-BP(I))
C**** BERROR IS THE DIFFERENCE BETWEEN THE EXPERIMENTAL AND THE           ****
C**** PREDICTED VALUE OF D                                                ****
  108 CONTINUE
      CALL SDEV(BD,N,BDMEAN,SDBD)
C**** BDMEAN IS THE MEAN VALUE OF BD                                      ****
C**** SDBD IS THE S.D. OF BD                                              ****
      CALL SDEV(BERROR,N,EBMEAN,SDBERR)
C**** EBMEAN IS THE MEAN VALUE OF BERROR                                  ****
C**** SDBERR IS THE S.D. OF BERROR                                        ****
      WRITE(2,18)
      WRITE(2,8)(IT(I),B(I),BP(I),BERROR(I),I=1,N)
      WRITE(2,9)BDMEAN,SDBD,EBMEAN,SDBERR
C**** THE MAIN GRAPH IS NOW PLOTTED                                       ****
      CALL C1051N
C**** NOMINATE THE GRAPH PLOTTER                                          ****
      CALL AXIPLO(0,200.0,140.0,3,3,10,7,0.0,300.0,0.0,7.0,'TIME(MINUTES
     1)',13,'CONC.(M-MOLES)',14)
C**** PLOT THE AXIS                                                       ****
      CALL GRASYM(T,A,N,7,0)
      CALL GRASYM(T,C,N,1,0)
      CALL GRASYM(T,D,N,2,0)
C**** PLOTS EXPERIMENTAL VALUES OF A, B, C, AND D AS SYMBOLS           ****
      DO 109 I=1,N
      XPT=T(I)
      YPA1=ADP(I)
      YPA2=ADN(I)
      YPB1=BDP(I)
      YPB2=BDN(I)
      YPC1=CDP(I)
      YPC2=CDN(I)
      CALL GRAMOV(XPT,YPA1)
      CALL GRALIN(XPT,YPA2)
      CALL GRAMOV(XPT,YPB1)
      CALL GRALIN(XPT,YPB2)
      CALL GRAMOV(XPT,YPC1)
      CALL GRALIN(XPT,YPC2)
C**** PLOTS THE DEVIATIONS OF THE EXPERIMENTAL DATA A, B, AND  C       ****
  109 CONTINUE
      CALL GRACUR(T,AP,N)
      CALL GRACUR(T,AP,N)
      CALL GRACUR(T,BP,N)
      CALL GRACUR(T,BP,N)
      CALL GRACUR(T,CP,N)
      CALL GRACUR(T,CP,N)
      CALL GRACUR(T,DP,N)
      CALL GRACUR(T,DP,N)
C**** PLOTS THE PREDICTED VALUES OF A, B, C, AND D AS CURVES           ****
      CALL DEVEND
C**** CALLS END OF GRAPH PLOTTER                                       ****
  110 CONTINUE
    1 FORMAT(I0)
    2 FORMAT(I0,7F0.0)
    3 FORMAT(///,1H ,'THE EXPERIMENTAL DATA ARE :',//,1H ,'TIME',6X,'A',
     110X,'AD',9X,'B',10X,'BD',9X,'C',10X,'CD',9X,'D',/)
    4 FORMAT(/,1H ,13,7(5X,F6.4))
    5 FORMAT(/////,1H ,'THE RESULTS FOR A ARE :',/)
    6 FORMAT(/,1H ,'THE CORRELATION COEFFICIENT IS =',F8.6,//,1H ,'THIS
     1VALUE WAS OBTAINED USING ',I2,' POINTS',//,1H ,'THIS GIVES K =',F1
     20.6,'  FROM WHICH ,',//,1H ,'K1 =',F10.6,'    AND K2 =',F10.6,/)
    7 FORMAT(//,1H ,'TIME',6X,'A(EXPT)',7X,'A(PRED)',7X,' ERROR ',/)
    8 FORMAT(/,1H ,I3,7X,F7.5,7X,F7.5,7X,F7.5)
    9 FORMAT(/,1H ,'THE MEAN OF DEVIATIONS =',F9.6,2X,'THE S.D. OF THE D
     1EVIATIONS =',F9.6,//,1H ,'THE MEAN ERROR IS  =',F9.6,6X,'THE S.D.
     2OF THE ERROR IS    =',F9.6,/)
   10 FORMAT(2F0.0)
   11 FORMAT(/////,1H ,'THE RESULTS FOR C ARE :',/)
   12 FORMAT(/,1H ,'THE ORDER OF REACTION IS =',F10.6,//,1H ,'THE CONSTA
     1NT K3 =',F10.6,/)
   13 FORMAT(/,1H ,'TIME',6X,'C(EXPT)',7X,'C(PRED)',7X,' ERROR ',/)
   14 FORMAT(/////,1H ,'THE RESULTS FOR D ARE :',/)
   15 FORMAT(/,1H ,'THE ORDER OF REACTION IS =',F10.6,//,1H ,'THE CONSTA
     1NT K4 =',F10.6,/)
   16 FORMAT(/,1H ,'TIME',5X,'D(''EXPT'')',6X,'D(PRED)',7X,' ERROR ',/)
   17 FORMAT(/,1H ,'THE MEAN ERROR IS  =',F9.6,5X,'THE S.D. OF THE ERROR
     1 IS   =',F9.6,/)
   18 FORMAT(/////,1H ,'THE RESULTS FOR B ARE :',//,1H ,'TIME',6X,'B(EXP
     1T)',7X,'B(PRED)',7X,' ERROR ',/)
   30 FORMAT(/,1H ,'FAILED WITH C , IFAIL =',I1,/)
```

```
      STOP
      END
```

```
      SUBROUTINE CCMAX
C**** THIS ROUTINE FINDS THE MAXIMUM VALUE OF THE CORRELATION       ***
C**** COEFFICIENTS IN THE ARRAY CC                                  ***
C**** IT ALSO RETURNS THE CORRESPONDING REGRESSION COEFFICIENT      ***
C**** AND THE NUMBER OF POINTS USED TO OBTAIN THIS VALUE            ***
      REAL CC(6),XK(6)
      COMMON/BL1/CC,XK/BL2/NPTS,CCBEST,XKBEST
      NPTS=1
      CCBEST=CC(1)
      DO 102 I=2,6
      IF(CC(I).LE.CCBEST)GO TO 101
      GO TO 102
  101 NPTS=I
      CCBEST=CC(I)
  102 CONTINUE
      CCBEST=ABS(CCBEST)
C**** THIS IS THE ABSOLUTE VALUE OF THE MAXIMUM CORRELATION COEFF:  ***
      XKBEST=XK(NPTS)
C**** THIS IS THE CORRESPONDING REGRESSION COEFFICIENT              ***
      NPTS=NPTS+3
C**** THIS GIVES THE NUMBER OF POINTS USED TO OBTAIN THE COEFFICIENT***
      RETURN
      END
```

```
      SUBROUTINE SDEV(X,NN,XMEAN,SD)
C**** THIS SUBROUTINE CALCULATES THE MEAN AND THE STANDARD DEVIATION***
C**** OF THE NN VALUES IN THE ARRAY X                               ***
      REAL X(11)
      SUM=0.0
      DO 101 I=1,NN
      SUM=SUM+X(I)
  101 CONTINUE
      XMEAN=SUM/FLOAT(NN)
C**** XMEAN IS THE MEAN VALUE OF X                                  ***
      SUMXDS=0.0
      DO 102 I=1,NN
      XDIFF=X(I)-XMEAN
      XDIFFS=XDIFF*XDIFF
      SUMXDS=SUMXDS+XDIFFS
  102 CONTINUE
      SD=SQRT(SUMXDS/FLOAT(NN))
C**** SD IS THE STANDARD DEVIATION OF X                             ***
      RETURN
      END
      SUBROUTINE REGPLOT(MM,XK,X,Y)
C**** THIS SUBROUTINE PLOTS THE 9 POINTS USED IN THE LINEAR           ****
C**** REGRESSION ANALYSIS TO FIND K                                   ****
C**** IT ALSO PLOTS A STRAIGHT LINE THROUGH THE ORIGIN USING          ****
C**** MM POINTS , WHERE MM IS THE NUMBER OF POINTS USED TO OBTAIN     ****
C**** THE BEST VALUE OF THE REGRESSION COEFFICIENT                    ****
      REAL X(9),Y(9)
      XPT1=X(MM)
      YPT1=XK*XPT1
C**** XPT1 AND YPT1 ARE THE END-POINTS OF THE STRAIGHT LINE           ****
      CALL C1051N
C**** NOMINATE THE GRAPH PLOTTER
      CALL AXIPLO(0,200.0,150.0,3,3,8,5,0.0,240.0,0.0,-5.0,'TIME(MINUTES
     1)',13,'CONC.(M-MOLES)',14)
C**** DRAWS THE AXIS                                                  ****
      CALL GRASYM(X,Y,9,7,0)
C**** PLOTS THE 9 POINTS AS SYMBOLS                                   ****
      CALL GRAMOV(0.0,0.0)
      CALL GRALIN(XPT1,YPT1)
C**** DRAWS THE BEST STRAIGHT LINE THROUGH THE MM POINTS              ****
      CALL DEVEND
      RETURN



      SUBROUTINE LSFUN1(M,N,XC,FVECC)
C**** THIS SUBROUTINE EVALUATES THE RESIDUALS NEEDED BY THE           ****
C**** NAG ROUTINE E04FDF                                              ****
      INTEGER M,N
      REAL FVECC(M),XC(N)
      REAL T(11),C(11),D(11)
      COMMON/BL3/IVALUE/BL4/T,C,D
      IF(IVALUE.EQ.1)GO TO 102
C**** WHEN IVALUE=1  LSFUN1 CALCULATES RESIDUALS FOR D                ****
C**** WHEN IVALUE=0  LSFUN1 CALCULATES RESIDUALS FOR C                ****
      DO 101 I=1,M
      FVECC(I)=XC(1)*T(I)**XC(2)-C(I)
  101 CONTINUE
      GO TO 104
  102 DO 103 I=1,M
      FVECC(I)=XC(1)*T(I)**XC(2)-D(I)
  103 CONTINUE
C**** FVECC IS THE VALUE OF THE RESIDUAL                              ****
  104 CONTINUE
      RETURN
      END
      FINISH
DOCUMENT DATA
10
0     6.173    0.000    0.000    0.000    0.000    0.000    0.000        RE27
15    4.180    0.185    2.210    0.040    0.000    0.000    0.000
30    2.330    0.045    3.800    0.130    0.000    0.000    0.000
45    1.700    0.095    4.610    0.110    0.090    0.078    0.000
60    1.300    0.120    4.870    0.040    0.090    0.079    0.000        pH7
90    0.900    0.140    4.960    0.050    0.150    0.100    0.163
120   0.800    0.025    4.600    0.250    0.300    0.150    0.473
130   0.700    0.010    4.130    0.200    0.500    0.053    0.893
240   0.650    0.010    3.200    0.100    0.450    0.130    1.823
300   0.600    0.030    2.100    0.100    0.800    0.120    2.673
10
0     6.173    0.000    0.000    0.000    0.000    0.000    0.000        RE28
15    3.853    0.138    2.588    0.049    0.094    0.071    0.000
30    2.303    0.128    3.795    0.138    0.098    0.062    0.000
45    1.485    0.043    4.485    0.175    0.105    0.023    0.000
60    1.222    0.075    4.830    0.251    0.125    0.073    0.000        pH6
90    0.963    0.035    4.658    0.203    0.188    0.062    0.364
120   0.894    0.093    4.485    0.252    0.266    0.082    0.528
180   0.795    0.103    3.709    0.312    0.359    0.078    1.310
240   0.752    0.083    2.846    0.211    0.469    0.091    2.106
300   0.623    0.135    1.725    0.113    0.560    0.102    3.265
11
0     6.173    0.000    0.000    0.000    0.000    0.000    0.000        RE31
15    3.705    0.155    1.724    0.174    0.000    0.000    0.000
30    2.160    0.010    2.845    0.085    0.000    0.000    1.168
45    1.440    0.060    3.320    0.390    0.125    0.027    1.288
60    1.020    0.028    3.410    0.130    0.141    0.031    1.602
90    0.990    0.040    3.450    0.210    0.203    0.052    1.530        pH 7.5
120   0.740    0.050    3.450    0.230    0.220    0.032    1.762
150   0.680    0.032    3.410    0.300    0.250    0.038    1.963
180   0.665    0.003    3.280    0.283    0.312    0.032    1.786
240   0.605    0.005    2.810    0.320    0.385    0.058    2.373
300   0.505    0.015    2.030    0.300    0.491    0.033    3.147
10
0     6.173    0.000    0.000    0.000    0.000    0.000    0.000        RE32
15    3.230    0.030    2.717    0.302    0.063    0.000    0.163
30    1.780    0.050    3.963    0.173    0.078    0.000    0.347
45    1.135    0.095    4.658    0.690    0.126    0.063    0.223
60    0.920    0.110    4.313    0.690    0.157    0.063    0.814
90    0.775    0.105    3.839    0.129    0.213    0.039    1.332
120   0.725    0.095    3.795    0.110    0.321    0.024    1.342        pH 5
180   0.645    0.085    2.243    0.345    0.360    0.109    2.925
240   0.540    0.070    1.812    0.087    0.531    0.078    3.290
300   0.455    0.065    1.294    0.173    0.625    0.094    3.799
****
0.000645       1.24
0.01   0.5
0.00467        0.83
0.000052       1.94
0.00332        0.87
0.01   0.5
0.01   0.5
0.01   0.5
```

6.5 PUBLICATIONS

Communication

Material for publication as a Communication must be on an urgent matter and be of obvious scientific importance. Rapidity of publication is enhanced if diagrams are omitted, but tables and formulae can be included. Communications should not be simple claims for priority: this facility for rapid publication is intended for brief descriptions of work that has progressed to a stage at which it is likely to be valuable to workers faced with similar problems. A fuller paper may be offered subsequently, if justified by later work.

Manuscripts are not subjected to the usual examination by referees and inclusion of a Communication is at the Editor's discretion.

Application of Transition-metal Salts as Ion-pair Reagents in the Liquid Chromatography of Dithiocarbamates

Keywords: *Dithiocarbamate analysis; liquid chromatography; ion-pair reagents; transition-metal salts*

N-Alkyl- and *NN*-dialkyldithiocarbamates and their salts and disulphides are widely used as fungicides, rubber vulcanisers and pharmaceuticals. As part of a study of the degradation of the cyclic fungicide dazomet (3,5-dimethyltetrahydro-1,3,5-thiadiazine-2-thione), we wished to analyse *N*-methyldithiocarbamate (MDTC), one of the primary decomposition products.[1,2] Existing techniques based on ultraviolet spectroscopy, total degradation to carbon disulphide and methylamine and thin-layer, paper or gas - liquid chromatography[3] either could not differentiate MDTC from other components of the reaction or could not be used to monitor the other components in the same analysis.

Although reversed-phase liquid chromatography could be used to analyse dazomet and neutral degradation products, MDTC was not retained even with aqueous phosphate buffer (pH 5) as solvent. Eluents with a lower pH could not be used to suppress ionisation as dithiocarbamates are very unstable at pH below 4.[4] The ion-pair reagents tetrabutylammonium hydroxide and cetrimide were tried, but appeared to result in the degradation of MDTC.

As the transition-metal salts of a number of dithiocarbamates have been successfully separated using liquid chromatography,[5] we investigated the use of transition-metal ions as ion-pair reagents. The results of our study using the direct injection of the sample into an eluent containing transition-metal ions suggest that this approach could be a versatile and powerful method for the analysis of dithiocarbamates.

Experimental and Results

Samples of 10 μl were separated on a 5-μm Hypersil-ODS column (10 cm × 5 mm i.d.) using a Waters Associates 6000 pump and an ALC 202 detector at 254 nm, with a solvent flow-rate of 1.5 ml min^{-1}. The samples of the sodium salts of MDTC and *N*-ethyldithiocarbamate (prepared according to Klopping and Van der Kerk[6]), *NN*-dimethyldithiocarbamate (Aldrich) and *NN*-diethyldithiocarbamate (Sigma) were prepared as solutions in methanol - water (7 + 3). Analytical-reagent grade nickel sulphate and cobalt nitrate were used as ion-pair reagents at a concentration of 0.1%

The capacity factors of the dithiocarbamates and some related thiuram disulphides with the different metal ions are given in Table I. The peaks were readily detected without background interference from the metal ions in the solvent. The efficiency of the column was unchanged in comparison with unpaired eluents, suggesting that complex formation was rapid.

As the dazomet degradation studies were carried out in phosphate buffer, it was necessary to use methanol - phosphate buffer (pH 5) (3 + 7) instead of water, to prevent precipitation of transition-metal phosphates on injection. Using these conditions with 0.1% cobalt(II), the peak-height response of MDTC was linear from 100 to 1 000 μg ml^{-1}.

An attempt to analyse disodium ethylenebisdithiocarbamate was unsuccessful, probably because it forms polymeric metal salts.

TABLE I

CAPACITY FACTORS OF DITHIOCARBAMATES WITH TRANSITION-METAL ION-PAIR REAGENTS

	Solvent			
		Methanol - water (6 + 4)		
	Methanol - water (7 + 3), ion pair 0.1% Ni(II)	Ion pair 0.1% Ni(II)	Ion pair 0.1% Co(II)	Methanol - buffer* (3 + 7), ion pair 0.1% Co(II)
Dithiocarbamate (Na salt)—				
N-Methyl (MDTC)	0.57	1.25	0.71	11.8
N-Ethyl	1.28	3.37	2.71	
NN-Dimethyl	1.57	4.12	3.85	
NN-Diethyl	8.28	32.1	—†	
Thiuram disulphide—				
NN'-Tetramethyl (thiram)	0.85	1.25	1.57	
NN'-Tetraethyl (disulfiram)	3.71	11.8	12.7	

* 0.05 M phosphate buffer adjusted to pH 5.
† Peak not observed.

Discussion

Little previous use has been made of metal ions as reagents in liquid chromatography, with the exception of the interaction of silver(I) and olefinic bonds, to give a complex with increased polarity (*e.g.*, ref. 7). In this study, the transition-metal ions in the solvent form a neutral non-aqueous soluble ion-pair complex with the injected dithiocarbamates, enabling them to be resolved from the solvent front and to be eluted under similar conditions to the corresponding disulphides.

Although only nickel(II) and cobalt(II) metal ions have been studied in this way, the work by Schwedt[5] on the separation of pre-formed complexes of diethyldithiocarbamate suggests that lead(II), mercury(II), copper(II) (and possibly others in less polar solvents) should act in a similar way. As each of these complexes has a different capacity factor, it should be possible, by selection of the ion-pair reagent, to adjust the retention of the complex so that it can be resolved from other components of a mixture. The order of elution of the complexes of lead, nickel, cobalt, copper and mercury was the same for diethyldithiocarbamate and tetramethylenedithiocarbamate,[5] but does not agree with the present results in which cobalt(II) complexes are eluted before nickel(II) complexes. Other metal ion salts of diethyldithiocarbamate, such as iron(III), molybdenum(II), chromium(VI) and vanadium are reported[5] to give many peaks on direct injection and would probably be unsuitable for pairing.

Using cobalt(II) as the ion-pair reagent, this technique was applied to the study of dazomet and permitted the determination of dazomet and its degradation products, MDTC, methyl isothiocyanate and *NN*-dimethylthiuram disulphide, in the same run.[8]

Further studies into the scope and applications of this technique are in progress.

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Determination of Dithiocarbamates by Liquid Chromatography Using Transition-metal Salts as "Ion-pair" Reagents

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A technique has been developed for the liquid-chromatographic determination on an octadecylsilyl silica reversed-phase column of *N*-alkyl- and *NN*-dialkyldithiocarbamates by adding transition-metal salts of cobalt(II) or nickel(II) to the mobile phase. The unsuccessful use of mercury(II), copper(II) and lead(II) is discussed and the mixed complexes formed when two dithiocarbamates are injected are described. The retentions of the dithiocarbamates are compared with those of related thiuram disulphides.

Keywords: *Dithiocarbamate determination*; *thiuram disulphide determination*; *liquid chromatography*; *complex formation*; *transition-metal salts*

A number of methods have been developed for the determination of *N*-alkyl- and *NN*-dialkyldithiocarbamates and their salts and related thiuram disulphides,[1] because of their widespread use as fungicides, rubber vulcanisers and as pharmaceuticals.[2] However, many of these methods, including ultraviolet spectroscopy, colorimetry with copper salts and the widely used acid degradation to give carbon disulphide, are specific only for the dithiocarbamate group and cannot distinguish between compounds with different *N*-alkyl substituents.

As part of a study of the degradation of the cyclic fungicide dazomet (3,5-dimethyltetrahydro-1,3,5-thiadiazine-2-thione), we developed and briefly reported a liquid-chromatographic technique in which the individual dithiocarbamates were separated using a solvent containing nickel or cobalt salts as "ion-pair" reagents.[3] Although the sodium salts were unretained on chromatography, the non-aqueous soluble metal-ion complexes formed in the eluent were readily separated on a reversed-phase column. In this paper we describe our qualitative and quantitative studies in detail and discuss the examination of further metal ions as potential complexation reagents.

The interaction of dithiocarbamates, in particular *NN*-diethyl- and *NN*-tetramethylenedithiocarbamate, with transition-metal ions to form complexes extractable into organic solvents has been used in analytical chemistry for the separation[4] and spectrophotometric determination of a number of metals.[5] The complexes, after extraction, can be separated by thin-layer chromatography (*e.g.*, reference 6), gas chromatography (*e.g.*, reference 7) or liquid chromatography on silica gel,[8–14] bonded nitrile[15–16] or hydrocarbon (reversed-phase) columns.[17–19] In most instances the separation has been aimed at the determination of the metal ion rather than of the ligand.

Experimental

Apparatus

Liquid chromatography was carried out using a Water Associates 6000 pump connected to a Shandon Southern column (10 cm × 5 mm i.d.) packed with Hypersil-ODS and fitted with a Rheodyne 7010 valve injector with a 10-µl loop. The eluates were detected using an ALC 202 detector at 254 nm. The solvent flow-rate was 1.5 cm^3 min^{-1}.

Reagents and Standards

Metal salts. Analytical-reagent grade nickel sulphate, cobalt nitrate, copper nitrate, mercury(II) nitrate and lead nitrate were used.

Dithiocarbamates. The sodium salts of *N*-methyl-, *NN*-diethyl- and *NN*-dimethyldithiocarbamate were supplied by J. D. Cambell and Sons Ltd., Warrington, Sigma London Chemical Co. Ltd., Poole, and Aldrich Chemical Co. Ltd., Gillingham, respectively. Ammonium *NN*-tetramethylenedithiocarbamate was supplied by Sigma London Chemical Co., Ltd., Poole. Sodium *N*-ethyldithiocarbamate and ammonium dithiocarbamate were synthesised from the corresponding amine and carbon disulphide.[2]

Thiuram disulphides. Thiram (*NNN'N'*-tetramethylthiuram disulphide) was supplied by Robinson Bros. Ltd., West Bromwich, and disulfiram (*NNN'N'*-tetraethylthiuram disulphide) was supplied by Sigma London Chemical Co. Ltd., Poole.

Methanol. HPLC grade from Fisons Scientific Apparatus, Loughborough.

Procedure

Solutions of the dithiocarbamates or thiuram disulphides, approximately 500 mg dm^3 in methanol - water, were injected on to the column. Different ratios of methanol and water, containing 0.1% *m*/*V* of the metal salts, nickel sulphate, cobalt nitrate, copper nitrate or lead nitrate, were used as indicated for the eluent. In some runs 0.05 M phosphate buffer, pH 5, containing the metal ions was used.

As prolonged elution of methanol - water mixtures did not remove all the trace amounts of cobalt(II) from the column, a different column was used for each metal ion.

Results and Discussion

On examination of solutions of the sodium salts of *N*-methyl- or *NN*-diethyldithiocarbamic acid by liquid chromatography using an octadecylsilyl silica reversed-phase column, the solutes were eluted at the solvent front ($k' = 0$) even if water alone was used as the solvent. In some runs the sample appeared to interact with the column and no peaks were observed. Attempts to control the ionisation of the dithiocarbamates by using a phosphate buffer at pH 5 as the eluent had no effect. A lower pH was not used because the dithiocarbamates are sensitive to acid and are rapidly degraded to carbon disulphide and an amine.[5] The addition of the organic ion-pair reagents tetrabutylammonium hydroxide and cetrimide to the eluent was also studied but their use appeared to result in complete degradation of the sample. The thiuram disulphides thiram and disulfiram could be readily chromatographed without decomposition.

As a number of metal-ion complexes of *NN*-diethyl- and *NN*-tetramethylenedithiocarbamates have been successfully chromatographed on RP-8 and RP-18 reversed-phase columns,[19] it was decided to investigate the use of transition-metal ions as potential ion-pair reagents. It was hoped that on injection of the dithiocarbamate salts, the metal ions would form stable neutral complexes, suitable for chromatography. Despite the common use of organic ion-pair reagents to improve the separation or efficiency of polar compounds on liquid chromatography, there are few reports of the use of metal ions. Examples include the interaction of zinc ions with aminobenzoic acids,[20] nickel ions with aniline[21] and its metabolites and a zinc or cadmium C_{12}-dien complex with the dansyl amino acids and sulphonamides.[22,23] The more common addition of silver ions to solvents to alter the retention of olefins results not in the formation of a neutral ion-pair complex but of a polar π-complex with a shorter retention time on a reversed-phase column than the original olefin (*e.g.*, reference 24).

After initial trials using separately prepared complexes a range of solvents containing different proportions of methanol and 0.1% solutions of nickel(II) sulphate or cobalt(II) nitrate were examined. A series of sodium or ammonium salts of dithiocarbamates and related thiuram disulphides were individually injected and their capacity factors were determined (Table I). The complexes were readily detected at 254 nm, with no background interference from the metal salts in the solvent. The peak efficiencies of the complexes were comparable to those of uncomplexed eluates and suggested that mixing and complex formation occurred rapidly on injection.

A number of important fungicides, including zineb, maneb and mancozeb, are based on ethylenebisdithiocarbamic acid, $(CH_2NHCS_2)_2{}^{2-}$; however, its metal complexes are polymeric and attempts to examine the sodium salt using cobalt as a reagent were unsuccessful. Many dithiocarbamates form stable silver(I) salts but a trial using silver nitrate appeared to result in decomposition of the ethylenebisdithiocarbamate.

TABLE I

CAPACITY FACTORS OF DITHIOCARBAMATES AND THIURAM DISULPHIDES ON LIQUID CHROMATOGRAPHY WITH NICKEL AND COBALT REAGENTS

	Solvent					
	Methanol - water containing 0.1% Co(II) as nitrate				Methanol - water containing 0.1% Ni(II) as sulphate	
	30+70*	50+50	60+40	70+30	60+40	70+30
Dithiocarbamate—						
N-H_2-	0.25	—	—	—	—	—
N-Methyl-†	11.8	1.25	0.71	0.50	1.25	0.57
N-Ethyl-	—	7.25	2.71	1.37	3.37	1.28
NN-Dimethyl-	—	10.6	3.85	2.25	4.12	1.57
NN-Diethyl-	—	—	>30	24.7	32.1	7.14
NN-Tetramethylene-	—	—	26.5	11.8	—	—
Thiuram disulphide—						
NNN'N'-Tetramethyl (thiram)	—	2.75	1.75	0.88	1.25	0.86
NNN'N'-Tetraethyl (disulfiram)	—	—	12.7	6.37	11.8	3.71

* Methanol - 0.05 M phosphate buffer adjusted to pH 5.

† These results were obtained using methanol - water containing 0.1% Ni(II) as sulphate: 50+50, k' = 2.86; 40+60, k' = 10.4; 30+70, k' = 25.4.

In addition to the cobalt and nickel complexes, Schwedt also successfully chromatographed the complexes of copper, mercury and lead, although the last was unstable.[19] As each of the complexes has a different capacity factor, it appeared that it would be possible by selection of the appropriate metal ion to adjust the retention times of dithiocarbamates, when they are present in a mixture, to give the optimum resolution. The complexes of most other metal ions including iron(III), manganese, silver, chromium(III), molybdenum and vanadium were found either to decompose or to give several bands.[19]

Although the reaction with diethyldithiocarbamate followed by extraction and spectrophotometry of the complex is a widely used assay for copper(II),[2,5] when copper nitrate was used as a reagent the results were erratic and reproducible peaks could not be obtained. In addition, a strong interaction between the copper ions and thiuram disulphides appeared to take place, presumably similar to the reactions that cause colour changes on mixing aqueous solutions of disulphides and copper(II) ions,[1] whereas with nickel or cobalt ions any effect was minimal. In order to determine the product that was being formed on injection, a solution of copper(II) ions was mixed with diethyldithiocarbamate and the precipitate was dissolved by the addition of methanol. The resulting solution has a $\lambda_{max.}$ at 386 nm, whereas if the copper - dithiocarbamate complex was extracted from the aqueous solution into chloroform and then diluted with methanol - water the absorbance of the solution had a $\lambda_{max.}$ at 434 nm. The latter band corresponds to the neutral ML_2 complex, whereas the former is very similar to the ML^+ water-soluble complex formed with *NN*-diethanolaminodithiocarbamate and copper ions (ML^+, $\lambda_{max.}$ at 380 nm, and ML_2, $\lambda_{max.}$ 435 nm).[25] Thus, when used as a reagent the copper(II) ions, which are in excess, appear to form the ML^+ complex rather than the neutral ML_2 complex, which can be chromatographed. Samples of the ML_2 copper complex prepared by extraction could therefore be readily chromatographed.

Mercury(II) ions are reported to form the strongest complexes with diethyldithiocarbamate and will displace all other metal ions from complexes.[26] However, attempts to chromatograph mercury(II) diethyldithiocarbamate were unsuccessful and no peaks were obtained. Although the extracted complex is very stable, the equilibrium constant for the reaction $2HgL^+ \rightleftharpoons HgL_2 + Hg^{2+}$ is 0.[27] Therefore, the complex forms stepwise so that in the presence of an excess of mercury(II) ions only HgL^+ will be present.

In contrast to mercury and copper, lead ions form much weaker complexes[26] and it was felt that they would be a useful comparison. Schwedt found the complexes to be unstable,[19] and in two papers reported very different retentions, relative to the other complexes.[18,19] In this study an attempt to use lead nitrate as a reagent was unsuccessful, and the complex when prepared by extraction was not eluted on chromatography. A recent report has suggested that the lead complex decomposes in solution in isobutyl methyl ketone to give the free metal after 2–3 h[28] although other papers[29] considered the complex to be stable under these conditions. Extraction of lead ions by ammonium tetramethylenedithiocarbamate was also found to fail with both high and low proportions of dithiocarbamate.[28] The disulphides chromatographed essentially unaltered in the presence of lead ions.

Two previous studies have examined in detail the separation of nickel(II) complexes on silica gel columns[8,12] and as in our degradation studies the cobalt complex gave a more suitable retention time relative to the other components in the mixture, a detailed examination of the use of cobalt(II) was carried out.

The pH of the aqueous solution was not critical and was usually not controlled except for the degradation studies.[3] In the degradation studies, as the samples were in phosphate buffers at a range of pH values it was necessary to use a pH 5 phosphate buffer in the solvent to prevent precipitation of cobalt phosphate. In extraction studies both cobalt and nickel complexes are completely extracted between pH 2 and 12.[4] Different concentrations of cobalt nitrate, 0.01, 0.5 and 0.1%, gave the same capacity factors and it is assumed that complex formation is essentially complete.

As part of the degradation study a calibration graph was prepared for *N*-methyldithiocarbamate using caffeine as an internal standard (Table II). The graph is linear from 100 to 1000 μg ml^{-1}, but a small negative intercept, also found on other calibration runs, suggested a small but constant amount of decomposition.

TABLE II

CALIBRATION OF *N*-METHYLDITHIOCARBAMATE USING METHANOL - pH 5 PHOSPHATE BUFFER [0.1% Co(II) AS NITRATE] (30+70) AS SOLVENT

	Peak height, scale units		
Sample concentration*/ μg ml^{-1}	*N*-Methyl-dithiocarbamate†	Caffeine (internal standard)†	Ratio, sample × 10^3 to internal standard
100	6	183	32
200	14.5	183	79
300	23	183	126
400	31	177.5	175
500	40.5	177	229
600	49	178	275
700	58	179	324
800	65	178.5	364
1000	79	177	446

Slope 0.4682
Intercept . . . . . . −11.5
Correlation coefficient 0.9991
Standard deviation/μg ml^{-1} . . 5.894

* 10-μl injection of a sample solution containing caffeine (800 μg ml^{-1}) in pH 7.0 phosphate buffer.
† For caffeine $k' = 2.0$ and for *N*-methyldithiocarbamate $k' = 18.8$.

In all the studies so far, only one dithiocarbamate was injected at a time. In view of the exchange of ligands found with nickel complexes the effect of injecting mixtures of two dithiocarbamates was determined (Table III). Four peaks were obtained in each instance corresponding to a random formation of mixed complexes of cobalt(III). The observation confirmed that, as in the test-tube reaction between cobalt and dithiocarbamates, the cobalt(II) reagent was being oxidised to yield the very stable cobalt(III) complex. This reaction is reported to occur spontaneously by oxidation with atmospheric oxygen[15,30] and explains the marked colour change from pink to green on complex formation. The mass spectrum of the extracted complex is reported to agree with the formation of a cobalt(III) - tridithiocarbamate complex.[15] The four peaks found in this work with cobalt ions can therefore be assigned to MX_3, MX_2Y, MXY_2 and MY_3 complexes.

Although a mixture of tetramethyl- and tetraethylthiuram disulphides gave two peaks on chromatography immediately after mixing, on re-examination after standing for 90 min an intermediate peak, presumably the mixed *NN*-diethyl-*N′N′*-dimethylthiuram disulphide, was also present, an effect that had also been noted earlier[31] (Table III).

TABLE III

CAPACITY FACTORS OF MIXED DITHIOCARBAMATE METAL COMPLEXES FORMED BY INJECTION OF TWO DITHIOCARBAMATES

	Dithiocarbamates		Capacity factors			
Solvent	Sample (X)	Sample (Y)	MX_3	MX_2Y	MXY_2	MY_3
30 + 70*	$-NH_2$	$-NHMe$	0.25	1.37	4.90	1.47
50 + 50†	$-NHEt$	$-NMe_2$	7.25	7.89	9.00	10.6
	$-NHMe$	$-NMe_2$	1.25	2.63	5.25	10.6
	$-NHMe$	$-NHEt$	1.25	2.50	4.12	7.25
70 + 30†	$-NHMe$	$-N(CH_2)_4$	0.50	1.25	4.00	11.8
	$-NHEt$	$-N(CH_2)_4$	1.37	2.87	5.87	11.8
	$-NMe_2$	$-N(CH_2)_4$	2.00	3.62	6.75	11.8
	$-N(CH_2)_4$	$-NEt_2$	11.8	15.0	19.2	24.7
	$-NHMe$	$-NHEt$	0.50	0.75	1.00	1.37
	$-NHEt$	$-NEt_2$	1.37	2.75	10.0	24.7

* 30 + 70 methanol - 0.05 M phosphate buffer (pH 5) containing 0.1% Co(II) as nitrate.
† Methanol - water containing 0.1% Co(II) as nitrate.

If a disulphide and dithiocarbamate were mixed and injected (Table IV), interchange apparently also occurred, leading to all possible combinations of disulphides, mixed disulphides and mixed complexes, although some of the assignments are tentative as not all the possible standards were available. Whether this exchange occurs via oxidation, reduction and cleavage of the S—S bond or by amine exchange[32] is not known.

TABLE IV

CAPACITY FACTORS FOR MIXTURES OF THIURAM DISULPHIDES AND DITHIOCARBAMATES

	Disulphide		Dithio-carbamate	Capacity factors of complexes							
Solvent*	A–A	B–B	X	A–A	A–B	B–B	MX_3	MX_2A	MXA_2	MA_3	Other
50 + 50	$-NMe_2$	—	$-NMe_2$	2.75			10.6				
	$-NMe_2$	—	$-NHMe$	2.75						10.5	
70 + 30	$-NMe_2$	$-NEt_2$	—	0.75		6.25					
	(after 90 min)	(after 90 min)		0.75	2.25	6.25					
	$-NMe_2$		$-N(CH_2)_4$	—	1.5†	3.0†	1.75	3.5	6.5	11.8	
	$-NEt_2$		$-NMe_2$	6.25	—	—	2.12	2.62	4.75	—	1.12
	$-NEt_2$		$-N(CH_2)_4$	6.37	4.37†	3.0†	11.8	14.4	19.1	24.7	8.4

* Methanol - water containing 0.1% Co(II) as nitrate.
† A tentative assignment assuming that tetramethylenedithiocarbamate is yielding the corresponding disulphide (B—B) and a mixed disulphide (A—B).

Hence, the use of nickel or cobalt ions as complexation reagents is a useful technique for the determination of individual dithiocarbamates but the interpretation of the results can be complicated if dithiocarbamates or thiuram disulphides with different amino functions are present in the sample.

The method has been successfully applied to the determinations of *N*-methyldithiocarbamate formed during the degradation of dazomet *in vitro*, in cell cultures and in bacterial cell cultures under the same chromatographic conditions used to monitor dazomet and methyl isothiocyanate.[33]

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