Molecular structure and antibacterial function of hop resin materials

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STATEMENT

The work described in this thesis was carried out in the laboratories of the Brewing Research Foundation and the School of Biological and Chemical Sciences, Thames Polytechnic between May 1988 and January 1991. Except where otherwise indicated it is entirely the work of the author.

AIMS

The aims of the study were (i) to elucidate the mechanism by which compounds derived from hops inhibit growth of beer-spoilage lactic acid bacteria, and (ii) to identify aspects of the molecular structure of hop-derived materials which affect antibacterial activity.

CONTENTS

CHAPTER 2: Molecular structure and ionisation behaviour of hop compounds and hop-derived compounds

CHAPTER 3: Factors affecting antibacterial activity of hop compounds and hop-derived compounds

CHAPTER 4: The mechanism by which *trans*-isohumulone inhibits growth of *Lactobacillus brevis* IFO 3960

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Finally, I thank the Director of the Brewing Research Foundation, Professor Bernard Atkinson, for allowing me to submit this thesis for examination.

ABSTRACT

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Spoilage of beer by lactic acid bacteria (*Lactobacillus* spp., *Pediococcus* spp.) has considerable economic significance. Compounds derived from the hop (*Humulus lupulus*) possess antibacterial activity but their role in prevention of beer spoilage has been disputed. Prior to the present study, the mechanism by which such compounds inhibited growth of beer-spoilage lactic acid bacteria was unknown.

A range of hop compounds (colupulone, (-)-humulone, (-)-cohumulone) and hop-derived compounds (*trans*-isohumulone, *trans*-isocohumulone, *trans*-humulinic acid, dehydrated humulinic acid) was prepared and analysed using several spectroscopic techniques (UV, IR spectrometry; mass spectroscopy; ^{1}H-NMR, ^{13}C-NMR spectroscopy). The ability of these weak acids to ionise in solution was studied. The lack of agreement between pKa values measured using potentiometric, conductimetric, spectroscopic and solubility methods suggests that, in aqueous solution, the compounds formed covalent hydrates.

Antibacterial activity of the compounds against *Lactobacillus brevis* IFO 3960 was pH-dependent: the undissociated form of each compound was the active antibacterial moiety and the ionised form had little activity. The antibacterial activity of the undissociated forms of each of the compounds was similar. In the case of the undissociated form of *trans*-isohumulone, 0.1-0.4μM was sufficient to inhibit growth of *Lact.brevis* IFO 3960 in a modified version of de Man Rogosa Sharpe medium over the pH range 4.0-7.0.

Trans-isohumulone acted as an ionophore of the mobile-carrier type. In lactic acid bacteria, H^{+} was exchanged for Mn^{2+} in an electroneutral process that required the presence of a second monovalent cation (*e.g* K^{+}, Na^{+}).

ABBREVIATIONS

ATP	adenosine 5′-triphosphate
ATPase	adenosine 5′triphosphatase
b.	broad
BSA	bovine serum albumin
CCCP	carbonyl cyanide *m*-chlorophenylhydrazone
cfu	colony forming unit
CMC	critical micelle concentration
COSY	correlated spectroscopy
d.	doublet
DEPT	distortionless enhancement by polarisation transfer
DMG	3,3′-dimethylglutarate
EDTA	ethylenediamine tetra-acetic acid
Hepes	*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid
J	Coupling constant
m.	multiplet
MIC	minimum inhibitory concentration
mod. MRS	modified de Man Rogosa Sharpe medium
M_r	relative molecular mass
MRS	de Man Rogosa Sharpe medium
NADH	nicotinamide adenine dinucleotide (reduced form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NOE	nuclear Overhauser effect
NOESY	nuclear Overhauser effect difference spectroscopy

pH_{ext}	extracellular pH
pH_{int}	intracellular pH
pKa_{equil}	equilibrium pKa
q.	quartet
s.	singlet
TCA	trichloroacetic acid
TMS	tetramethylsilane
TPP^{+}	tetraphenylphosphonium ion
VALIN	valinomycin
v.b.b.	very broad band
δ	chemical shift value
Δp	proton-motive force
ΔpH	transmembrane pH gradient
$\Delta\psi$	membrane potential

CHAPTER 1

Introduction to hop compounds, hop-derived compounds and the action of antibacterial agents

The continuance of the drink
is alwaie determined after
the quantitie of the hops,
so that being well hopped
it lasteth longer

(Harrison, Description of England)

1.1. BEER, HOPS, AND HOP COMPOUNDS

1.1.1. Beer, beer-spoilage, and lactic acid bacteria

Beer is a fermented alcoholic beverage made primarily from water, malted barley, hops and yeast. Like other foodstuffs it is at risk from microbial attack. Unlike those associated with many other foods, none of the microorganisms capable of growth in beer are pathogenic. Beer contains ethanol (typically 3-6% w/v), compounds derived from hops, has a low concentration of nutrient material and a low pH value (typically pH 3.8-4.4). Although pathogenic microorganisms are unable to grow under such conditions, beer is still subject to attack from a specialised group of spoilage organisms, including yeasts of the genera *Saccharomyces, Kloeckera, Pichia,* and *Hansenula,* and bacteria of the genera *Lactobacillus, Pediococcus, Acetobacter, Gluconobacter, Zymomonas, Pectinatus* and *Megasphaera* (Lawrence 1988). Lactic acid bacteria belonging to the genera *Lactobacillus* and *Pediococcus* are the most prevalent of beer-spoilage bacteria (Shimwell 1935; Kulka 1953; Rainbow 1957; Kulka 1960; Kleyn & Hough 1971). These organisms are Gram-positive, catalase-negative, fermentative bacteria which produce lactic acid as a major end product. Their role in beer-spoilage has been reviewed (Ault 1965; Hough *et al.* 1982; Lawrence 1988).

The economic cost of actual or potential beer spoilage is considerable. While losses due to beer spoilage in the UK are relatively low, the cost of carrying out microbiological quality control tests on beer prior to despatch from the brewery is high. Work relating to aspects of the natural resistance of beer to bacterial spoilage, such as that described in this thesis, is likely to be of significant economic benefit.

1.1.2. The hop plant

The hop plant, *Humulus lupulus*, is grown throughout the temperate regions of the world with the virtually exclusive purpose of meeting the demands of the brewing industry. Hops are climbing plants with separate male and female plants, and it is the female flowers which are usually dried, compressed and used in brewing. The flower consists of a cone of greenish bracts on the surface of which are small yellowish glands which can easily be rubbed off (Bishop 1949). These glands (known as lupulin glands) contain the resins and essential oils of importance in brewing (Hough *et al.* 1982).

1.1.3. The use of hops in brewing: an historical perspective

Antibacterial compounds derived from hops, are consumed in significant quantities by man (apparently without ill-effect), and this has been the case for several hundred years. At present, world-wide consumption of hop-derived compounds is in excess of 2.6×10^7 Kg/annum. The use and study of hops and hop-derived compounds has a long and interesting history. A synopsis of such historical aspects is recorded below.

Beer as we know it today evolved from a beverage made from malted grain, water and yeast and known to the Babylonians of two thousand years ago as 'Boozah'. Such a drink would be barely recognisable to us since it contained none of the familiar bitter-tasting compounds derived from hops. In his *Natural History* written in about A.D. 60, the elder Pliny referred to the hop as '*Lupus saliactarius*' and described it as an appetising ingredient for salads (Sanvill 1915). Other early historians mention that hops were employed for treatment of skin diseases and fever. Hops did not appear in

Western Europe until some time after Pliny's description, the first documented mention of their cultivation being in A.D. 798, when Pepin le Bref gave the monastery of St. Denis, near Paris, certain 'homularias' or hop gardens (Sanvill 1915). During the following centuries, hop cultivation spread to other parts of France and to Austria, Germany and the Netherlands.

At one time beer was flavoured with a mixture of herbs which included bog myrtle, rosemary and yarrow, known as gruit; beer produced with the mixture was known as gruit beer (Corran 1975). In addition, laurel, ivy and Dutch myrtle were used as preservatives, specifically to prevent (microbially-induced) souring of beer (Herz 1964). Spices employed as beer flavourings have included cinnamon and cloves (Corran 1975).

Hopped beer was probably first brewed in England in the reign of Henry VII by immigrant Dutch brewers (Thatcher 1887). Very quickly, this new hopped 'beer' replaced the more traditional English unhopped ales (Bishop 1949). According to Sanvill (1915), Henry VII took a strong dislike to what he termed the *'wicked weed'* and passed an act of Parliament prohibiting the use of hops in beer production. Although the act was rigorously enforced by his successor Henry VIII, the flavour of hopped beer was greatly appreciated by Edward VI and upon succeeding Henry VIII he introduced legislation designed to encourage hop usage. As a result, production and use of hops increased substantially but not at equal rates and, as demand outstripped supply, foreign hops were imported to make up the deficit. In the reign of Queen Anne, the hop industry had reached such importance that it was possible to place an excise duty of 10 shillings per hundred-weight on home-grown hops and an import duty of £3 per hundred-weight on foreign hops (a further incentive to use of home-grown hops) (Sanvill 1915).

To ensure that no revenue was lost from the use of other bittering agents, an act of Parliament was passed forbidding brewers "*to use any other bitter, or drug, or any other ingredient whatsoever except hops for the impregnation of their sale worts*" (Thatcher 1887).

Legislation aside, the reasons why the use of hops in beer production became so ubiquitous, remain obscure. Indeed, it is not clear if the change was consumer-led or producer-led. From a producer's viewpoint, the justifications for the use of hops given by Moritz and Morris (1891) were,

> "*1. To give the beer the distinctive bitter flavour and aroma.*
>
> *2. To precipitate certain nitrogenous constituents of the wort.*
>
> *3. To clarify the wort, not only by separation of the above constituents but by the clarifying property of the hop leaves when agitated in the copper and by the formation of a filter bed for the filtration of wort in the hop-back.*
>
> *4. To preserve the beer by the antiseptic influence of some of their constituents.*
>
> *5. To assist in the sterilization of the wort.*"

These principles remain valid today and could be incorporated into any textbook of brewing science although a further role of hop derived compounds should be added to the list; namely their ability to promote beer foam formation (Bishop *et al.* 1974; Asano & Hashimoto 1976). It is likely that the pioneers of hopped beer had two main objectives: (i) to improve the flavour of beer; (ii) to improve the keeping qualities of beer. The first objective does have some merit since, in the past, beers had been flavoured with spices or herbs such as cinnamon, cloves, ivy or bog myrtle (Herz 1964; Corran 1975). But flavour enhancement was not the exclusive role of

additives. For example, common salt (sodium chloride) and wheat flour were added to beer in the nineteenth Century partly to mask off-flavours and partly to promote beer consumption (Moritz 1892). The origins of this practice, however, lie in the seventeenth Century belief that salt could ward off the evil influences of witchcraft (Moritz 1892). An equally important function of many additives was to improve the keeping qualities of the beer and in this respect hops certainly make a positive contribution. Indeed, Harrison, in his "Description of England" published in 1577 wrote that "*The continuance of the drink is alwaie determined after the quantitie of the hops, so that being well hopped it lasteth longer*".

1.1.4. Terminology, properties and structure of hop compounds and their derivatives

Suggestions for the nomenclature of the various fractions derived from hops have been made by the European Brewery Convention (E.B.C.) and the American Society of Brewing Chemists (E.B.C & A.S.B.C. 1957). Their nomenclature is briefly outlined below (Trolle 1969).

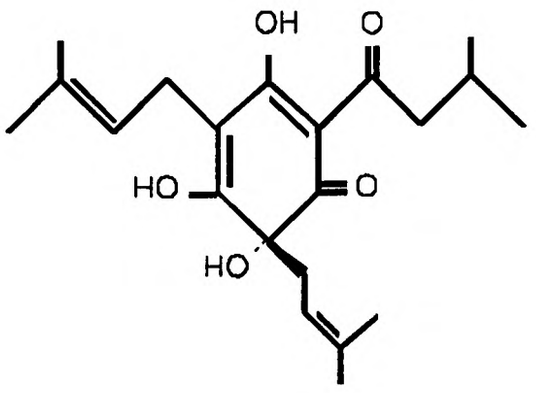
The portion of the ether extract of hops which is soluble in cold methanol, or the portion of the cold methanol extract which is soluble in ether, is termed the total resin fraction. This can be divided into the hard resin fraction and the total soft resin fraction on the basis of the solubility of the respective groups in low-boiling point paraffinic hydrocarbons (the hard resins being the insoluble portion). Of the soft resins, the most important are the alpha-acids (α-acids) which are characterised by their ability to form a lead salt which is insoluble in methanol. The beta-acids (β-acids) do not form a lead salt but may be crystallised from aqueous

methanol. The uncharacterised soft resin material chiefly comprises that which neither forms a lead salt nor crystallises from methanol.

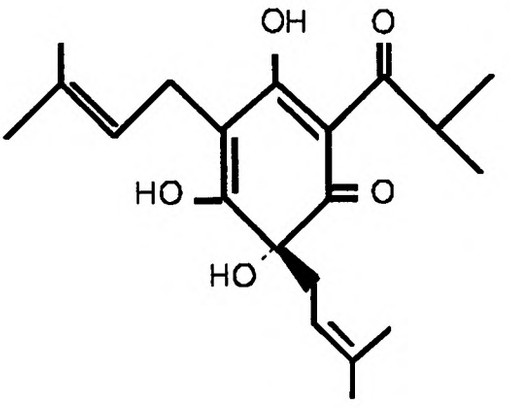
Many of the compounds encountered in hop chemistry have cyclic dione and trione nuclei in their structure which can potentially enolise to form several different tautomers. The compounds discussed below are represented in the accompanying figures in a single tautomeric form. The structure presented is not necessarily the only, or even the main tautomer present.

The* α*-acids

Humulone ($C_{21}H_{30}O_5$) is the major homologue of the α-acids and the first of such compounds to be characterised. It was isolated in 1904 by Lintner and Schnell as a bitter-tasting, laevorotatory, enolic acid, which reduced Fehling's and Tollem's reagents, but which did not produce carbonyl derivatives. Humulone is now known to form complexes with lead and with *o*-phenylenediamine (Hough *et al.* 1982). The chemical structures of humulone, and its congeners cohumulone and adhumulone are shown in Figure 1.1. Together these compounds make up about 95% of the α-acids fraction of fresh hops. It has been shown that, whereas the proportion of adhumulone is fairly constant (10-15%), that of humulone and cohumulone show wide variance (20-65%). The proportions are related to the variety of hop from which the compounds are isolated (Howard & Tatchell 1953a; Stevens 1967). The remaining compounds present in the α-acids fraction of fresh hops are homologues and analogues of humulone, cohumulone and adhumulone. The 1:1 complex formed between humulone and *o*-phenylenediamine, facilitates purification of humulone from other hop compounds since, after repeated crystallisation, humulone can be recovered from the complex by treatment with dilute hydrochloric



I



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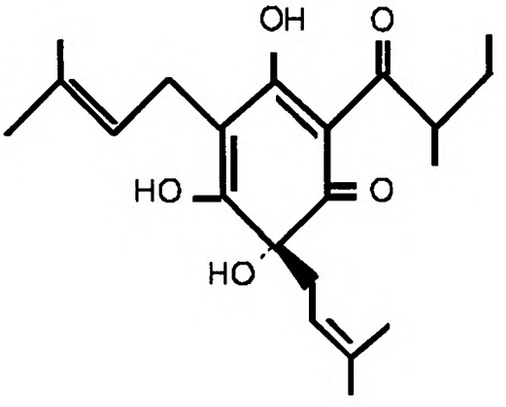


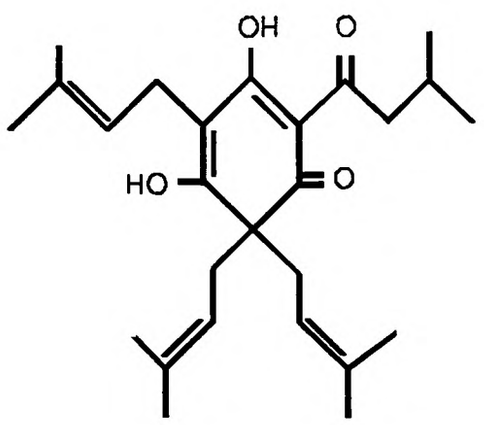
III

Figure 1.1 Structure of humulone (I), cohumulone (II) and adhumulone (III). In each case only one tautomeric form is shown. Other tautomers may be present in solution. The α-acids are optically active; (-)-humulone is the naturally-occurring form.

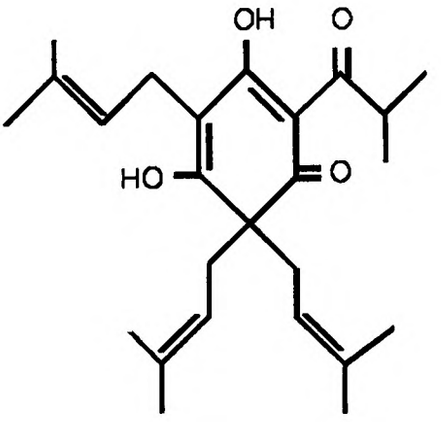
acid (Hough *et al.* 1982). Cohumulone is more soluble in non-polar organic solvents than is humulone, so recrystallisation of humulone from such solvents permits further purification (Howard & Tatchell 1953b). However, even 'pure' preparations of humulone, obtained after multiple recrystallisation, still contain a significant proportion of cohumulone (at best >1%, though more typically about 5%) (Verzele 1986). Although α-acids can be synthesised, the yields obtained are invariably low and it is therefore usually simpler to isolate the compounds from hops (Stevens 1967).

The* β-*acids

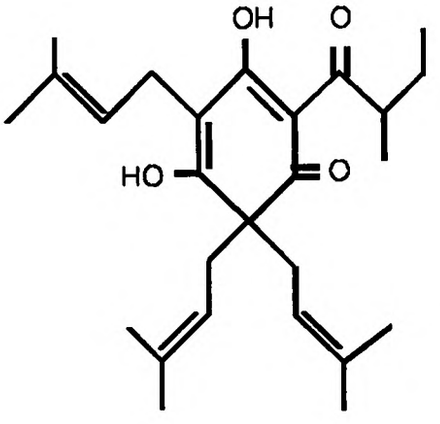
Unlike the α-acids, the β-acids do not form lead salts or complexes with *o*-phenylenediamine (Hough *et al.* 1982). Lupulone ($C_{26}H_{38}O_4$) was the first β-acid to be isolated from hops by Lermer in 1863 (Clarke *et al.* 1976). While lupulone can be crystallised from suitable extracts of continental European hops, British and American hops yield colupulone as the major β-acid. Early workers in the area of hop chemistry thought that they were dealing with lupulone (Walker 1949; Carson 1951) but it was subsequently shown that they were, in fact, dealing with colupulone (Howard *et al.* 1955; Riedl & Nickl 1956). Like the α-acid fraction, the β-acid fraction consists primarily of three compounds differing only in the structure of the side chain. The structures of these compounds, lupulone, colupulone and adlupulone are shown in Figure 1.2. Small amounts of β-acids (typically 5nM) survive the brewing process to remain in beer (McMurrough *et al.* 1988).



IV



V



VI

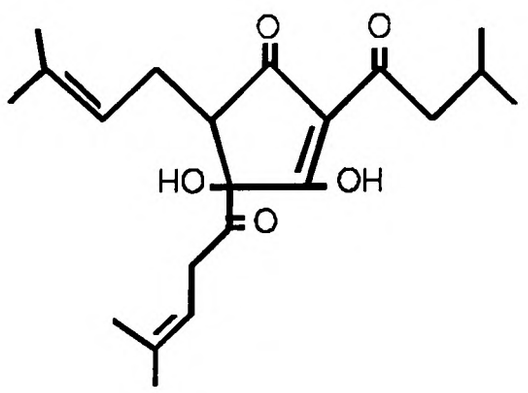
Figure 1.2 Structure of lupulone (IV), colupulone (V), and adlupulone (VI). In each case only one tautomeric form is shown. Other tautomers may be present in solution. Adlupulone is the only β-acid of the three which is optically-active.

The iso-alpha-acids

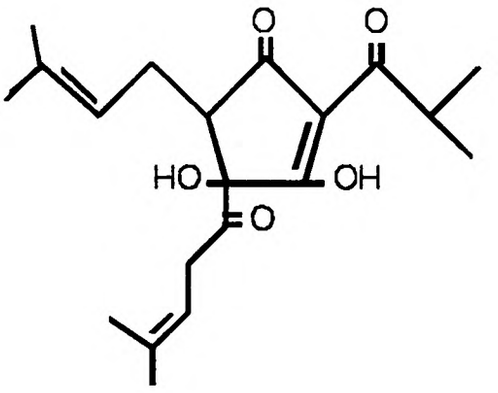
Although humulone and its congeners are among the most important compounds found in hops, only small quantities are found in finished beer due to their very low solubility in aqueous media. Typically <15μM α-acids are found in beer (McMurrough *et al.* 1988). The bitterness of beer is mainly due to a group of compounds derived from the α-acids and known as iso-alpha-acids (iso-α-acids). These are formed from α-acids during the wort boiling process. This group of compounds, originally termed resin A or isohumulone A, consists of two isomeric products for each of the α-acids. Thus, humulone yields *trans*-isohumulone and *cis*-isohumulone, cohumulone yields *trans*-isocohumulone and *cis*-isocohumulone, and adhumulone yields *trans*-isoadhumulone and *cis*-isoadhumulone. In this respect, the term *trans* refers to the orientation of the 3-hydroxy and 4-isopentenyl substituents. The structures of these compounds are shown in Figure 1.3. A system of nomenclature which has been used previously (Stevens 1967) described the compounds on the basis of the relative orientation of the larger groups on each of the 3 and 4 carbon atoms in the ring. This nomenclature describes photoisohumulone (*trans*-isohumulone) as *cis-isohumulone*; care must therefore be taken when surveying the literature to note which system has been used. Typically about 69μM isohumulone is found in beer (Verzele 1986) although values range from 22-165μM. *Trans*- and *cis*-isohumulone are equally bitter (Aitken *et al.* 1970).

The humulinic acids

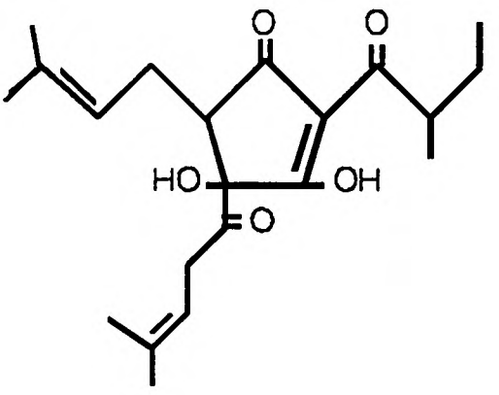
During conversion of α-acids to iso-α-acids under basic conditions, humulinic acids are formed in hydrolysis reactions. These compounds (*trans*-humulinic acid,



VII



VIII



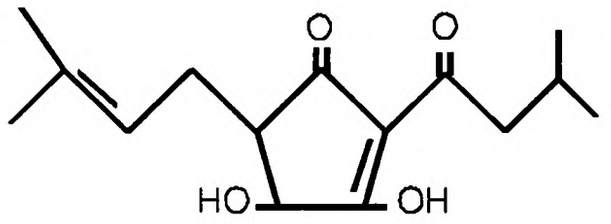
IX

Figure 1.3 Structure of isohumulone (VII), isocohumulone (VIII) and isoadhumulone (IX). Each compound occurs as both *trans*- and *cis*-isomers (the prefix refers to the orientation of the 3-hydroxy and 4-isopentenyl substituents). Only one tautomeric form is shown. Other tautomers may be present in solution.

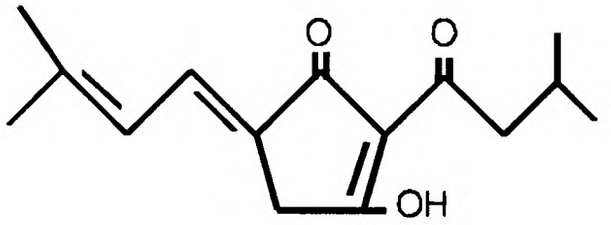
cis-humulinic acid, *trans*-cohumulinic acid *etc.*) are derived from the corresponding iso-α-acid but differ from them in that the isopentenyl side chain at C-4 is absent. Under certain conditions (*e.g.* in the laboratory, or in the production of hop extracts) humulinic acids can become dehydrated to form dehydrated humulinic acids. Since the dehydration reaction involves substitution on C-4, the *cis-/trans-* stereoisomerism is lost. Dehydrated humulinic acids have been shown to cause gushing, a phenomenon in which carbon dioxide is lost, with great force, from beer (Laws & McGuinness 1972; Outtrup 1980). Neither the humulinic acids nor their dehydrated counterparts impart bitter flavour. Their structures are shown in Figure 1.4.

The hard resins

The hard resins are soluble in cold methanol and ether, but insoluble in light petroleum. Ion-exchange chromatography has revealed the complex nature of this fraction, the major component of which is xanthohumol (Stevens 1967). Xanthohumol ($C_{21}H_{22}O_5$), a yellow crystalline compound, is the only naturally-occurring methylated hop resin (Stevens 1967). Analogues such as iso-xanthohumol are also present in hops (Ashurst 1967). Hard resins have little significance in the brewing process, since they are poorly soluble in aqueous media. However, Verzele (1986) has pointed out that xanthohumol has oestrogenic properties and could therefore have affected the menstrual cycle of female hop pickers. (Nowadays hops are mostly picked by machine.) Although xanthohumol is found in fresh hops in significant quantities (up to 1%), it disappears rapidly on storage through oxidation (Verzele 1986).



X



XI

Figure 1.4 Structure of humulinic acid (X) (which occurs as both *trans*- and *cis*-isomers) and dehydrated humulinic acid (XI). Only one tautomer is shown. Other tautomers may be present in solution.

The essential oils

Hops contain 0.5-2.5% of essential oil which is a complicated mixture comprising over 200 components. There are two main groups of essential oils in hops, the hydrocarbon fraction and the oxygenated fraction. The hydrocarbon fraction comprises mainly monoterpenes such as myrcene ($C_{10}H_{16}$) and sesquiterpenes such as humulene ($C_{15}H_{24}$). The oxygenated fraction is complex and includes alcohols, aldehydes, acids and terpene esters (Hough *et al.* 1982). The essential oils have a considerable influence on the aroma and flavour of beer and are responsible for the 'late hopped' flavour of some lager beers and the 'dry hopped' flavour of traditional ales.

1.1.5. Early studies of the antibacterial function of hop compounds and hop-derived compounds

The empirical view of the brewer prior to the application of science to brewing was that hops improved the keeping qualities of beer, and the antibacterial activity of compounds derived from hops has been the subject of scientific study for more than a Century (Thatcher 1887; Briant & Meacham 1893; Brown & Morris 1893; Brown & Clubb 1913; Kolbach 1925; Walker 1925; Shimwell 1937a; Rohne & Rische 1952; Kulka 1958). As a result of experiments carried out at the end of the nineteenth Century, Brown and Ward (1910) were able to state that the shelf life (or keeping quality) of beer was *'mainly controlled by the antiseptic properties of the hop'*.

At first it was not clear which compounds were responsible for the preservative effect. If antibacterial activity resided in the tannin fraction, then it would have been advantageous to use the same batch of hops repeatedly in order to obtain maximum

extraction of these compounds (Briant & Meacham 1893). If, instead, the antibacterial effects were due to resins, then re-use of hop material would be futile, since the resin-like material was extracted from hops early on in the boiling process. In 1885, Hayduck showed that antibacterial activity was associated with the resin fraction of hops and that a differentiation could be made between soft resins, which had a preservative action, and hard resins, which comprised oxidation and polymerisation products of the soft resin fraction. Within a short time, biological techniques for the estimation of hop 'preservative activity' had developed to such an extent that they were used to provide an indication of the suitability of hops for use in brewing. The wide range of techniques developed for this purpose in the period 1887-1941 has been critically reviewed by Hudson (1960) and MacRae (1964). In brief, methods for estimating 'preservative value' relied upon separation of the biologically-active compounds from the hops in a form approximating to that in which they occurred in beer and then assessing their activity by evaluating their effect on the microorganisms responsible for beer spoilage.

The first method used for this purpose consisted of an evaluation of the effect of an aqueous extract of hops on a bacterial test strain (Brown & Ward 1910). Various amounts of this extract were mixed with an extract of malt, inoculated with a bacterial culture, and incubated at 30°C for 18h. The volume of hop extract required to prevent visible growth of the bacteria was taken as a measure of the preservative activity of the hop. A variety of improvements to this method were later proposed (see Hudson 1960). In addition, a substantial amount of work was carried out (mostly under the auspices of the Institute of Brewing Research scheme) in which the relative antibacterial activity of each of the resin

fractions from hops and a range of compounds derived from such resins was examined. In many cases the results of such studies were controversial; conflicting results were obtained by different groups of workers.

The arguments concerning the relative preservative value of the antibacterial constituents of hops and the compounds derived from them, far from being resolved, were left to gather dust when relatively satisfactory methods for the analysis and quantification of hop compounds and hop-derived compounds in hops and in beer became available in the 1950's. In addition, a number of studies carried out since that time claimed to show that, in the past, the antibacterial activity of hop compounds and hop-derived compounds had been seriously overrated (*e.g.* Richards & MacRae 1964; Garrick *et al.* 1968; Jährig & Schade 1981). It will be shown later in this thesis that although the experimental data on which such conclusions were based were sound, the interpretation of the data was flawed. *Compounds derived from hops have a very significant influence on the bacteriological stability of beer.*

1.2. ANTIBACTERIAL AGENTS

1.2.1. Potential sites and mechanisms of action of antibacterial agents

Some antibacterial agents display a degree of selectivity with respect to the organisms which they inhibit while others do not. For example, mercury ions inhibit growth of bacteria but are also poisonous to most other life forms. In contrast, penicillin has the ability to inhibit growth of bacteria while having no toxic effect on other forms of life. This selective effect has led to

the chemotherapeutic application of antibacterial substances and the control of many bacterial diseases.

The study of the selective action of an antibacterial agent is important from several points of view. For example, knowledge of the spectrum of organisms to which the inhibitory effects extend can suggest practical applications for the substances. Thus, the dawning of the *antibiotic era* coincided with the finding that antibacterial agents produced by certain fungi and streptomycetes had no deleterious effect on animal (and in particular human) physiology even when the antibiotic substances were administered intravenously. Similarly, the established use of several compounds as food preservatives, including benzoic acid, sorbic acid, acetic acid, and in the case of beer, hop compounds and hop-derived compounds, suggests that they have relatively little, if any, effect on human physiology.

Antibacterial agents can exert their effects on sensitive organisms by one, or more, of a number of mechanisms (for reviews see Hugo 1967; Hugo 1971; Gale *et al*. 1981; Russell & Chopra 1990). Potential sites of action (summarised in Table 1.1) include inhibition of the synthesis of cell wall material, cell membrane material, protein, DNA or RNA; inhibition of cellular metabolism (either by an inhibitory action specific to one or more enzymes or by gross inhibitory effects on biochemical reactions); inhibition of nutrient uptake; or impairment of the function of the plasma membrane. Indeed it is this selective action on cell processes which results in some antibacterial agents displaying a bactericidal action (in which the target organism is killed) while others display a bacteriostatic action (in which growth of the target organism is merely inhibited).

Antibacterial agents which inhibit a cellular process unique to the target organism provide the most

Table 1.1: Potential sites of action of antibacterial agents

Site	Process affected	Example
Cell wall	synthesis	penicillin
plasma membrane	integrity	polymyxin
	permeability	2,4-dinitrophenol
DNA	DNA replication	nalidixic acid
mRNA	RNA translation	rifampicin
ribosomes	protein synthesis	chloramphenicol
cytoplasm	specific enzyme reactions	sulphamethoxazole

efficient form of selectivity. For example, antibiotics such as penicillin, D-cycloserine, fosfomycin and vancomycin have in common an ability to inhibit synthesis of the peptidoglycan component of bacterial cell walls. Since this polymer is unique to bacteria, these antibiotics are almost without effect on eukaryotic cells. Selectivity is seldom absolute: vancomycin also has an ability to affect the function of both bacterial and mammalian plasma membranes and can therefore cause kidney damage as a result of therapeutic use.

One of the aims of the work described in this thesis was to identify the cellular site at which growth of beer-spoilage lactic acid bacteria is affected by hop compounds and hop-derived compounds. A second aim was to describe the way in which the action of the compounds on this sensitive site leads to inhibition of cell growth.

1.2.2. The chemiosmotic theory and the action of ionophores

The concept of the proton-motive force stems from the work of Mitchell on the chemiosmotic theory (Mitchell 1961a; Mitchell 1966). This theory provides a framework within which the energy-transducing mechanisms operative in cellular units as diverse as bacteria, mitochondria and chloroplasts can be explained. In the context of fermentative bacteria, such as lactobacilli, it seeks to explain how energy generated by catabolic reactions occurring in the cell cytoplasm can be utilised to facilitate uptake of nutrients from the extracellular medium.

The origins of the theory lie in the work of Mitchell and Moyle on vectorial group translocation (Mitchell & Moyle 1958). These workers attempted to explain how apparently spacially-directionless chemical

reactions could result in directional (vectorial) transport of solutes across the plasma membrane (Nicholls 1982). Mitchell proposed that the structure of proteins involved in the transport of solutes was such that substrates and products had to approach and leave the catalytic sites along defined pathways. Mitchell called this mechanism *vectorial group translocation*. One of the major tenets of the chemiosmotic theory is that energy generation is coupled to nutrient transport via an electrochemical gradient of protons. In order for the cell to be capable of establishing such an electrochemical gradient, several criteria must be met. First of all, the cell must possess a plasma membrane which has a very low conductivity with respect to protons and other ions. This is a general feature associated with lipid bilayer membranes. Secondly, the cell must possess a mechanism by which an electrochemical gradient of protons can be established. In lactic acid bacteria this role is mainly played by the proton-translocating ATPase, but in aerobic bacteria, such as *Bacillus subtilis*, the respiratory chain, which catalyses a series of oxidation-reduction reactions, is used to establish the gradient. Lactic acid is the major metabolic end-product of all lactic acid bacteria and a contribution to proton translocation is also made by the lactate/proton symport which utilises the energy stored in the transmembrane lactate gradient to translocate protons across the plasma membrane. The third criterion which must be satisfied is that the cell must possess a series of membrane-spanning enzymes which can utilise the electrochemical gradient of protons generated by this system to perform *vectorial translocation* of solutes.

Although the functions of proton-translocating enzymes, such as the proton-translocating ATPase, is to establish a proton gradient and to maintain an

appropriate intracellular pH, it should be realised that the activity of such enzymes is, in fact, constrained by the generation of a proton-motive force, especially the membrane potential component (Mitchell 1973). It can be calculated that for a spherical microorganism of 1μm diameter with an internal volume of 5 x 10^{-10}μl and a capacitance of 3 x 10^{-14}F approximately 40,000 protons must be expelled from the cell to generate a membrane potential of 200mV. If it is assumed that the cell has a buffering capacity of 50nmol of H^+ per pH unit per mg (dry weight), extrusion of this quantity of protons would raise the internal pH value by only 0.001 pH units (Booth 1985). Clearly, extrusion of a much larger number of protons is required to generate a pH gradient than that required to generate a membrane potential.

How then is a pH gradient established across the cell membrane of living microorganisms? The bulk of the evidence favours the hypothesis that a pH gradient is established as a result of the build-up of a compensating membrane potential. In bacteria, net potassium uptake is the predominant means by which this is achieved. Since potassium is the major cellular cation, uptake of this ion may be the major means by which the cell maximises its transmembrane pH gradient (Booth 1985).

While the chemiosmotic theory is now well accepted (Mitchell received a Nobel prize in 1978 for formulating the theory), several details still provoke considerable debate among those involved in the study of bio-energetics. Most of the debate concerns whether the energy available for transport depends on the *bulk-phase* activity of ions or whether localised differences in ion activity are responsible for driving nutrient transport. Much of the experimental evidence for the chemiosmotic theory, and in particular with regard to the argument over bulk-phase or localised proton gradients, has been

obtained from experiments on the response of cell suspensions and isolated membrane preparations to a group of compounds known as *ionophores*.

In general, ionophores have the ability to delocalise the charge on ions (such as H^+, Na^+, K^+ or Ca^{2+}) by forming a complex with one, or more, ions. In this way, the high activation energy required to insert such ions into the hydrophobic region of a lipid bilayer membrane can be overcome. As a result, in the presence of ionophores, the permeability of natural membranes to certain ions is increased (Harold *et al.* 1974).

Ionophores (sometimes referred to as complexones) are typically compounds of M_r 200-2000 which possess a hydrophobic exterior (to enhance their lipid solubility) and a hydrophilic interior (to permit the ion to bind) (Ovchinnikov 1974; Pressman 1976). There are two general mechanisms by which ionophores facilitate transfer of ions across lipid membranes. They may act as mobile carriers, diffusing within the cell membrane. In this way, they can catalyse the transfer of about 10^3 ions per second across the membrane. These mobile carriers are true ionophores as defined by Pressman (1976). Alternatively they may act as pore-forming ionophores, catalysing the transfer of up to 10^7 ions per second. This latter group, although possessing similar characteristics to true ionophores, are so different that they have been termed *quasi*-ionophores (Pressman 1976).

The two groups of ionophores have certain distinctive features. Mobile carriers are characterised by an ability to discriminate between certain ions (often to a very high degree). They can function across very thick membranes and are also affected by the fluidity of the membrane (thus imposing a temperature dependence on their activity). Channel-forming ionophores generally show poor discrimination between ions and, as described

above, are capable of catalysing very high rates of ion conductance across biological membranes (Pressman 1976).

In general, ionophores have no therapeutic value since they are toxic both to microorganisms and to higher forms of life, although some ionophores (monensin, lasalocid) are used as cattle-feed additives to reduce bacterial growth in the forestomach (Chow & Russell 1990). Ionophores, however, are widely employed in research laboratories where they are used to manipulate cellular ion gradients. Their use for this purpose has been reviewed by Denyer & Hugo (1983).

A wide range of ionophores have been characterised including valinomycin, monensin, nigericin and A23187 (Harold & Baarda 1967; Harold & Baarda 1968b; Reed & Lardy 1972). All of these ionophores are produced by microorganisms. It is of note that ionophoric antibiotics produced by plants have not been previously described. As will be shown in Chapter 4, the work described in this thesis constitutes the first documented report of a plant-derived substance which possesses ionophoric activity.

CHAPTER 2

Molecular structure and ionisation behaviour of hop compounds and hop-derived compounds

In den Einzelheiten

steckt der Teufel.

(the devil lurks in the details)

(German proverb)

2.1. INTRODUCTION

Textbooks of brewing science (*e.g.* Pollock 1979; Hough *et al.* 1982) indicate that the molecular structure of the main hop compounds and hop-derived compounds is well known. Furthermore, an understanding of their behaviour, with respect to ionisation and solubility, is tacitly inferred. Although much of the nuclear magnetic resonance (NMR) data which provided information concerning the molecular structure of such compounds was obtained using continuous-wave instruments at low magnetic field strength, the results of these studies were both valuable and incisive. Nevertheless, room for improvement, or at least confirmation, existed with the application of modern multi-pulse NMR techniques to the study of such compounds.

Of no little importance are the fallacious deductions made by those reviewing the chemistry of hop compounds and their derivatives with regard to their ionisation behaviour and the physicochemical behaviour resulting from ionisation. Much has been made of little data.

The aims of the work reported in this Chapter were; (i) to prepare a range of hop compounds and hop-derived compounds for use in biological experiments reported in subsequent chapters; (ii) to examine the molecular structure and purity of the materials using a range of spectroscopic techniques; and (iii) to make a detailed examination of the ionisation behaviour of the compounds and explore the effect of ionisation on their physicochemical behaviour.

2.2. EXPERIMENTAL

2.2.1. Materials

Chemicals were obtained from Sigma (UK) or BDH (UK) unless otherwise indicated and were at least of analytical reagent grade. Solvents used for spectroscopic measurements were AristaR grade. Deuterated solvents were obtained from Aldrich (UK). Liquid CO_2 hop extracts were obtained from Pauls Hop Products (UK).

2.2.2. Analysis methods

Melting point

Samples for analysis were transferred to glass capillary tubes (1mm int. ϕ) in a silicone oil heating bath. The temperature was slowly raised and the melting point (mp) recorded using a mercury bulb thermometer. Values were not corrected for the emergent stem of the thermometer.

Ultra-violet spectroscopy

Ultra-violet (UV) absorption spectra were determined in quartz cells of 1cm path length using a Philips PU 8720 UV/vis spectrophotometer (Philips, UK). Samples were dissolved in an appropriate solvent as indicated in each experimental section at 25°C then analysed at a scan rate of 50-200nm/min against a solvent blank.

Infra-red spectrometry

Infra-red (IR) spectra were determined using a Perkin Elmer IR spectrometer (Perkin Elmer, UK) in NaCl cells using CCl_4 as solvent against a CCl_4 blank. Sample concentration was 0.125-0.15M. Abbreviations used in presentation of IR data: s=strong; w=weak; b=broad.

Mass spectrometry

Mass spectra were determined using a VG-Trio 2 mass spectrometer (VG Ltd., UK) equipped with a Waters model 590 pump (Waters Associates, USA). The solvent employed for delivery of the sample was MeOH, buffered with ammonium acetate (0.13M) and acetic acid (0.167M). Samples were dissolved in MeOH and a portion (10µl) of each applied using a loop injector (flow rate 1ml/min). A thermospray ionisation mode (ionisation potential 70eV), probe temperature of 245°C and acceleration voltage of 500V was employed. These conditions were optimal with respect to maximising detection of the molecular ion of hop compounds and hop-derived compounds.

Optical rotation

Optical activity at 20°C was determined using a Stanley photoelectric polarimeter. Samples were dissolved in MeOH and the optical rotation of the sample, at the D line of sodium, calculated after correction for the optical rotation of the solvent.

Measurement of pH

pH values were determined by potentiometry using a Corning Delta 220 general purpose pH meter or Delta 140 pH meter (Ciba Corning Ltd., UK). Both meters were equipped with a calomel reference electrode (Ciba Corning Cat. no. 476350), pH electrode (Ciba Corning Cat. no. 476280) and temperature compensation and calibrated using buffer solutions (BDH, UK) of pH 4.00 (potassium hydrogen phthalate, 0.05M: a primary standard buffer) and pH 7.00 (phosphate buffer). In addition, a secondary standard buffer solution (pH 1.18) containing sulphamic acid (1% w/w: Albert & Serjeant 1984) was used to assure accurate calibration of the pH meter at acidic pH values.

Conductivity measurements

Conductivity was measured at 25°C using a Conductometer E 518 conductivity meter (Metrohm, Switzerland) equipped with a platinum electrode and calibrated with KCl.

Analytical high performance liquid chromatography (HPLC)

The identity and purity of (-)-humulone and (-)-cohumulone was assessed using a NovapaK 4μm cartridge column (100 x 8mm, Waters Associates, USA) used with a radial compression module and a mobile phase which consisted of (% w/v) MeOH (85), water (15) and H_3PO_4 (0.025) delivered through a Waters 6000A pump (Waters Associates, USA) at a flow rate of 2ml/min at room temperature (Buckee & Baker 1987). The compounds were identified using a UV detector (λ=313nm) and quantified using an electronic integrator (TriVector Trio computing integrator, TriVector, UK) calibrated by means of an external α-acids standard.

The identity and purity of *trans*-isohumulone and *trans*-isocohumulone was assessed in a similar way except that the mobile phase consisted of (% w/v) MeOH (70), water (30) and PicA reagent (tetrabutylammonium phosphate, 0.17) and detection was at λ=280nm. Calibration was performed by means of an external *trans*-isohumulone standard.

NMR spectroscopy

^{1}H-NMR (270MHz), ^{13}C-NMR (67.5MHz) and two-dimensional (2-D) NMR spectra were obtained using a JEOL JNM-GX270 FT NMR spectrometer. Each sample (25-50mg) was dissolved in $CDCl_3$ (0.7ml) containing tetramethylsilane (TMS, *ca* 1g/l) as internal standard. Chemical shifts are reported as parts per million (ppm) downfield from TMS. Spectra were recorded under the following conditions. ^{1}H-NMR - Pulse duration (3.2μsec); Pulse delay (2.27sec); Acquisition

time (2.73sec); Number of data points (zero-filled) 65536. ^{13}C-NMR - Pulse duration (3.7μsec); Pulse delay (1.0sec); Acquisition time (0.901sec); Number of data points (zero-filled) 65536. For discrimination of CH_3, CH_2, CH and C-resonances Distortionless Enhancement by Polarisation Transfer (DEPT) experiments were employed. DEPT 90° spectra were recorded at 67.5MHz (in $CDCl_3$ solution) using a 29.6μsec pulse, 2.0sec pulse delay and 0.901sec acquisition time. DEPT 135° spectra were recorded using a 40.2μsec pulse. Coupling between carbon atoms and their attached protons was studied using heteronuclear Correlated Spectroscopy (^{1}H-^{13}C COSY). Coupling between spin-coupled protons was studied using homonuclear Correlated Spectroscopy (^{1}H-^{1}H COSY). Stereochemical aspects were investigated using Nuclear Overhauser Effect difference Spectroscopy (NOESY). The identity of the carbonyl and enolic NMR resonance lines of *trans*-isohumulone and *trans*-humulinic acid was deduced using ^{1}H-coupled ^{13}C NMR (optimised for detection of long-range ^{13}C-^{1}H couplings). The way in which these methods were used to assign the NMR spectra of each of the compounds is summarised in Figure 2.1.

Measurement of surface tension

The surface tension of (-)-humulone and *trans*-isohumulone solutions was measured (at 21°C) in 0.1M NaDMG buffer at pH values of 4 and 7 using a DuNoüy tensiometer (Cambridge Instrument Co., UK). Aqueous, rather than alcoholic, stock solutions of the compounds were used to avoid any contribution of the solvent to depression of the surface tension. Since the solubility of both (-)-humulone and *trans*-isohumulone at pH 4 was very low, stock solutions for experiments at this pH were prepared in pH 7 buffer. The pH of the experimental buffer system was not affected by this procedure. In a control

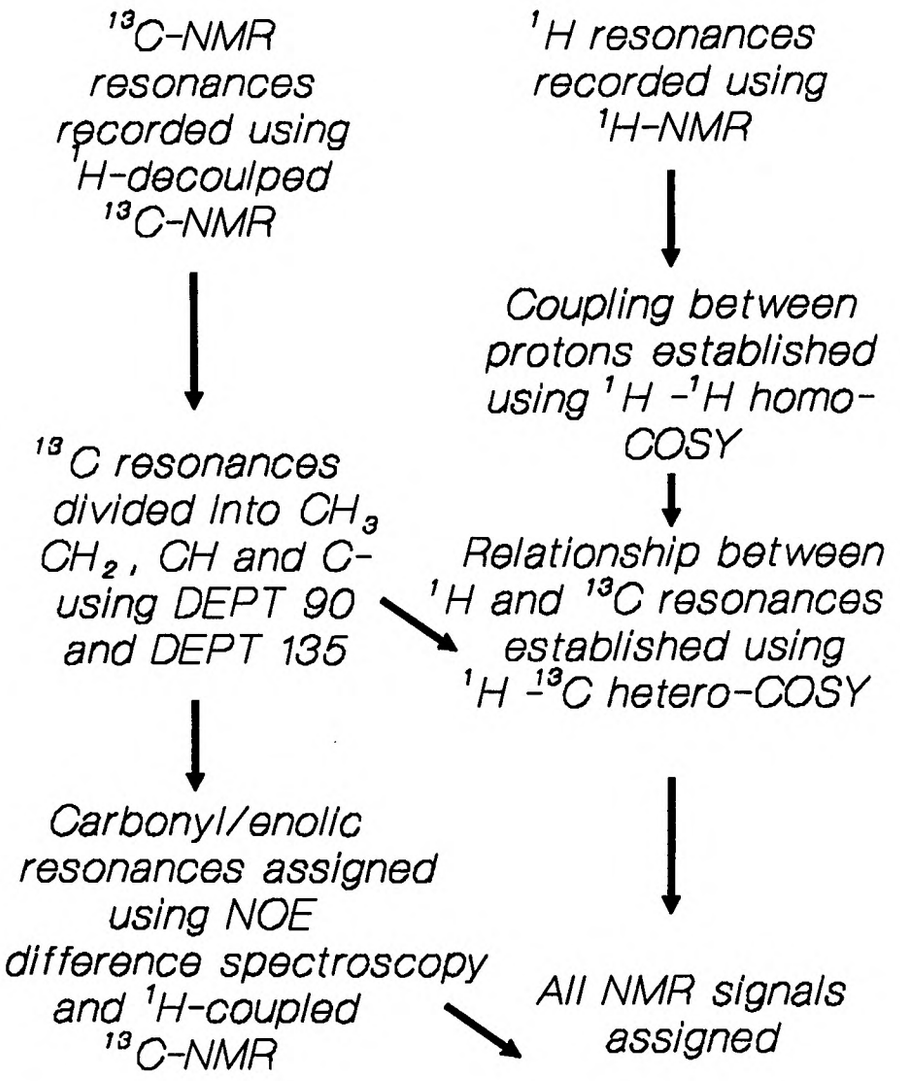


Figure 2.1 Flow chart showing techniques used for assigning the NMR signals of hop compounds and hop-derived compounds.

experiment, the surface tension of the quaternary ammonium compound benzethonium chloride was measured in NaDMG buffer at pH 7.

2.2.3. Preparation of test compounds

Preparation of colupulone

Colupulone was isolated from a liquid CO_2 hop extract enriched in β-acids by recrystallising the substance from two grades of light petroleum (bp 80-100°C then bp 60-80°C). After several crystallisations white crystals were obtained; mp 92°C (lit.94°C [Drewett & Laws 1970]); $[\alpha]_D^{20}$=0° (MeOH; *c* 0.75%); λ_{max} (acidic MeOH) 203nm (ε=23260), 227nm (ε=11160), 279nm (ε=6060), 334nm (ε=8000); λ_{max} (alkaline MeOH) 223nm (ε=20680), 356nm (ε=16700); $\upsilon_{max}^{CCl_4}$ (/cm) 3560(w), 3350 (b,w), 2980 (s), 2940 (s), 2920 (s), 2880 (s), 2750 (w), 1710 (w), 1660 (s), 1650 (s), 1525 (b), 1450 (s,b), 1380 (s), 1235, 1190, 1100 (b), 980 (b), 850; MS (Thermospray), m/z (%), $[M]^+$ (100), [M] + 18 (n.d.), [M] (n.d.), 423 (14.4), 402 (34.6), 387 (6.6), 275 (7.0), 243 (10.2), 242 (51.1).

Preparation of (-)-humulone and (-)-cohumulone

(-)-Humulone was prepared from a liquid CO_2 α-acid extract by complexation with *o*-phenylenediamine (OPD) (McGuinness 1974). Free (-)-humulone was regenerated from the complex by addition of concentrated HCl then recrystallised several times from cyclohexane (at -20°C) to give pale yellow crystals; mp 62°C (lit. 64-65°C [Clarke & Hildebrand 1965]); $[\alpha]_D^{20}$=-209° (MeOH; *c* 1%) (lit. -211° [Alderweireldt *et al.* 1965]) ; λ_{max} (acidic MeOH) 204nm (ε=16280), 236nm (ε=11160), 286nm (ε=7420), 322nm (ε=7220), 350nm (ε=5800); λ_{max} (alkaline MeOH) 226nm (ε=16900), 327nm (ε=12880), 360nm (ε=10700); $\upsilon_{max}^{CCl_4}$ (/cm) 3500, 3400 (s,b), 2980 (s), 2940 (s), 2920 (s), 2880, 2750 (w), 1675 (s), 1620, 1540 (b), 1475 (b), 1360, 1350,

1305, 1260, 1240, 1190, 1140, 1110, 1080, 1060, 1040, 1000, 960, 920, 850. MS (Thermospray), m/z (%), $[M]^+$ (37.8), [M] + 18 (5.3), [M] (n.d) 453 (3.0), 385 (4.9), 364 (9.2), 345 (4.9), 307 (3.0), 295 (4.5), 243 (16.5) 242 (100), 223 (5.7).

(-)-Humulone and (-)-cohumulone were also isolated from a liquid CO_2 hop extract using high performance liquid chromatography. The compounds were separated from a liquid CO_2 extract of high α-acid content using an APEX ODS 10μm column (250 x 10mm, Jones Chromatography, UK) with a mobile phase which consisted of (% v/v) acetonitrile (57.8): water (42) and formic acid (0.17) delivered through a Waters 6000A pump (Waters Associates, USA) at ambient temperature. Fractions containing either (-)-humulone or (-)-cohumulone (detected at λ=313nm) were collected then re-chromatographed until pure. (-)-Humulone prepared via the OPD complex contained *ca* 8% cohumulone and 1% adhumulone, while that prepared by HPLC was essentially free of contaminants (as assessed by HPLC and ^{1}H-NMR). (-)-Humulone prepared by HPLC was reserved for structural analysis while that prepared with OPD was used for preparation of *trans*-isohumulone by photoisomerisation (see below). (-)-Cohumulone, which does not form crystals, was obtained as a yellow oil after evaporation of the solvent under reduced pressure (30°C, 50mm Hg).

Preparation of* trans-*isohumulone and* trans-*isocohumulone

Trans-isohumulone was prepared by photoisomerisation of (-)-humulone as described by Clarke & Hildebrand (1965). Recrystallisation from 2,2,4-trimethylpentane at -20°C gave white crystals: mp 63°C (lit. 62-63°C [Clarke & Hildebrand 1965]); $[\alpha]_D^{20}$=-7.4° (MeOH; *c* 0.5%) (lit. =-7.6° [Clarke & Hildebrand 1965]); $\upsilon_{max}^{CCl_4}$ (/cm) 3520 (w,b), 3450 (w,b), 2980 (s), 2940, 2920, 2880, 1700 (s), 1615 (s),

1590 (s,b), 1465, 1445, 1380, 1280, 1235, 1150, 1110, 965, 915, 840 (w). MS (Thermospray, m/z (%), $[M]^+$ (100), [M] + 18 (12.5), [M] (10.1), 385 (20.7), 364 (18.2), 345 (32.1), 249 (19.7).

Trans-isohumulone and *trans*-isocohumulone were also isolated from a photoisomerised liquid CO_2 hop extract. The compounds were separated by HPLC using the conditions described for separation of α-acids and detected by their absorption at λ=280nm. Fractions containing each compound were collected in glass tubes and frozen immediately in a mixture of solid CO_2 and acetone. Several fractions of each compound were thawed, pooled, extracted with methylene chloride then dried over sodium sulphate. The compounds were freed of solvent by rotary evaporation (30°C; 50mm Hg) and by flushing with N_2 gas, then recrystallised from 2,2,4-trimethylpentane at -20°C. This procedure gave *trans*-isohumulone which was identical to that described above. *Trans*-isocohumulone was obtained as a yellow oil after removal of solvent under reduced pressure (30°C, 50mm Hg).

Preparation of* trans-*humulinic acid

Trans-humulinic acid was prepared by alkaline hydrolysis of (-)-humulone (Ashurst 1966) and recrystallised several times (at 4°C) from light petroleum (bp 60-80°C) to yield white crystals; mp 92.5°C (lit. 94°C [Dierckens & Verzele 1969]); $[\alpha]_D^{20}=0°$ (MeOH; *c* 1%); λ_{max} (acidic MeOH) 204nm (ε=16280), λ_{max} (alkaline MeOH) 226nm (ε=11243), 267nm (shoulder) (ε=16957); $\upsilon_{max}^{CCl_4}$ (/cm) 3560(w,b), 3440(w,b), 2980(s), 2930, 2870, 1790(s), 1630(s), 1580(s), 1460, 1440, 1385, 1370, 1360, 1320, 1280, 1220, 1160, 1100, 1030(w), 980(w), 960, 955, 850(w), 840(w).

Preparation of dehydrated humulinic acid

Dehydrated humulinic acid was prepared by the method of Outtrup (1980) to give yellow-orange crystals: mp 85°C (lit. 87°C [Outtrup 1980]); λ_{max} (acidic MeOH) 259nm (ε=6900), 355nm (ε=34000); λ_{max} (alkaline MeOH) 274nm (ε=18000), 324nm (ε=28500); $\upsilon_{max}^{CCl_4}$ (/cm) 3390(w), 2980, 2925, 2865, 1700(s), 1635, 1610(s), 1560(s), 1440(s), 1400, 1380, 1365, 1275, 1225, 1210, 1165, 1130(w), 1115(w), 1080, 990, 950(w), 915, 855, 655(w), 640, 625(w). MS (Thermospray), m/z (%), $[M]^+$ (100), [M] + 18 (n.d.), [M] (12.8), 272 (3.1), 271 (18.3), 250 (19.1), 242 (7.4).

2.2.4. Methods used for study of the ionisation of hop compounds and hop-derived compounds

Potentiometric determination of pKa values

The pKa values of colupulone, (-)-humulone, *trans*-isohumulone, *trans*-humulinic acid and dehydrated humulinic acid were measured by potentiometry (Albert & Serjeant 1984). In a control experiment, the pKa value of benzoic acid was determined under the same conditions.

Preparation and standardisation of titrants:- Carbonate-free NaOH solution (0.1M) was prepared from a commercially-available volumetric solution (BDH, UK), diluted using freshly deionised water and calibrated by titration against potassium hydrogen phthalate using phenolphthalein as indicator. (Potassium hydrogen phthalate was dried to constant weight at 120°C before use.) Standard solutions of HCl were prepared from a commercially-available volumetric solution (BDH, UK), diluted using freshly deionised water and calibrated by titration against a standard NaOH solution using phenolphthalein as indicator.

Titration procedure:- Each test compound was weighed, dissolved in MeOH (1ml), made up to 100ml with freshly deionised water then stirred using a magnetic stirrer until dissolved (up to 3h was required in some cases). Oxygen-free nitrogen gas was bubbled through the solution, which was protected from light, to ensure that the compound did not undergo change. All solutions were maintained at 25°C. The pH was recorded, then a portion of titrant (NaOH) added. Brief magnetic stirring after each addition ensured that titrant and sample were well mixed. The sample pH was recorded once a constant value had been reached. In some cases up to 15min was required for the pH to reach equilibrium. This procedure was repeated for each of a further nine incremental additions of titrant. In most cases the sample was back-titrated with HCl then titrated once again with NaOH, recording the results in each case.

Calculation of results:-Calculations of pKa were performed as described by Albert & Serjeant (1984): activity corrections were applied on account of the low concentration and relatively low pKa values of the compounds tested.

Spectrophotometric determination of pKa

The spectrophotometric method for measurement of pKa depends on determination of the ratio of undissociated to ionised molecules in a series of non-absorbing buffer solutions of known pH. The spectrum of the undissociated species in a buffer solution of appropriate pH value is first obtained then compared to that of the pure ionised species isolated at another pH value. A wavelength is chosen at which differences between the absorbances of the two species are maximal. Under conditions in which both species obey Beer's law, the absorbance (A) at the

analytical wavelength is the sum of the absorbances of the ionised (A_I) and undissociated (A_U) species. Thus,

$$A = A_I + A_U \qquad (2.1)$$

pKa can therefore be determined by measuring the absorbance of solutions in which an equilibrium mixture of ionised and undissociated species is present and substituting the values into the following equation (Albert & Serjeant 1984),

$$pKa = pH + \log\frac{A_I - A}{A - A_U} \qquad (2.2)$$

The pKa values of *trans*-isohumulone, (-)-humulone and colupulone were determined in aqueous buffer at 25°C. Three buffers were used: (i) 0.01M 3,3′-dimethylglutaric acid, (ii) 0.01M chloroacetic acid, or (iii) 0.01M each of chloroacetic acid, formic acid, succinic acid, citric acid, acetic acid, phosphoric acid, tris, borax and sodium hydrogen carbonate. None of the buffer components significantly absorbed light in the region of the spectrum examined. Buffers i and ii were used to determine the ionisation constant of *trans*-isohumulone. Buffer iii was used to determine the ionisation constants of (-)-humulone (both pKa_1 and pKa_2) and colupulone. Typically a set of seven determinations was made for each pKa analysis. Control experiments established that Beer's law was obeyed for each of the species examined. In addition, pKa values for *trans*-isohumulone were determined in solutions containing 5, 50 and 90% (v/v) MeOH.

Determination of the solubility of hop compounds and hop-derived compounds at different pH values

In 1918, the solubility of humulone and lupulone was determined by Wöllmer who titrated solutions of phosphate

buffer with alcoholic solutions of the hop substances until turbidity was observed (Wöllmer 1918). After abortive attempts to apply more sophisticated methods, this same turbidimetric method was adopted for assessment of the solubility of colupulone, (-)-humulone and *trans*-isohumulone at 25°C. Stock solutions of each compound (0.1M final concentration) were prepared in MeOH. The buffer used for the experiments contained 0.1M of each of the following: chloroacetic acid, formic acid, succinic acid, citric acid, acetic acid, phosphoric acid, tris, borax and sodium hydrogen carbonate. Ionic strength was adjusted by addition of 0.1M NaCl, then the pH adjusted using solid NaOH or concentrated HCl. Portions of each stock solution were added in increments to 50ml buffer in a 100ml Erlenmeyer flask. The volume required to attain a standard level of turbidity in the test solution (assessed by two independent operators) was noted and converted to concentration units to give an apparent solubility value. Although such values are higher than the true solubility values (since they represent a standard state of supersaturation) the method permits the relative solubility of the compounds at a range of pH values to be simply estimated. Determinations were made at pH values ranging from pH -1 to pH 8.47.

Determination of partition coefficients of hop compounds and hop-derived compounds at different pH values

The partition of colupulone, (-)-humulone, *trans*-isohumulone and dehydrated humulinic acid between a buffered aqueous phase and 2,2,4-trimethylpentane was determined. The aqueous phase consisted of 0.01M each of chloroacetic acid, formic acid, succinic acid, citric acid and acetic acid adjusted within the pH range 2.2-6.7 using solid NaOH. When it was required to determine partition coefficients at pH values <2.2, 0.01M

chloroacetic acid, acidified with concentrated HCl, was used. The test compound (200μl, 2.5mM-10mM in MeOH) was added to buffer (25ml) in a 100ml glass-stoppered Erlenmeyer flask. 2,2,4-Trimethylpentane (25ml) was added and the mixture shaken for 10min at *ca* 5Hz on a wrist-action shaker. For each measurement, a control sample to which MeOH, instead of the test compound, had been added provided a reference blank. A positive control sample was prepared by adding the test compound to 2,2,4-trimethylpentane. The concentration of the test compound in the 2,2,4-trimethylpentane phase was estimated from the absorbance of each sample at λ=280nm (*trans*-isohumulone, (-)-humulone) or λ=340nm (colupulone, dehydrated humulinic acid) in cells of 1cm path length against the appropriate solvent blank. Partition coefficients (P) were estimated using the expression

$$P = \frac{a}{b-a} \qquad (2.3)$$

where *a*=concentration of test compound in 2,2,4-trimethylpentane layer after extraction and *b*=concentration of test compound prior to extraction.

Additional control experiments established that, (i) the pH of the buffer solutions was not affected by the solvent extraction process; (ii) the absorbance of the 2,2,4-trimethylpentane extract did not exceed 0.003 when compared against a 2,2,4-trimethylpentane blank; and (iii) the test compound was not altered by the extraction procedure, as indicated by a comparison of its UV absorption spectra in 2,2,4-trimethylpentane with and without extraction.

Determination of the pKa of* trans-*isohumulone by conductimetry

The pKa of *trans*-isohumulone was determined in water at 25°C using the conductimetric method described by Albert & Serjeant (1984). In brief, the conductivity of a series of *trans*-isohumulone solutions in water was measured using a conductivity meter equipped with a platinum electrode. The conductivities of a series of sodium *trans*-isohumulate solutions were also measured in order to calculate the limiting conductance of the *trans*-isohumulone ion. The ionisation factor (α) was calculated from

$$\alpha = \frac{\Lambda_c}{\Lambda_o} \qquad (2.4)$$

where Λ_c=molar conductance of *trans*-isohumulone at concentration C and Λ_o=limiting molar conductance of *trans*-isohumulone ion. The dissociation constant (K_a) was calculated from

$$K_a = \frac{\alpha^2 C}{1 - \alpha} \qquad (2.5)$$

where C=concentration of *trans*-isohumulone (M). pKa was derived from K_a in the usual way. The concentration of *trans*-isohumulone was 5 x 10^{-4}M - 5 x 10^{-2}M in the case of limiting conductance evaluations and 5 x 10^{-4}M - 1.25 x 10^{-3}M in the case of free acid determinations. In a control experiment, the pKa of acetic acid was determined.

2.2.5. NMR spectroscopy of *trans*-isohumulone in acid and alkaline MeOD/D_2O mixtures

The molecular structure of each of the predominant species of *trans*-isohumulone in acidic and alkaline aqueous MeOD was determined using ^{1}H-NMR and ^{13}C-NMR

spectroscopy. Samples (final concn. 0.12M) were dissolved in a mixture of deuterated MeOH and deuterated water (50% each) with, or without, NaOH (0.15M). ^{1}H-NMR, ^{13}C-NMR and DEPT (90° & 135° pulse angles) spectroscopy were used to determine the molecular structure of the major species of *trans*-isohumulone present in each solvent at 21°C.

2.2.6. UV spectroscopy of *trans*-isohumulone in piperidine

The absorption spectrum of *trans*-isohumulone (2 x 10^{-4}M) in piperidine (Aldrich Chemicals, UK) was determined in quartz cells of 1cm path length against a solvent blank.

2.2.7. Demonstration of complex formation between *trans*-isohumulone and Mn^{2+} and determination of the stoichiometric ratio of the complex

The interaction between *trans*-isohumulone and Mn^{2+} in MeOH was studied by UV spectroscopy. $MnCl_2$ was added as an aqueous solution (5µl) to 3ml methanolic *trans*-isohumulone solution (50µM). Control *trans*-isohumulone solutions received 5µl water. Absorption spectra in the region λ=200-400nm were measured against a solvent blank prepared in an identical way (in the absence of *trans*-isohumulone) in quartz cells of 1cm path length. The stoichiometry of complex formation was estimated as described by Pfeiffer *et al.* (1974). Changes in absorbance of *trans*-isohumulone at 245nm (ΔA_{245}) were plotted against [Mn^{2+}] at fixed [*trans*-isohumulone]; the intersect between the Mn^{2+}-dependent increase in ΔA_{245} and the maximum ΔA_{245} obtained at saturating [Mn^{2+}] provided an indication of the stoichiometry of the complex.

2.3. RESULTS

2.3.1. Identity and purity of hop compounds and hop-derived compounds

Melting point, UV absorption properties, IR absorption properties, optical rotation, mass spectra, ^{1}H-NMR and ^{13}C-NMR spectra were all in agreement with the available literature values.

Purity was assessed in several ways. In some cases (*e.g.* that of *trans*-isohumulone) agreement between the literature and experimental mp suggested that the compound was pure. However, a better indication of the identity and quantity of contaminants was provided by HPLC (Figure 2.2) and by NMR spectroscopy (Figure 2.3). The purity of the compounds, together with the identity and quantity of contaminants is shown in Table 2.1.

2.3.2. NMR spectroscopy of hop compounds and hop-derived compounds

The assignment of the NMR signals of the test compounds in $CDCl_3$ solution is described below. The way in which the assignments was arrived at is given in detail in the case of *trans*-humulinic acid. The other compounds were assigned in a similar way, but for the sake of brevity, the detail has been omitted.

Trans-*humulinic acid*

^{13}C-NMR spectroscopy of *trans*-humulinic acid revealed 15 major signals since the experiment was carried out under conditions in which each carbon atom gave rise to one signal (Table 2.2, Figure 2.4). The resonances were divided on the basis of their substitution (-C-, CH, CH_2, CH_3) using DEPT. Two experiments, one using a pulse angle of 90°, the other a pulse angle of 135°, were

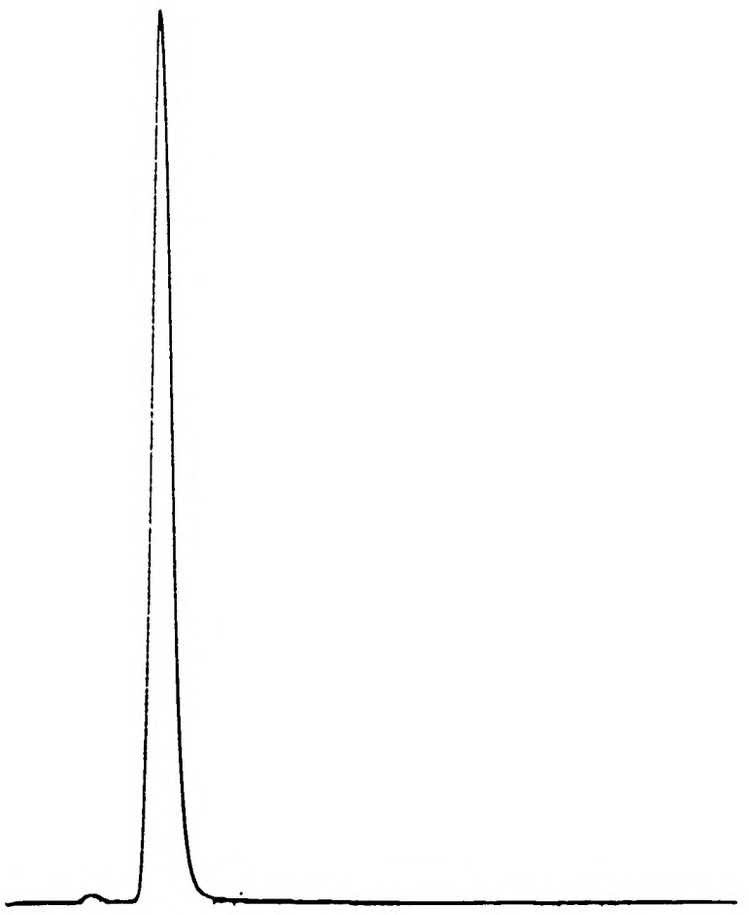


Figure 2.2 Determination of the identity and purity of *trans*-isohumulone by HPLC. The trace shows the response of the UV detector (λ=280nm) to a sample of *trans*-isohumulone (83μM) in a mobile phase which consisted of (%) MeOH (70), water (30) and tetrabutylammonium phosphate (0.17). The analysis was performed at ambient temperature. The trace indicates that the sample contained <0.05% isocohumulone as contaminant. Other batches were free of this compound.

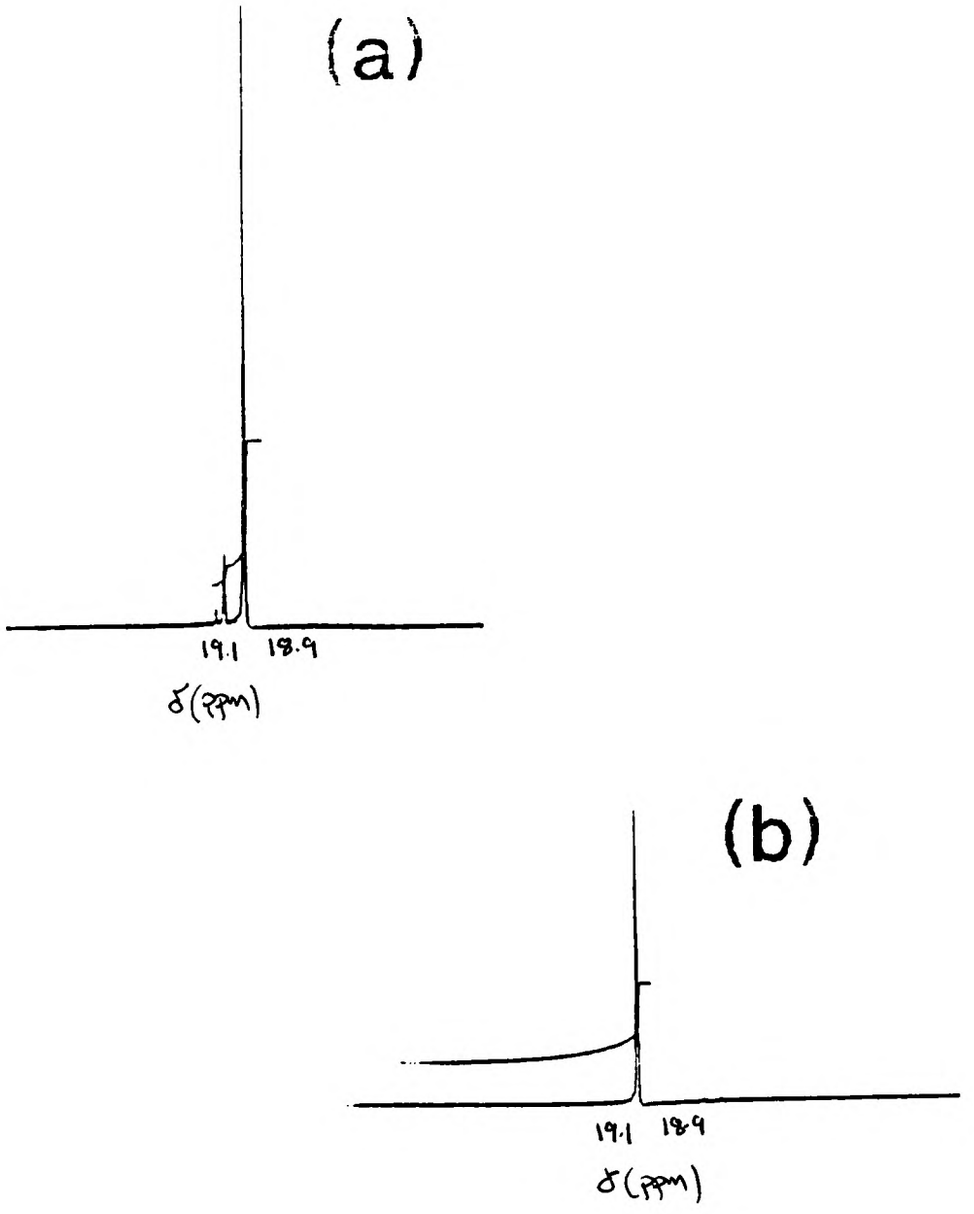


Figure 2.3 Determination of the identity and purity of (-)-humulone by ^{1}H-NMR spectroscopy. The high-field NMR signals provide a good indication of the extent of contamination by related congeners. (a) (-)-Humulone preparation containing cohumulone (8%) and adhumulone (1%) as contaminants; (b) Pure (-)-humulone. Samples were dissolved in $CDCl_3$ and analysed using a JEOL JNM-GX270 FT NMR spectrometer.

Table 2.1: Purity of hop compounds and hop-derived compounds

Compound	Contaminant(s)[1]
colupulone	lupulone (10), adlupulone (<1)
(-)-humulone	cohumulone (8), adhumulone (1)
(-)-humulone*	solvent only[2]
(-)-cohumulone*	solvent only[2]
trans-isohumulone	none
trans-isohumulone*	solvent only[2]
trans-isocohumulone*	solvent only[2]
trans-humulinic acid	*cis*-humulinic acid (8)
dehydrated humulinic acid	humulinic acid (*cis* and/or *trans*) (1)

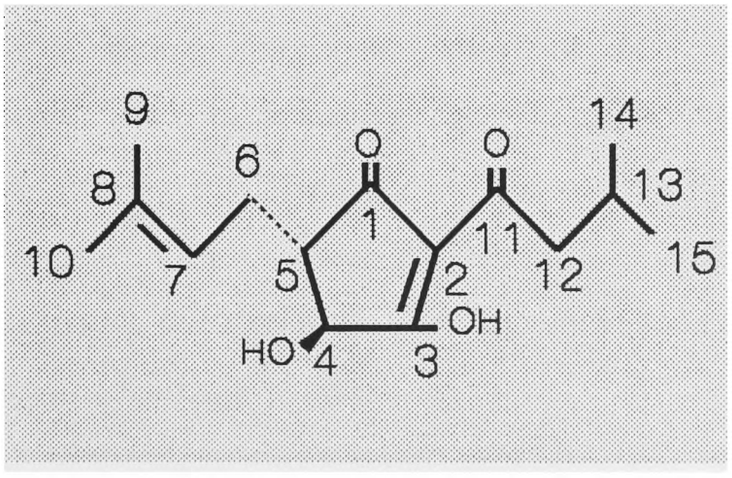
* Prepared by HPLC.

1. Numbers in parenthesis indicate the amount of each contaminant present on a mole *per cent* basis.

2. Solvents detected included 2,2,4-trimethylpentane, MeOH, $MeCl_2$, and $CDCl_3$.

Table 2.2: NMR spectral data of *trans*-humulinic acid in $CDCl_3$

Position	^{1}H-NMR		^{13}C-NMR	
	δ (ppm)	Coupling Pattern	δ (ppm)	Multi-plicity (DEPT)
1	-	-	203.70	-C-
2	-	-	111.54	-C-
3	-	-	199.43	-C-
4	4.06	d. J=4.8	76.05	CH
5	2.88	d.d.d. J=6.8,6.8,4.8	49.62	CH
6	α2.46 β2.64	d.d.d. J=14.0,6.8,6.8 d.d.d. J=14.0,6.8,6.8	26.44	CH_2
7	5.14	m.	118.78	CH
8	-	-	135.79	-C-
9	1.72	s.	25.84	CH_3
10	1.67	s.	17.96	CH_3
11	-	-	200.46	-C-
12	α2.73 β2.83	d.d. J=17.4,6.8 d.d. J=17.4,6.8	46.01	CH_2
13	2.20	nonet J=6.8	25.60	CH
14	0.99	d.J=6.8	22.49	CH_3
15	0.98	d.J=6.8	22.55	CH_3



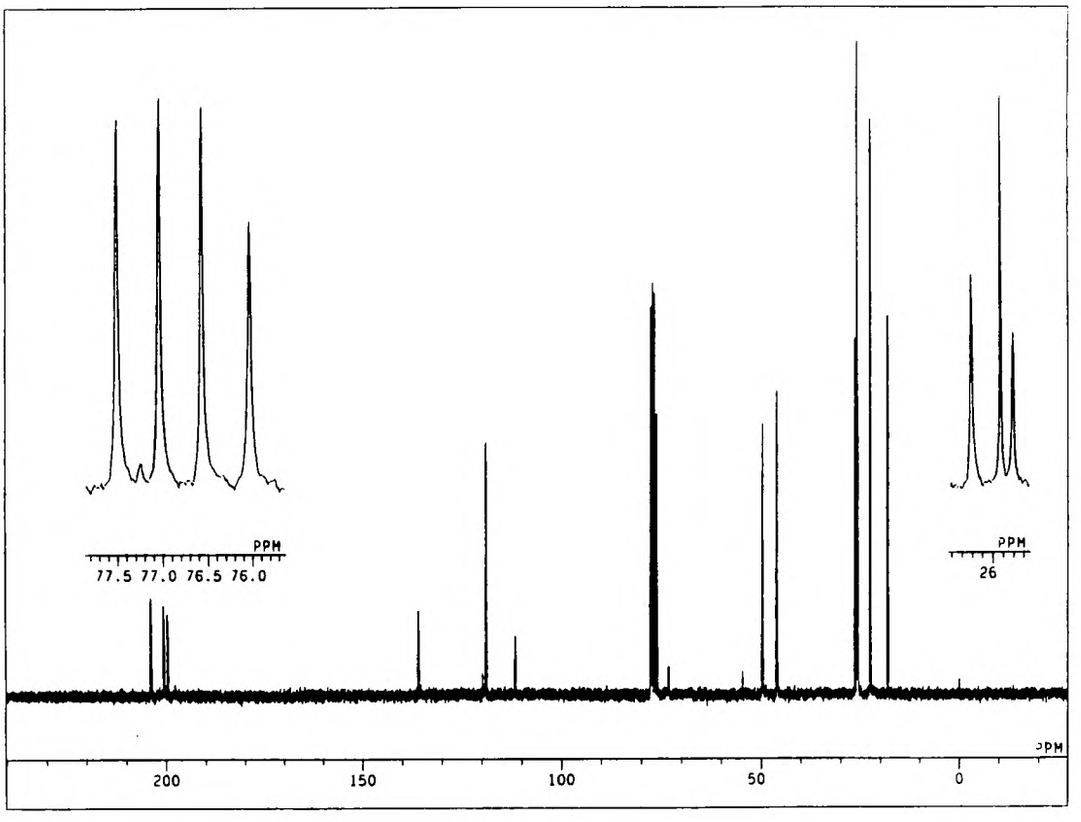


Figure 2.4 ^{1}H-decoupled ^{13}C-NMR spectrum of *trans*-humulinic acid. The spectrum was recorded at 67.5MHz (in $CDCl_3$ solution at 20°C) using a 3.7μsec pulse, 1.0sec pulse delay, 0.901sec acquisition time. Data shown represent the Fourier transform of 3267 scans. With the exception of the peak at 0ppm (TMS), peaks at 77.51ppm, 77.05ppm and 76.57ppm ($CDCl_3$ solvent) and peaks at 54.53ppm, 73.05ppm, 77.25ppm and 119.70ppm (minor contaminants), each peak arose from one carbon atom.

employed. In the DEPT 90° spectrum (Figure 2.5a) only CH resonance lines appeared. In the DEPT 135° spectrum (Figure 2.5b) CH, CH_2, CH_3 resonance lines appeared: CH_3 and CH resonance lines appeared above the baseline while CH_2 resonances appeared below the baseline. A comparison of 90° and 135° spectra allowed the identity of all CH, CH_2 and CH_3 groups to be deduced. Finally, quaternary, carbonyl and enolic carbon atoms could be identifed by the fact that they appeared on the unedited ^{13}C-NMR spectrum (Figure 2.4) but did not appear on either DEPT spectrum (Figure 2.5).

The chemical shift of attached protons was identified using ^{1}H-^{13}C COSY. Analysis of the spectrum in Figure 2.6 revealed the coupling pattern reported in Table 2.2. ^{1}H-^{13}C COSY experiments provide spectra which consist of a ^{1}H-NMR spectrum on one axis and a ^{13}C-NMR spectrum on the other axis. Coupling between the ^{1}H resonances and ^{13}C resonances is indicated by the presence of cross peaks on the spectrum which connect the ^{1}H and ^{13}C lines on the individual spectra, *e.g.* the line drawn from the ^{1}H-NMR peak at 0.99ppm intersects, at a cross peak, a line drawn through the ^{13}C-NMR peak at 22.49ppm.

The carbonyl and enolic resonances of *trans*-humulinic acid have not been previously assigned. Using a combination of two techniques, NOE difference spectroscopy and ^{1}H-coupled ^{13}C-NMR spectroscopy (optimised for long range couplings) the assignments in Table 2.2 were arrived at. The identity of the carbonyl and enolic resonances at δ=199.43, 200.46, 203.70ppm was deduced as follows. Irradiation of the methylene protons at C-12 (δ=2.78ppm) induced an NOE at the carbon atoms with δ=200.46 and 203.70ppm. No NOE was evident at the carbon atom with δ=199.43ppm. Irradiation of the proton at C-5 (δ=2.88ppm) elicited an NOE at these same carbon atoms thus indicating their identity as C-1 and C-11.

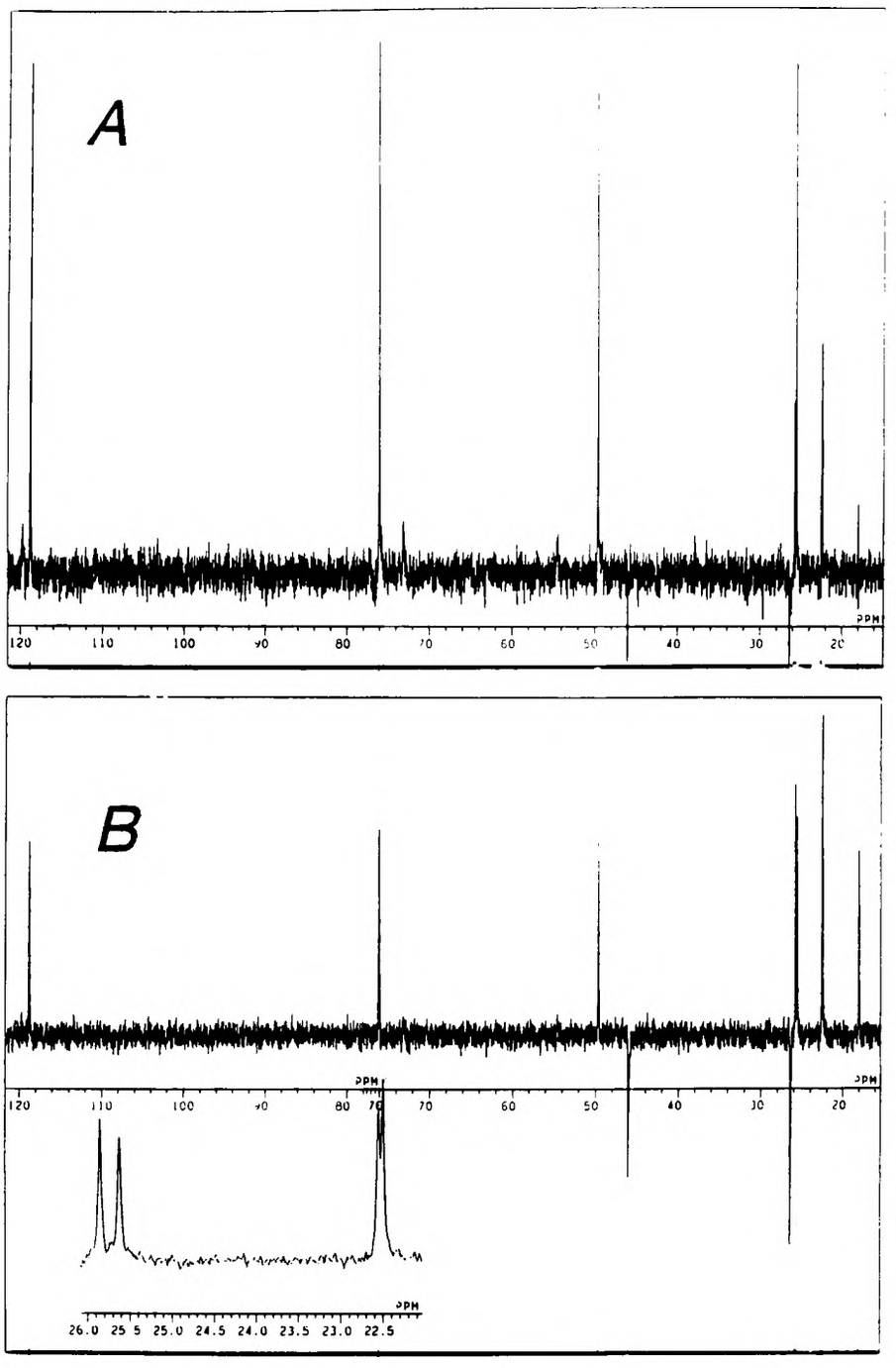


Figure 2.5 Editing of ^{13}C-NMR resonances using DEPT. DEPT 90° spectrum (a) was recorded at 67.5MHz (in $CDCl_3$ solution at 20°C) using a 29.6μsec pulse, 2.0sec pulse delay, 0.901sec acquisition time. The DEPT 135° spectrum (b) was recorded using a 40.2μsec pulse, 2.0sec pulse delay, 0.901sec acquisition time. Data shown represent the Fourier transform of 596 scans (DEPT 90°) and 342 scans (DEPT 135°).

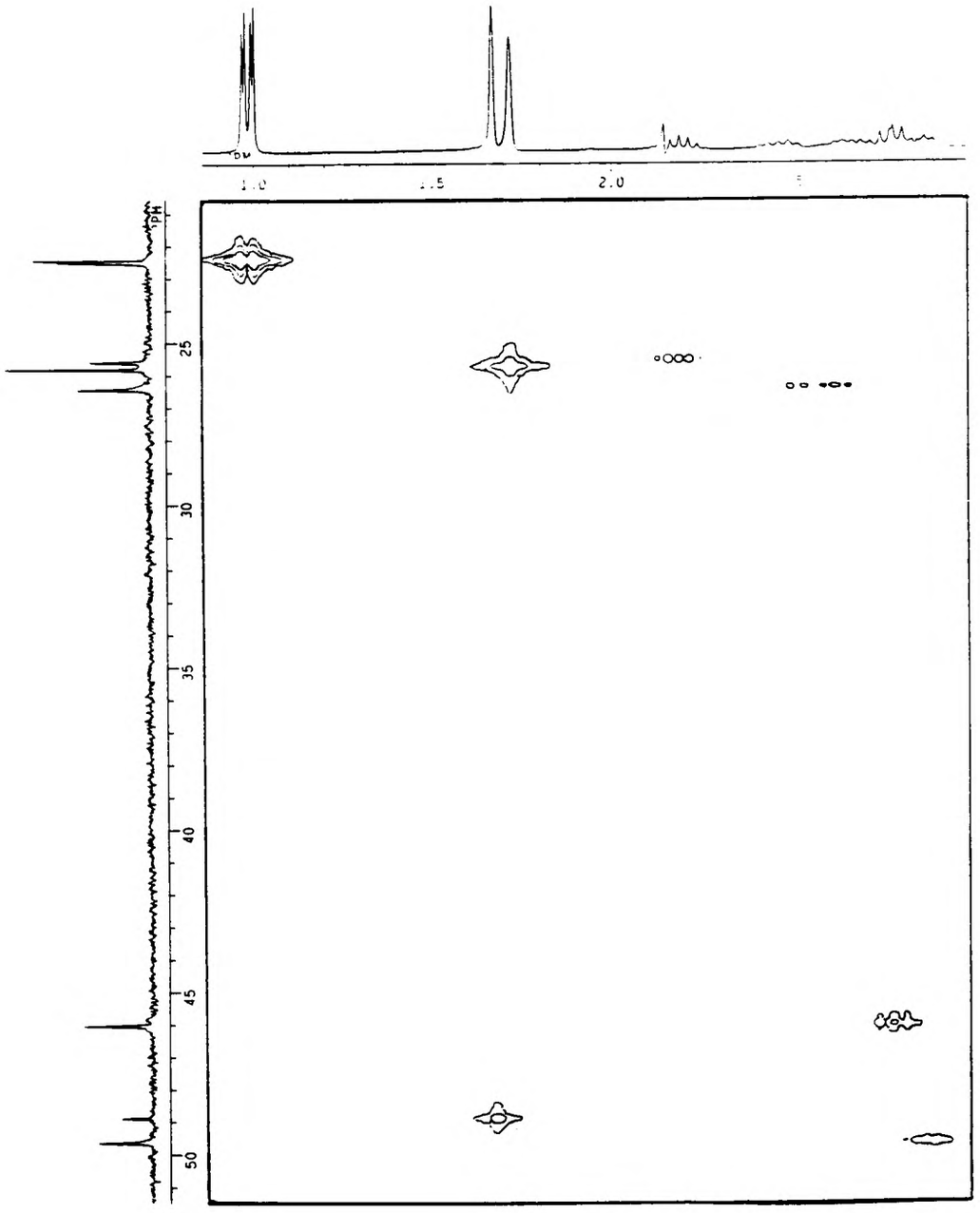


Figure 2.6 ^{1}H-^{13}C COSY. The spectrum of *trans*-humulinic acid (in $CDCl_3$ solution) at 20°C was recorded. ^{1}H-^{13}C COSY spectra consist of a ^{1}H-NMR spectrum on one axis and a ^{13}C-NMR spectrum on the other axis. Coupling between ^{1}H resonances and ^{13}C resonances is indicated by the presence of cross peaks on the spectrum which connect the ^{1}H and ^{13}C lines on the individual spectra. Only the low field portion of the spectrum is shown. The peak at 48.8ppm is a folded peak (an artifact induced by the restricted spectral range examined). Its correct position is at 17.96ppm.

The size of the NOE suggested that C-1 possessed δ=203.70ppm and C-11 possessed δ=200.46ppm. In $CDCl_3$ solution, association of the isopentenyl and isovaleryl side chains seems to occur, as indicated by the presence of a large NOE at δ=46.01ppm (C-12) on irradition of the ring proton at C-5 (δ=2.88ppm). Assignment of the carbonyl/enolic resonances was supported by analysis of the ^1H-coupled ^{13}C-NMR spectrum (not shown). The peak at δ=203.70ppm was a complex multiplet, consistent with its identity as a carbonyl function (C-1) split by protons at C-5, C-4, C-6, and C-7.

The ^1H-NMR spectrum of *trans*-humulinic acid is shown in Figure 2.7. The identity of the ^1H resonances was confirmed in several ways. Firstly, peak areas were integrated. The number of protons determined by integration agreed with those expected from the ^1H-^{13}C coupling pattern. Secondly, coupling between directly-bonded protons was established using ^1H-^1H COSY. Coupling to protons linked by one, two, three or four bonds was indicated although the intensity of the coupling was, in general, reduced in proportion to the number of bonds. Through-space coupling does not appear on ^1H-^1H COSY. (Nuclear Overhauser Effect difference Spectroscopy is used to probe through-space coupling, see below.)

The ^1H-^1H COSY spectrum shown in Figure 2.8 was interpreted as follows. The ^1H-NMR spectrum of the compound is shown on each axis. The diagonal line running through the spectrum represents the same ^1H-NMR spectrum but is presented in the form of a contour plot. Coupling between ^1H-NMR signals is indicated by the presence of peaks which are displaced from the diagonal. The identity of the coupled peaks can be deduced by drawing a line parallel to each through both the displaced peak and the diagonal. In the example shown in Figure 2.8, coupling between the ring proton at C-4,

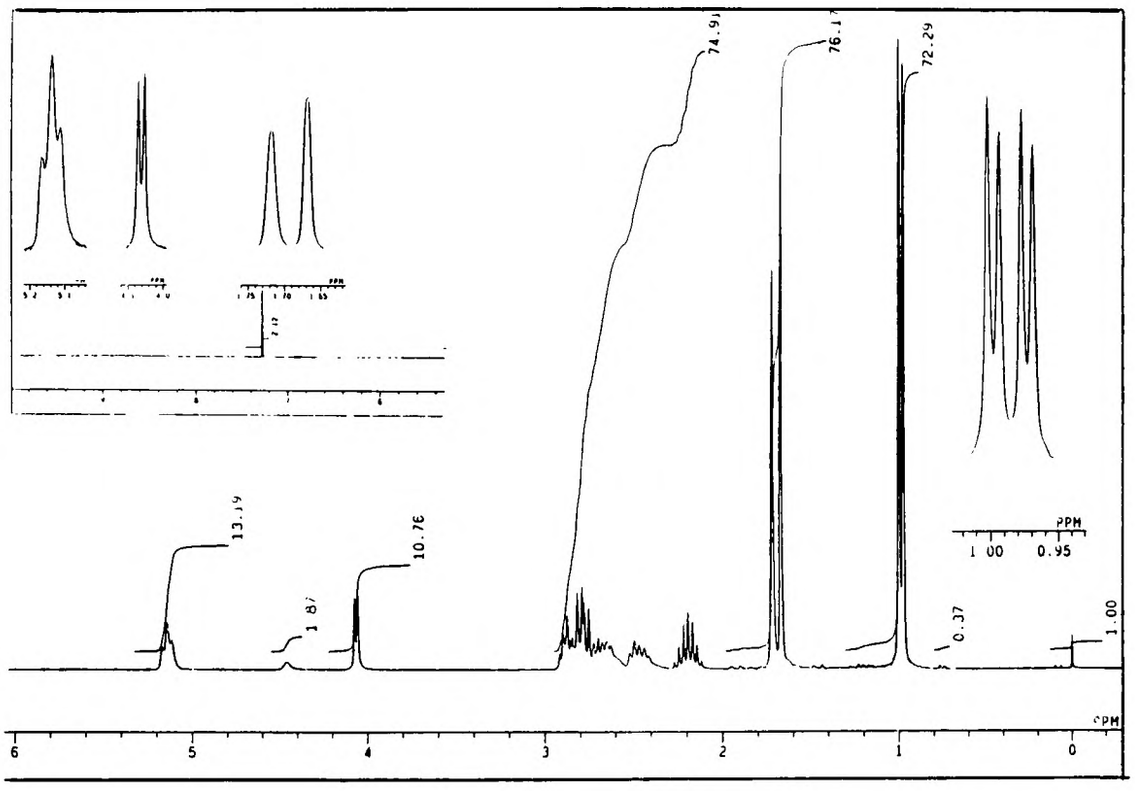


Figure 2.7 ^{1}H-NMR spectrum of *trans*-humulinic acid. The spectrum was recorded at 270MHz (in $CDCl_3$ solution at 20°C) using a 3.2μsec pulse, 2.27sec pulse delay, 2.73sec acquisition time. The data shown represent the Fourier transform of 16 scans. The peak at 7.28ppm represents the proton of $CHCl_3$ which invariably contaminates the deuterated form of this solvent. The small peak at 4.45ppm represents the C-4 proton of contaminant *cis*-humulinic acid. The numbers above the resonance lines are the integrals of the area under each peak. For this spectrum, a single proton gives an integral of about 12.

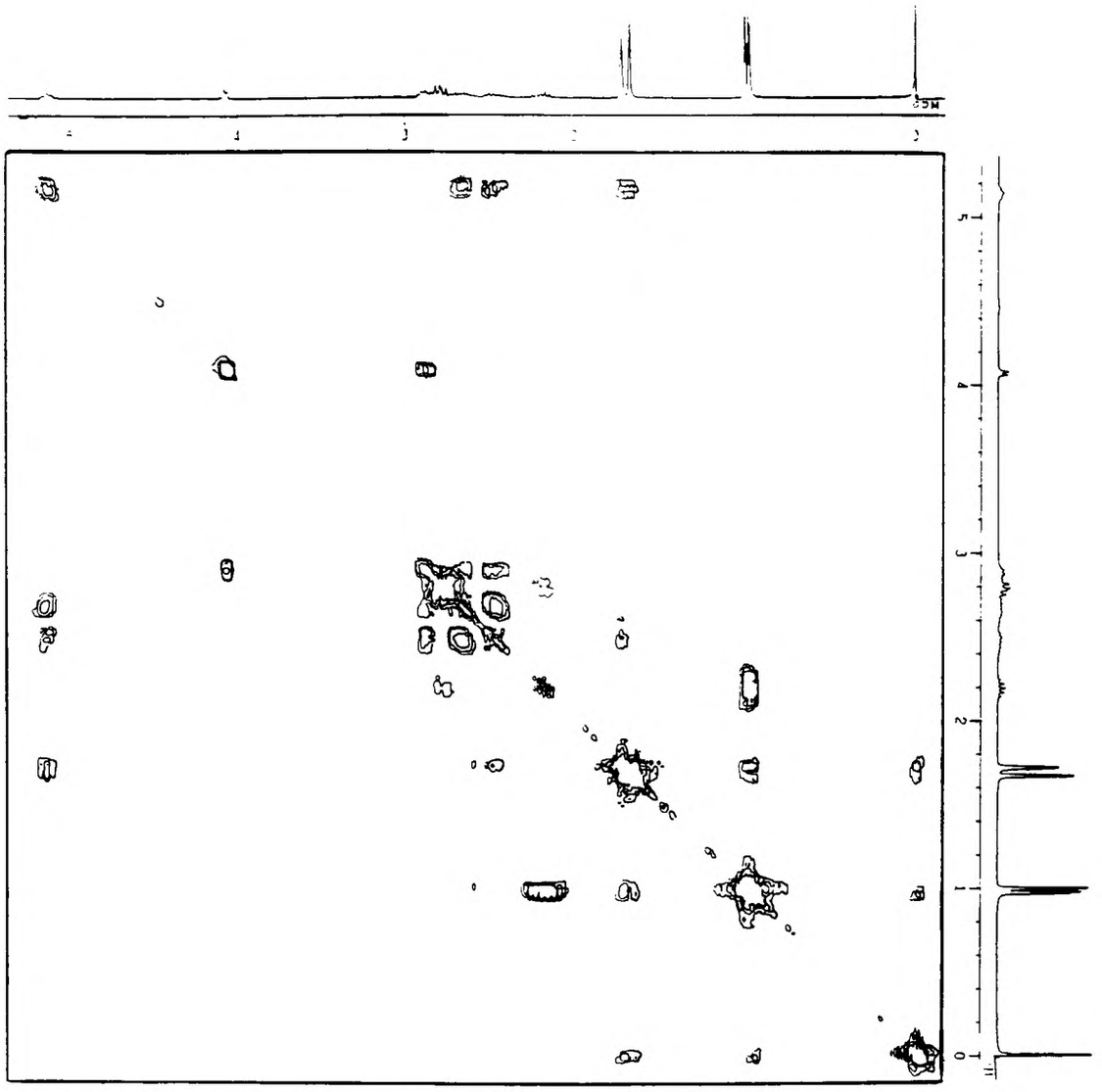


Figure 2.8 ^{1}H-^{1}H COSY. The spectrum of *trans*-humulinic acid (in $CDCl_3$ solution at 20°C) was recorded. The ^{1}H-NMR spectrum of the compound is shown on each axis. The diagonal line running through the spectrum represents the same ^{1}H-NMR spectrum but is represented in the form of a contour plot. Coupling between ^{1}H-NMR signals is indicated by the presence of peaks which are displaced from the diagonal.

(δ=4.06ppm) and the ring proton at C-5 (δ=2.88ppm) is indicated. The fact that the C-4 ring proton has no other off-diagonal peaks indicates that this proton is not directly coupled to any other protons.

Two coupling pathways can be traced in *trans*-humulinic acid. Beginning at the *gem*-dimethyl grouping (C-14, C-15) of the isovaleryl side chain (δ=0.98, 0.99ppm), coupling to the olefinic proton (C-13, δ=2.20ppm) was observed. This olefinic proton was coupled to each of the two diastereotropic methylene protons (C-12) at δ=2.73 and 2.83ppm which did not display any other couplings since the bonding pathway was interrupted by the presence of the carbonyl (C-11) and quaternary (C-2) carbon atoms which were displaced by one or two bonds respectively. Tracing the coupling pathway of the isopentenyl side chain the *gem*-dimethyl protons (C-9, C-10) could be seen to be coupled to the olefinic proton with δ=5.14ppm (C-7). Weak coupling to the methylene protons (6_α, 6_β ; δ=2.46 and 2.64ppm respectively) was also indicated. The olefinic proton was strongly coupled to these methylene protons and also to the ring proton (C-5) which had δ=2.88ppm. An expansion of the COSY spectrum (not shown) indicated that the methylene protons were strongly coupled both to each other and to the ring proton at C-5. This ring proton, in turn, was coupled to the proton at C-4 (δ=4.06ppm) which does not display any other couplings on account of the presence of an enolic function at C-3 and the quaternary carbon at C-2 which were displaced by one and two bonds respectively.

Both sets of *gem*-dimethyl groups appeared to be weakly-coupled to the methyl protons of TMS (δ=0ppm) and to each other. Such artifacts, common in ^1H-^1H COSY experiments, can be simply rejected by comparison with other spectral features. The coupling constants measured

from the ^{1}H-NMR spectrum (Figure 2.7) were, without exception, in agreement with the coupling pattern presented and are reported in Table 2.2.

Dehydrated humulinic acid

^{13}C-NMR spectroscopy revealed 15 major signals (Table 2.3). DEPT experiments indicated the presence of four methyl carbons (resonating at 19.20, 22.60 [2xCH_3], 27.14ppm), two methylene carbons (resonating at 37.42, 47.93ppm), three methine carbons (resonating at 25.18, 121.94, 128.49ppm) and six quaternary carbons (resonating at 114.60, 125.62, 149.17, 190.46, 195.92, 202.54ppm).

^{1}H-NMR confirmed the presence of 12 methyl protons (resonating at 0.98 [2x3H], 1.96, 1.97ppm), four methylene protons (resonating at 2.83, 3.05ppm) and three methine protons (resonating at 2.19, 6.00, 7.30ppm). A broad signal, representing that from one enolic proton, was detected at δ=14.6ppm.

Trans-*isohumulone and* trans-*isocohumulone*

^{13}C-NMR spectroscopy of *trans*-isohumulone and *trans*-isocohumulone revealed 21 and 20 major signals respectively (Tables 2.4, 2.5). DEPT experiments indicated the presence in *trans*-isohumulone of six methyl carbons (resonating at 17.77, 17.86, 22.50, 22.79, 25.76, 26.00ppm), three methylene carbons (resonating at 23.34, 38.73, 44.44ppm), four methine carbons (resonating at 26.5, 55.34, 114.64, 120.16ppm) and eight quaternary, enolic, or carbonyl carbons (resonating at 90.7, 110.37, 134.65, 136.19, 195.64, 197.84, 204.95, 206.87ppm). Coupling between the carbon atoms of *trans*-isohumulone and protons separated by one, two or three bonds is indicated in Table 2.4. ^{13}C-^{1}H couplings in this compound, which are reported for the first time, were in agreement with the expected values. Experiments on

Table 2.3: NMR spectral data of dehydrated humulinic acid in $CDCl_3$

Position	^{1}H-NMR		^{13}C-NMR	
	δ (ppm)	Coupling Pattern	δ (ppm)	Multiplicity (DEPT)
1	-	-	unassigned	-C-
2	-	-	114.6	-C-
3	14.6	v.b.b.	unassigned	-C-
4	3.05	d. J=1.7	37.42	CH_2
5	-	-	149.17	-C-
6	7.29	d.t. J=12.0,1.7	128.49	CH
7	5.99	d.t.q. J=12.0,1.7,1.7	121.94	CH
8	-	-	125.62	-C-
9	1.97	s.	27.14	CH_3
10	1.96	s.	19.2	CH_3
11	-	-	unassigned	-C-
12	2.83	d. J=6.8	47.93	CH_2
13	2.19	nonet J=6.8	25.18	CH
14	0.98	d.J=6.8	22.60	CH_3
15	0.98	d.J=6.8	22.60	CH_3

Carbonyl or enolic resonances detected at 190.5, 195.9, 202.5ppm.

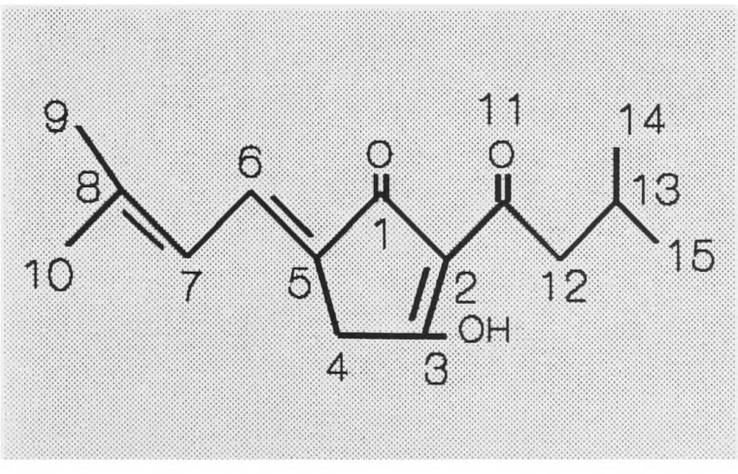


Table 2.4: NMR spectral data of *trans*-isohumulone in $CDCl_3$

Position	^{1}H-NMR		^{13}C-NMR				
	δ (ppm)	Coupling Pattern	δ (ppm)	Multiplicity (DEPT)	Coupling Pattern	J_1 $^{13}C-^1H$ (Hz)	J_2,J_3 $^{13}C-^1H$ (Hz)
1	-	-	204.95[a]	-C-	m.	-	U
2	-	-	110.37	-C-	s.	-	-
3	-	-	195.64	-C-	s.	-	-
4	-	-	90.7	-C-	m.	-	U
5	3.03	d.d. J=9.5,6.0	55.34	CH	d.t.	130.4	U
6	α2.33 β2.6	d.t. J=15.4,9.5 m.	23.34	CH_2	t.d.d.	126.5	5.2
7	5.15	m.	120.16	CH	d.t.	160.8	5.8
8	-	-	134.65	-C-	d.t.	-	13.9,5.2
9	1.69	s.	25.69	CH_3	q.q.	125.7	4.2
10	1.54	s.	17.9	CH_3	q.q.d.	125.5	4.2
11	-	-	206.87[a]	-C-	m.	-	U
12	3.3	m.	38.73	CH_2	t.d.	127.3	3.1
13	5.21	m.	114.44	CH	d.t.	158.4	5.8
14	-	-	136.19	-C-	m.	-	U
15	1.73	s.	25.69	CH_3	q.q.d.	125.7	4.2
16	1.57	s.	18.69	CH_3	q.q.d.	125.5	4.2
17	-	-	197.84	-C-	t.d.	-	2.9,2.5
18	2.71	d. J=6.8	44.44	CH_2	t.d. sept.	130.3	4.4,4.9
19	2.16	nonet J=6.8	26.5	CH	d. nonets	130.4	4.2
20	0.95	d. J=6.8	22.58	CH_3	q.q.d.	122.7	4.2
21	0.98	d. J= 6.8	22.36	CH_3	q.q.d.	122.7	4.2

a = Interchangeable assignment

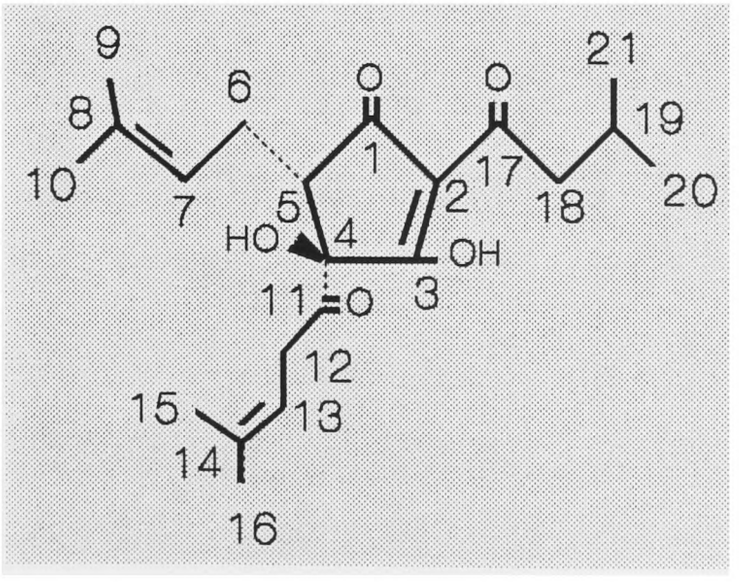
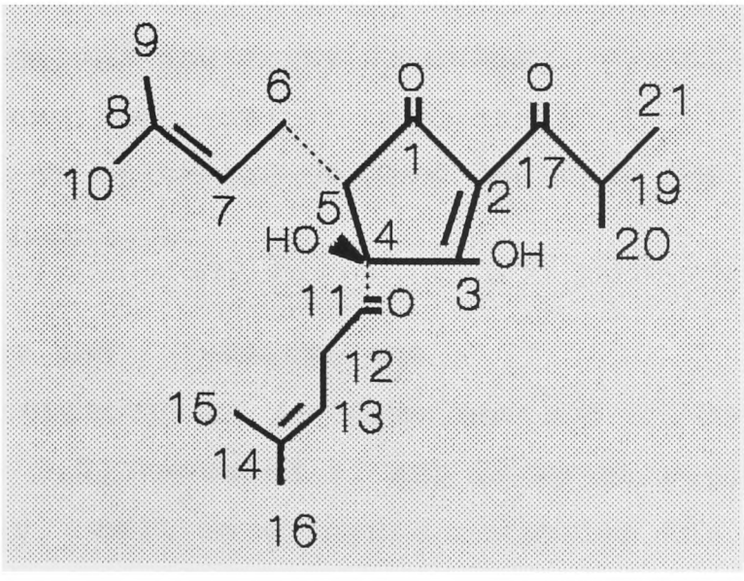


Table 2.5: NMR spectral data of *trans*-isocohumulone in $CDCl_3$

Position	^{1}H-NMR		^{13}C-NMR	
	δ (ppm)	Coupling Pattern	δ (ppm)	Multiplicity (DEPT)
1	-	-	205.03	-C-
2	-	-	109.3	-C-
3	-	-	195.67	-C-
4	-	-	90.38	-C-
5	3.05	b.m.	54.83	CH
6	α2.33 β2.58	m. m.	23.47	CH_2
7	5.13	t.m. J=6.0	120.14	CH
8	-	-	134.7	-C-
9	1.69	s.	25.7	CH_3
10	1.54	s.	18.14	CH_3
11	-	-	207.00	-C-
12	3.32	b.m.	38.75	CH_2
13	5.21	t.m. J=6.8	114.46	CH
14	-	-	136.22	-C-
15	1.57	s.	17.92	CH_3
16	1.73	s.	25.7	CH_3
17	-	-	203.51	-C-
18	+	-	+	-
19	3.48	b.m.	34.65	CH
20	1.14	d. J=6.9	18.17	CH_3
21	1.19	d. J=6.9	17.84	CH_3

(+) No atom present at this position.



trans-isocohumulone indicated the presence of six methyl carbons (resonating at 17.84, 17.92, 18.14, 18.17, 25.7 [2xCH_3]ppm), two methylene carbons (resonating at 23.47, 38.75ppm), four methine carbons (resonating at 34.65, 54.83, 114.83, 120.14ppm) and eight quaternary, enolic or carbonyl carbons (resonating at 90.38, 109.30, 134.70, 136.22, 195.67, 203.51, 205.03, 207.00ppm). Assignment was assisted by the use of ^{1}H-^{13}C COSY and ^{1}H-^{1}H COSY. The ^{13}C resonances of *trans*-isocohumulone (Table 2.5) were similar to those of *trans*-isohumulone, the major differences being the carbonyl (C-17), methine (C-19) and methyl (C-20, C-21) resonances of the isovaleryl side chain. In addition, *trans*-isohumulone gave a signal for the methylene carbon in this side chain at δ=44.44ppm.

^{1}H-NMR spectroscopy confirmed the presence in *trans*-isohumulone of 18 methyl protons (resonating at 0.96 [2x3H], 1.54, 1.57, 1.69, 1.73ppm), six methylene protons (resonating at 2.33, 2.6, 2.71, 3.30ppm) and four methine protons (resonating at 3.03, 3.48, 5.15, 5.21ppm). In *trans*-isocohumulone 18 methyl protons (resonating at 1.14, 1.19, 1.54, 1.57, 1.69, 1.73ppm), four methylene protons (resonating at 2.33, 2.58, 3.32ppm) and four methine protons (resonating at 3.05, 3.48, 5.13, 5.21ppm) were detected. No signals were detected from enolic protons in either compound. The methylene protons of the isopentenyl side chain of both *trans*-isohumulone and *trans*-isocohumulone were not equivalent and had separate chemical shift values of 2.33 and 2.60ppm in *trans*-isohumulone and 2.33 and 2.58ppm in *trans*-isocohumulone.

(-)-Humulone and (-)-cohumulone

^{13}C-NMR spectroscopy of (-)-humulone and (-)-cohumulone (Table 2.6, 2.7) revealed 21 and 20 major signals respectively. DEPT experiments indicated the presence in

Table 2.6: NMR spectral data of (-)-humulone in $CDCl_3$

Position	^{1}H-NMR		^{13}C-NMR	
	δ(ppm)	Coupling Pattern	δ(ppm)	Multiplicity (DEPT)
1	18.94	s.	unassigned	-C-
2	-	-	105.99	-C-
3	-	-	unassigned	-C-
4	-	-	78.91	-C-
5	-	-	unassigned	-C-
6	-		109.32	-C-
7	α3.03 β3.12	d.d. J=14.5,7.1 d.d. J=14.5,7.1	21.07	CH_2
8	5.13	t.m. J=7.1	121.01	CH
9	-	-	132.76	-C-
10	1.69	s.	26.0	CH_3
11	1.53	s.	17.77	CH_3
12	α2.46 β2.57	d.d. J=13.9,7.9 d.d. J=13.9,7.9	42.68	CH_2
13	4.99	t. J=7.9	115.66	CH
14	-	-	138.26	-C-
15	1.73	s.	25.76	CH_3
16	1.69	s.	17.86	CH_3
17	-	-	200.38	-C-
18	α2.73 β2.81	d.d. J=13.9,7.3 d.d. J=13.9,6.6	46.5	CH_2
19	2.13	nonet J=6.6	26.4	CH
20	0.97	d. J=6.6	22.79	CH_3
21	1.00	d. J=6.6	22.5	CH_3

OH ^{1}H-NMR signal at 8.04ppm. Carbonyl/enolic resonances detected at 167.83, 190.9, 195.32ppm

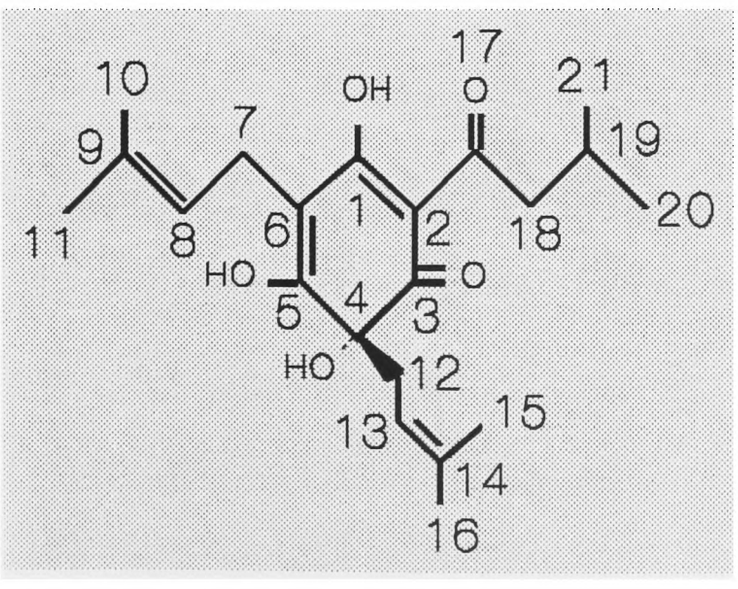
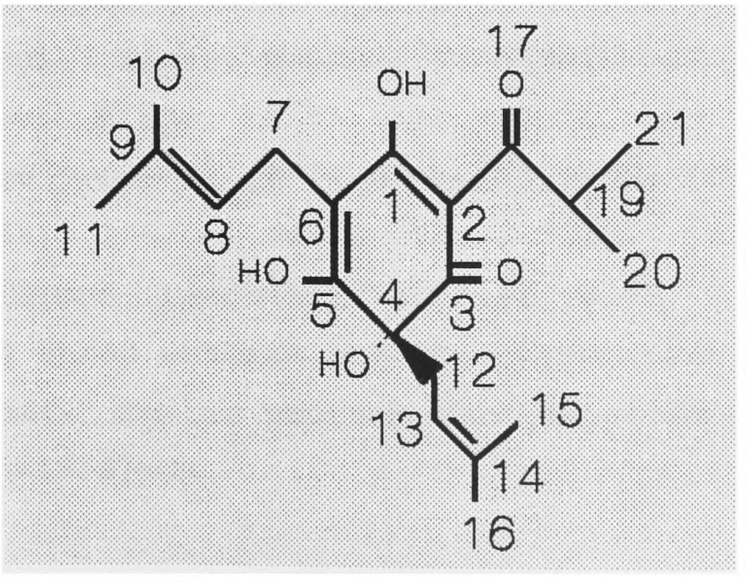


Table 2.7: NMR spectral data of (-)-cohumulone in $CDCl_3$

Position	^{1}H-NMR		^{13}C-NMR	
	δ (ppm)	Coupling Pattern	δ (ppm)	Multiplicity (DEPT)
1	19.07	s.	unassigned	-C-
2	-	-	104.47	-C-
3	-	-	unassigned	-C-
4	-	-	79.19	-C-
5	-	-	unassigned	-C-
6	-	-	109.16	-C-
7	α3.04 β3.13	d.d. J=14.5,7.2 d.d. J=14.5,7.2	21.06	CH_2
8	5.13	t.m. J=7.2	121.03	CH
9	-	-	132.69	-C-
10	1.68	s.	25.99	CH_3
11	1.52	s.	17.75	CH_3
12	α2.48 β2.59	d.d. J=13.9,8.9 d.d. J=13.9,8.9	42.61	CH_2
13	5.01	t.m. J=8.9	115.68	CH
14	-	-	138.20	-C-
15	1.73	s.	25.76	CH_3
16	1.68	s.	17.83	CH_3
17	-	-	205.66	-C-
18	+	-	-	+
19	3.70	sept.J=6.8	34.8	CH
20	1.20	d. J=6.8	18.4	CH_3
21	1.13	d. J=6.8	18.4	CH_3

(+) No atom present at this position. OH ^{1}H-NMR signal at 8.05ppm. Carbonyl/enolic resonances detected at 167.53, 191.3, 194.9ppm.



(-)-humulone of six methyl carbons (resonating at 17.77, 17.86, 22.50, 22.79, 25.76, 26.00ppm), three methine carbons (resonating at 26.4, 115.66, 121.01ppm) and nine quaternary, enolic, or carbonyl carbons (resonating at 78.91, 105.99, 109.32, 132.76, 138.26, 167.83, 190.9, 195.32, 200.38ppm). Experiments on (-)-cohumulone indicated the presence of six methyl carbons (resonating at 17.75, 17.83, 18.40, 19.60, 25.76, 25.99ppm), two methylene carbons (resonating at 21.06, 42.61ppm), three methine carbons (resonating at 34.80, 115.68 and 121.03ppm) and nine quaternary, enolic or carbonyl carbons (resonating at 79.19, 104.47, 109.16, 132.69, 138.20, 167.83, 190.9, 195.32, 205.60ppm).

With the exception of the enolic and carbonyl resonances these were assigned using ^{1}H-^{13}C COSY and ^{1}H-^{1}H COSY. ^{13}C resonances of each of the two congeners were very similar, the major differences being noted in the carbonyl (C-17) methine (C-19) and methyl (C-20, C-21), resonances of the isovaleryl side chain. In addition, (-)-humulone gave a signal for the methylene carbon in this side chain at 46.5ppm.

^{1}H-NMR spectroscopy confirmed the presence in (-)-humulone of 18 methyl protons (resonating at 0.99 [2x3H], 1.53, 1.69 [2x3H], 1.73ppm), six methylene protons (resonating at 2.46, 2.57, 2.73, 2.81, 3.03, 3.12ppm) and three methine protons (resonating at 2.13, 4.99, 5.13ppm). Enolic protons were detected at δ= *ca* 4.7ppm and δ=18.94ppm. In (-)-cohumulone, the spectrum indicated the presence of 18 methyl protons (resonating at 1.16 [2x3H], 1.52, 1.68 [2x3H], 1.73ppm), four methylene protons (resonating at 2.48, 2.59, 3.04, 3.13ppm) and three methine protons (resonating at 3.70, 5.01, 5.13ppm). Enolic protons were detected at δ= *ca* 7.5ppm and δ=19.07ppm.

^{1}H-NMR signals arising from C-8 and C-13 on each of the two congeners formed an overlapping multiplet at *ca* 5.0ppm. ^{1}H-^{1}H COSY showed that proton at C-13 had δ=4.99ppm and that at C-8 had δ=5.13ppm. The coupling constants for both single bond and allylic coupling between these protons and the methylene and methyl groups to which they were coupled confirmed this assignment.

Colupulone

^{13}C-NMR spectroscopy of colupulone revealed 22 major signals (Table 2.8) since the molecule had several coincident resonances. DEPT experiments indicated the presence of eight methyl carbons (resonating at 17.85, 17.92 [2xCH_3], 18.81, 18.96, 25.88 [3xCH_3]ppm), three methylene carbons (resonating at 21.07, 36.35, 37.63ppm), four methine carbons (resonating at 35.42, 117.89, 118.22, 121.72) and 10 quaternary, enolic or carbonyl carbons (resonating at 57.11, 102.73, 109.76, 134.94 [2xC-], 135.36, 172.48, 189.75, 195.45, 207.12ppm).

^{1}H-NMR spectroscopy confirmed the presence of 24 methyl protons (resonating at 1.12 [2x3H], 1.56 [3x3H], 1.78 [3x3H]ppm.), six methylene protons (resonating at 2.53, 2.68, 3.19ppm) and four methine protons (resonating at 4.03, 4.81 [2xH], 5.15ppm). Enolic protons were detected at δ=7.03, 9.58, 18.57 and 19.32ppm.

In the case of both ^{1}H-NMR and ^{13}C-NMR spectra, minor resonance lines, representing the alternative enolic tautomer of colupulone were also detected.

2.3.3. Surface activity of (-)-humulone and *trans*-isohumulone

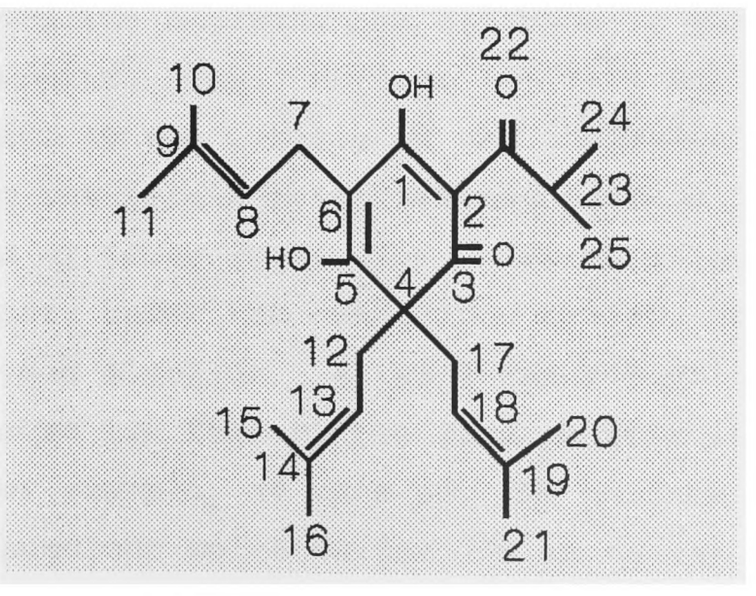
Hop compounds and hop-derived compounds possess surface-activity. A phenomenon associated with many surface-active compounds is an ability to form micelles when the

Table 2.8: NMR spectral data of colupulone in $CDCl_3$

Position	^{1}H-NMR		^{13}C-NMR	
	δ (ppm)	Coupling Pattern	δ (ppm)	Multiplicity (DEPT)
1	18.57[a]	s.	189.75[c]	-C-
2	-		102.73	-C-
3	19.32[a]	s.	195.45[c]	-C-
4	-	-	57.11	-C-
5	b	s.	172.48	-C-
6	-	-	109.76	-C-
7	3.19	d. J=7.3	21.07	CH_2
8	5.15	t.m. J=7.3	121.72	CH
9	-	-	135.36	-C-
10	1.56	s.	25.88	CH_3
11	1.78	s.	17.85	CH_3
12	2.68	d. J=7.7	37.63	CH_2
13	4.81	d.m. J=7.7	118.22	CH
14	-	-	134.94	-C-
15	1.78	s.	25.88	CH_3
16	1.56	s.	17.92	CH_3
17	2.53	d. J=7.7	36.35	CH_2
18	4.81	d.m. J=7.7	117.89	CH
19	-	-	134.94	-C-
20	1.56	s.	25.88	CH_3
21	1.78	s.	17.92	CH_3
22	-	-	207.12	-C-
23	4.03	sept. J=6.8	35.42	CH
24	1.12	d. J=6.8	18.96	CH_3
25	1.12	d. J=6.8	18.81	CH_3

a = Interchangeable assignments; c= Interchangeable assignments

b = enolic protons detected at 7.03ppm and 9.58ppm



critical micelle concentration (CMC) is exceeded. Micelles - molecular aggregates which are in dynamic equilibrium with free molecules (monomers) - continuously break down and reform and it is this property which distinguishes them from colloids (Florence & Attwood 1988). Micellisation has considerable relevance to antibacterial activity since it affects the number of discrete molecules or ions available in solution. If antibacterial activity is exerted solely by discrete molecules, then the potency of such compounds ceases to increase when the concentration is increased above the CMC (Lambert 1974). At, or above, the CMC the number of antibacterial agent molecules available to interact with bacterial cells remains constant as the total concentration is increased. Thus, it is important that the CMC (if any) of a surface-active antibacterial agent is measured so that the number of discrete molecules present at any given total concentration is known. For example, Lambert & Smith (1977) found that antibacterial activity of N-dodecyldiethanolamine was maximal at, or just below, the CMC but sharply diminished at higher concentrations.

Figure 2.9 shows that, at pH 7, both (-)-humulone and *trans*-isohumulone reduced the surface tension of NaDMG buffer. However, in comparison with the surface-active bactericide benzethonium chloride (a quaternary ammonium compound), their surface activity was small. More important is the absence of any inflexion in the line showing the relationship between surface tension and (-)-humulone or *trans*-isohumulone concentration. This indicates that, under the conditions tested, neither compound formed micelles. At pH 4 the solubility of each compound was so low that little surface activity was observed. For example, at this pH, the surface tension of a 10μM (-)-humulone solution (*ca* 20% in excess of the

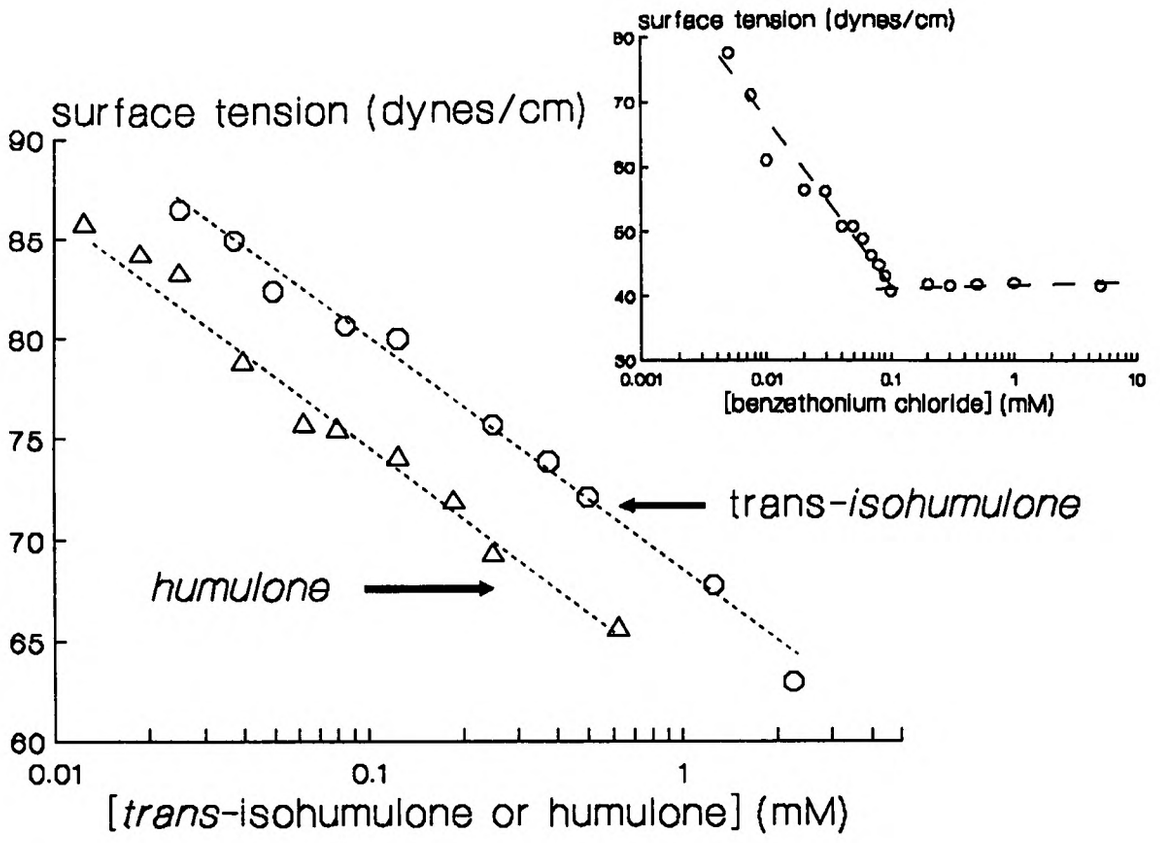


Figure 2.9 Surface activity of (-)-humulone and *trans*-isohumulone. Compounds were dissolved in 0.1M NaDMG buffer (pH 7.0) and measurements made using a DuNoüy tensiometer at 25°C. The inset figure shows the same relationship for the quaternary ammonium compound benzethonium chloride which displayed a critical micelle concentration (CMC) at 0.1mM.

solubility limit) was depressed by only 3.1dynes/cm. Similarly, the surface tension of a 0.11mM *trans*-isohumulone solution (slightly in excess of the observed solubility limit) was depressed by only 3.7dynes/cm. At both pH 7 and pH 4 *trans*-isohumulone exhibited less surface-activity than (-)-humulone. At pH 7 both compounds were predominantly (>99.9%) ionised. At pH 4 (-)-humulone was predominantly (*ca* 90%) undissociated while *trans*-isohumulone was predominantly (*ca* 90%) ionised (see section 2.3.4. for pKa values). Thus, factors other than ionisation must be important in determining the surface activity of such compounds.

2.3.4. Ionisation behaviour of hop compounds and hop-derived compounds

Potentiometric method

The pKa values of colupulone, (-)-humulone, *trans*-isohumulone, *trans*-humulinic acid and dehydrated humulinic acid were measured by potentiometry. This method generates a set of nine pKa values for each analysis. Albert & Serjeant (1984) state that for a set of results to be satisfactory 'no more scatter than 0.06 in a pKa value should be allowed for a set of readings in any one estimatation'. Scatter, calculated by taking the average antilogarithm of each pKa value, the logarithm of the average being expressed as the pKa, was defined as the largest deviation between the average pKa and any value in the set. Thus the pKa of benzoic acid (Table 2.9) is reported as 4.26$\pm$0.02.

In the case of hop compounds and hop-derived compounds acceptable precision was not attained. The most common causes of such errors are (i) the presence of impurities in the substance being titrated, and

Table 2.9: Determination of the pKa value of benzoic acid by potentiometry

Titrant added (equivalents)	pH	Calculated pKa
0.1	4.04	4.268
0.2	4.13	4.262
0.3	4.23	4.260
0.4	4.34	4.258
0.5	4.45	4.237
0.6	4.6	4.247
0.7	4.78	4.254
0.8	5.02	4.237
0.9	5.45	4.268
1.0	7.47	-

Mean pKa = 4.26

Maximum spread = 0.02

Values were determined at 25°C at sample concentration of 3.4×10^{-4}M. Titrant used was NaOH. Literature value for pKa of benzoic acid determined by potentiometry is 4.205 (King & Prue 1961). The small difference between the values is due to the fact that the experimentally-derived value is a 'concentration ionisation constant' while the literature value is an 'equilibrium ionisation constant'.

(ii) small inaccuracies in addition of the titrant (Albert & Serjeant 1984). Both faults are two aspects of a single error, namely a failure to maintain the correct ratio of titrant to test compound. In order to determine whether this was the cause of the imprecision, iterative calculations were performed by means of a computer program in which the contribution of such errors to precision was assessed. These calculations revealed that precision could be improved to a small extent by assuming that the weight of each compound had been overestimated by 3-8% (possibly due to the presence of residual solvent molecules). Values obtained under each set of conditions (reported without correction), together with their spread are given in Table 2.10. For reasons that will become apparent in section 2.4.3., it is appropriate to record the values as equilibrium pKa (pKa_{equil}) values. The pKa_{equil} of colupulone, *trans*-isohumulone and *trans*-humulinic acid was little altered by MeOH (50%), but that of (-)-humulone was substantially reduced.

Spectrophotometric method

Determination of pKa by spectrophotometry requires that absorption spectra of the undissociated and ionised forms of the test compound be obtained. Confirmation that spectra of the appropriate species have been isolated usually arises from the finding that identical spectra are obtained when each measurement is repeated at one pH unit higher or lower than that originally tested, depending on whether the undissociated or ionised forms of a weak acid are being investigated. In the case of colupulone, the spectrum of the putative undissociated form was obtained at all pH values tested below pH 4, that of the mono-ionised form at pH 8 and that of the di-ionised form at pH values above 9.5. In the case of (-)-humulone, the spectrum of the putative undissociated

Table 2.10: Equilibrium pKa values (pKa_{equil}) for hop compounds and hop-derived compounds determined by potentiometry

Compound	**pKa_{equil}**	
	water	MeOH/water (1:1)
colupulone	6.1 (5.82-6.33)	6.3 (6.26-6.75)
(-)-humulone	5.0 (4.94-5.12)	4.3 (4.19-4.42)
trans-isohumulone	3.1 (2.82-4.04)	3.2 (3.12-4.18)
trans-humulinic acid	2.7 (2.51-3.00)	2.8 (2.57-2.93)
dehydrated humulinic acid	n.d.	5.6 (4.83-5.64)

Values in parenthesis indicate the spread of results.

n.d. = not determined

form was obtained at all pH values tested below pH 3, while that of the mono-ionised form was obtained at pH 6.5-9.0. (Both colupulone and (-)-humulone are dibasic and have a second pKa [indicated by spectroscopic methods] at *ca* 8.8 in the case of colupulone and 11 in the case of (-)-humulone: see below).

The absorption spectrum of the putative undissociated form of *trans*-isohumulone was not easily obtained. In aqueous solution, coincident spectra were only obtained when the compound was exposed to highly acidic conditions (*e.g.* solutions containing 5% and 10% (v/v) HCl). The spectrum of the ionised form of *trans*-isohumulone was obtained at pH >3.0. The maximum difference in the absorbance values of the putative undissociated and ionised forms of *trans*-isohumulone was at λ=253nm. The relationship between the absorbance of *trans*-isohumulone and solution pH is shown in Figure 2.10. The absorbance at 253nm was not affected by pH changes in the region 2-6. Thus, although significant changes in the degree to which the compound was ionised occurred within this pH range (as indicated by pKa_{equil}=3.1-3.2 measured by potentiometry and by conductimetry) no absorbance changes were detected at any wavelength. Significant changes occurred only at pH values of one or more units below pKa_{equil}. From the spectroscopic data obtained, an apparent pKa (pKa_{app}) of 1.31$\pm$0.1 could be calculated.

In the case of (-)-humulone, which has two ionisable groups, changes could be monitored at two wavelengths. Substantial changes in the absorbance of (-)-humulone at λ=262nm occurred in the pH range 3.0-6.0 with pKa_{app}=4.08$\pm$0.1. Thus, in the case of the most acidic group, agreement between the value obtained and that derived from potentiometric measurements was poor. At high pH values, a second process, probably ionisation of

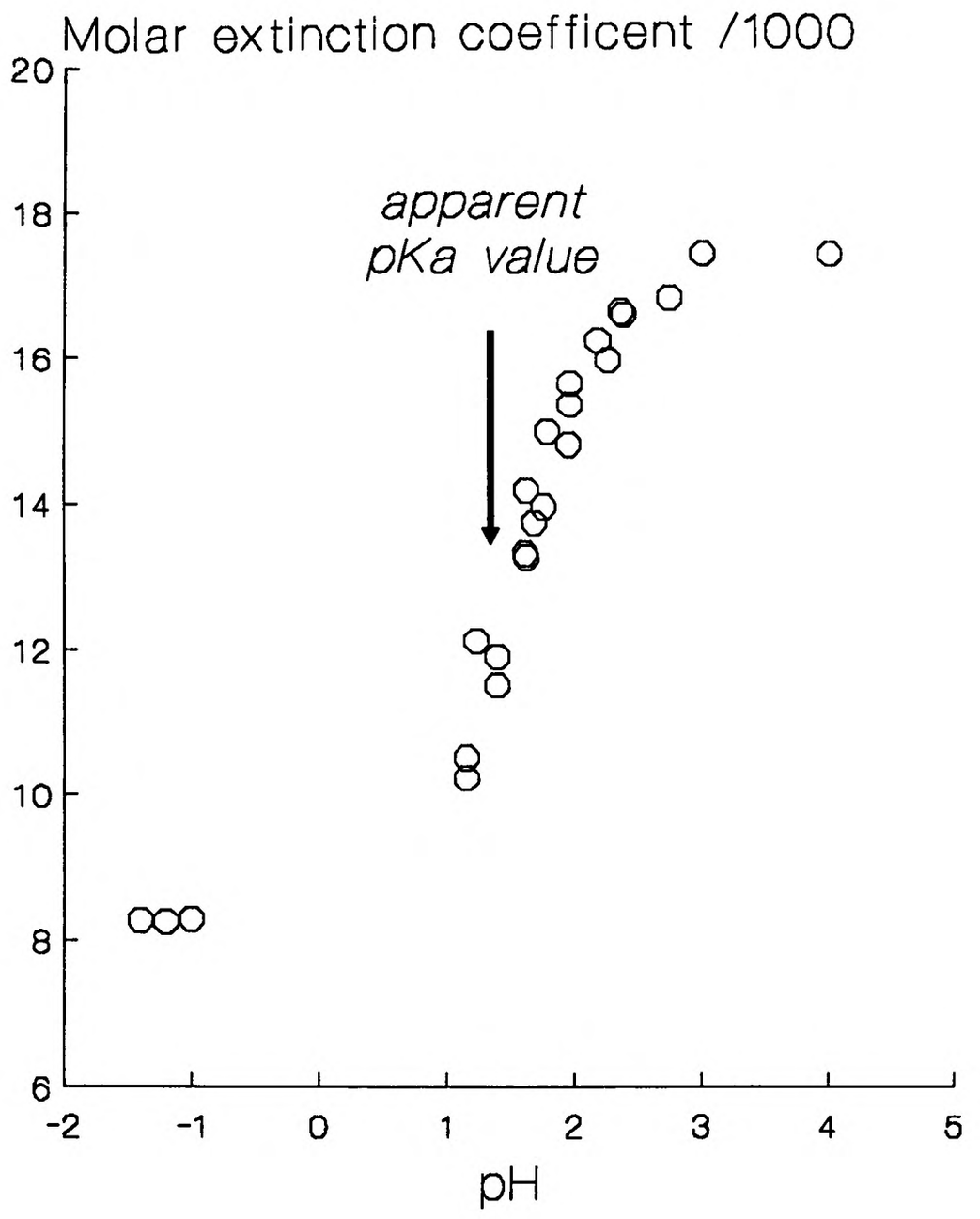


Figure 2.10 Variation of the extinction coefficient of *trans*-isohumulone with pH. *Trans*-isohumulone was dissolved in aqueous buffer of the pH value shown and the absorbance of the sample at 253nm measured against a sample of buffer of the same pH.

the more weakly acidic proton of (-)-humulone could be monitored. Although the process could be observed at λ=262nm, changes in absorbance were maximal at λ=325nm. This experiment indicated that the pKa_{app} of the more weakly acidic proton of (-)-humulone was *ca* 11.

Solubility method

The solubility of a weak acid in aqueous media is a function of three parameters, (i) the ionisation constant (pKa) of the acid; (ii) the intrinsic solubility of the neutral molecule (S_i) and the ionised molecule (S_o); and (iii) the pH of the solution. It is usually found that if the solution pH is known and the intrinsic solubility of the neutral molecule is determined (by measuring its solubility in acidic media; the solubility of the ionised form is always considerably greater than that of the undissociated form and is usually not considered in the calculations) the pKa of the compound may be calculated. Because dissolved ions influence solubility, the determinations were performed at constant ionic strength by addition of 0.1M NaCl (Albert & Serjeant 1984).

The apparent solubility of the compounds is shown in Figure 2.11. Limiting solubility values (S_i, the solubility of the least soluble species) at 25°C for colupulone, (-)-humulone and *trans*-isohumulone were 8μM, 8μM and 40μM respectively. In theory, pKa can be calculated by means of the following expression,

$$pKa = pH \quad \log[(S_o/S_i)-1] \qquad (2.6)$$

where S_o is the measured solubility at any given pH and S_i is the solubility of the neutral molecule. (pKa can also be deduced graphically by reading the value from the pH axis below the intersect of the two straight lines.)

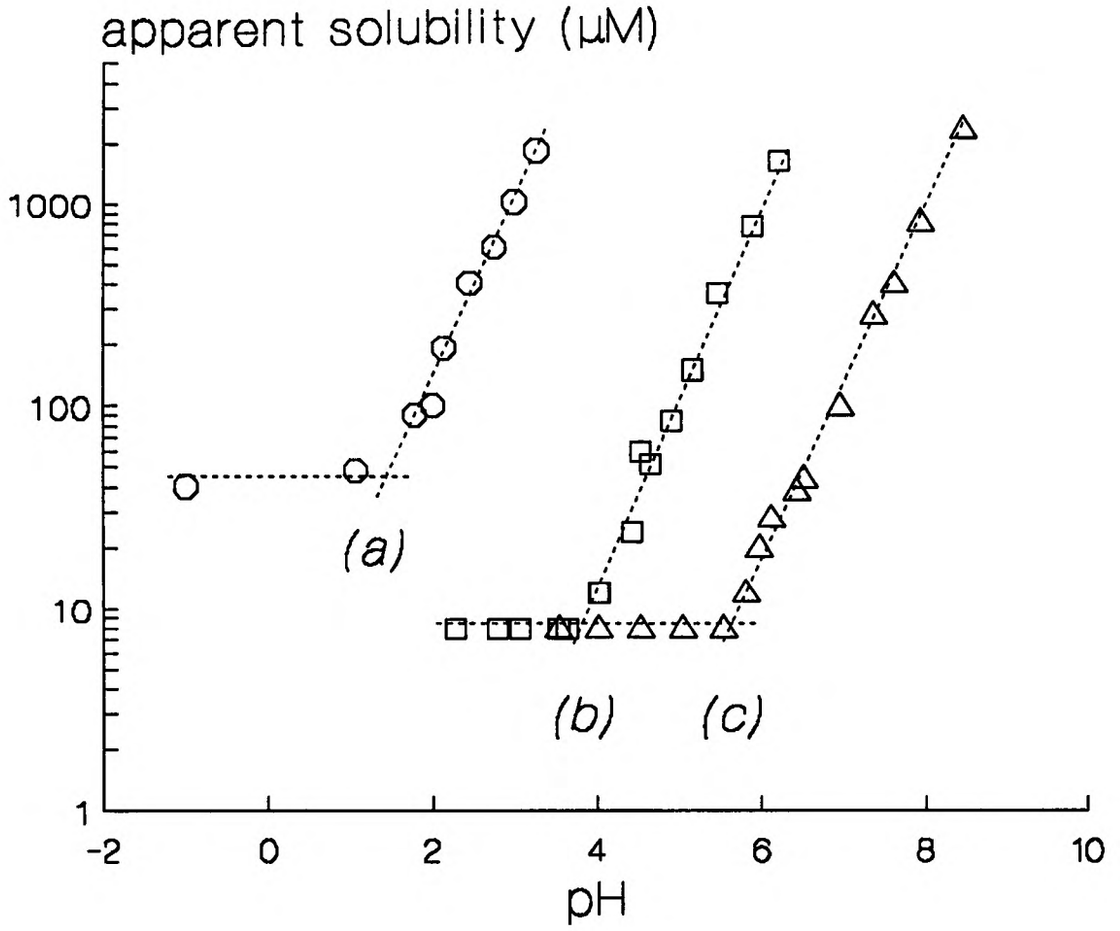


Figure 2.11 Relationship between the apparent solubility of hop compounds and hop-derived compounds and pH. *Trans*-isohumulone (*a*), (-)-humulone (*b*) or colupulone (*c*) were added to aqueous buffers of constant ionic strength (0.1M NaCl) at different pH values. Apparent solubility was determined visually. The intersects indicate, from their position with respect to the x axis, the apparent pKa values of the test compounds.

Manipulation of the data using equation (2.6) gave the following pKa values: colupulone, 5.89±0.12; (-)-humulone, 3.88±0.25; *trans*-isohumulone, 1.28±0.11. Figure 2.12 also shows that in the pH range at which ionisation of each compound occurred (as indicated by potentiometry and conductimetry) there was no inflexion in the solubility curve. This indicates that, (i) ionisation of the test compound did not influence its apparent solubility; and (ii) the undissociated form was not the least soluble species present.

Solvent partition method

The ability of the ionised and undissociated forms of a weak acid to partition between aqueous buffer and a non-polar hydrocarbon solvent usually differs. Because it carries a negative charge, the anionic form of a weak lipophilic acid preferentially accumulates in the polar phase since its partition into the non-polar organic phase is unfavourable. When a lipophilic compound, such as *trans*-isohumulone, is dissolved in a buffer solution of at least two pH units *below* pKa, it would be expected to almost completely partition into the organic hydrocarbon phase. If the compound is dissolved in buffer of at least two pH units *above* pKa, it would be expected to partition into the aqueous phase. Table 2.11 shows that (-)-humulone, colupulone and dehydrated humulinic acid had a strong affinity for the non-polar 2,2,4-trimethylpentane phase as indicated by their high log P. The effect of pH changes on the partition coefficent of (-)-humulone could not be determined since log P was so high. The effect of pH on the partition coefficent of *trans*-isohumulone is shown in Figure 2.12.

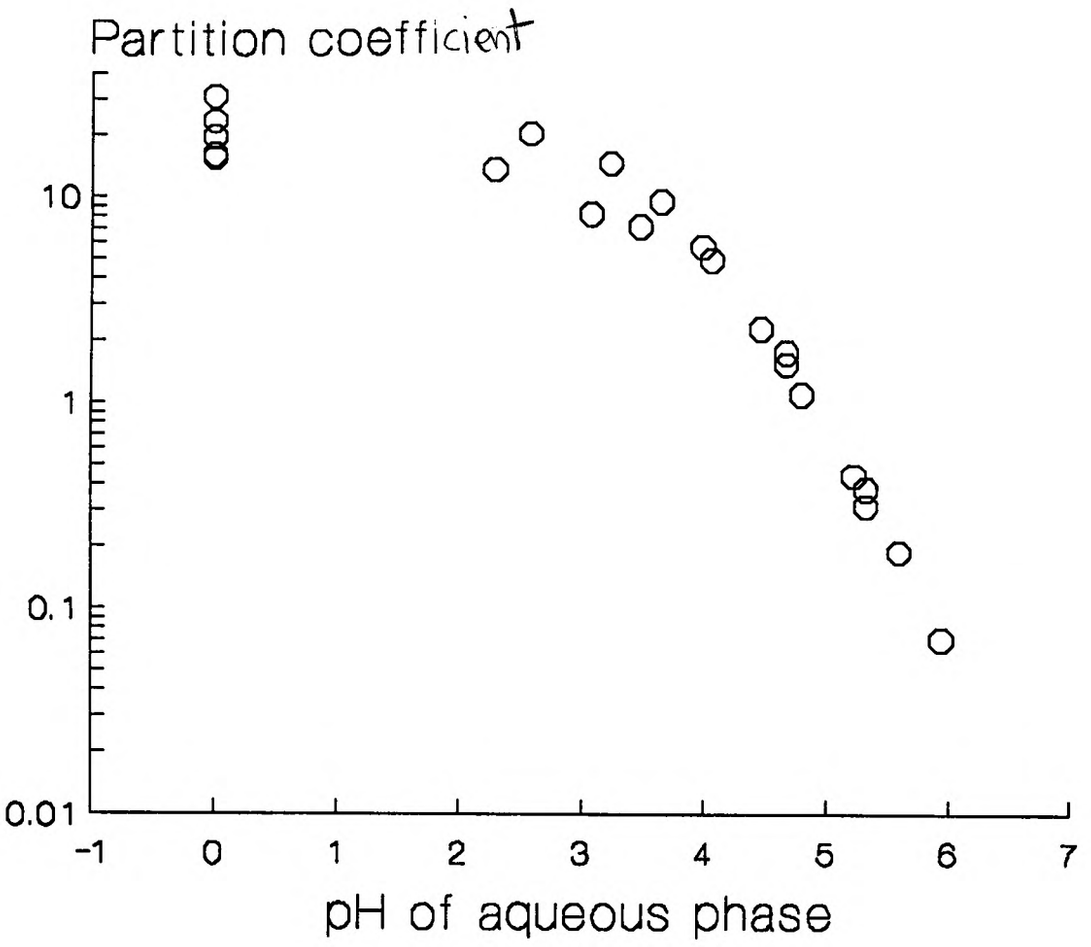


Figure 2.12 Relationship between the partition coefficient (P) of *trans*-isohumulone and pH. *Trans*-isohumulone was shaken in a two-phase solvent system, consisting of aqueous buffer and 2,2,4-trimethylpentane, for 10min at *ca* 5Hz. The amount of the compound in the 2,2,4-trimethylpentane phase was then determined by spectrophotometry. P = [*trans*-isohumulone in organic phase]/[*trans*-isohumulone in aqueous phase].

Table 2.11: Effect of buffer pH on partition coefficient (P) of hop compounds and hop-derived compounds

Test compound	log P* at pH			
	2.99	4.11	4.98	6.02
(-)-humulone	>2.3	>2.3	>2.3	>2.3
colupulone	2.17	2.18	1.76	1.29
dehydrated humulinic acid	>2.3	>2.3	>2.3	0.84

* Determined in an aqueous buffer/2,2,4-trimethylpentane system. Initial concentration of each compound in the aqueous phase was 80μM.

Conductimetric method

The pKa of *trans*-isohumulone measured in water by conductimetry was 3.23±0.17 (determined from a set of six measurements). The pKa value of acetic acid, determined in a control experiment (set of six measurements) was 4.72±0.27 (lit. 4.75 [Albert & Serjeant 1984]).

Effect of solvent composition on ionisation behaviour of trans-isohumulone

Determinations of the pKa of hop compounds and hop-derived compounds have previously been carried out in MeOH/H_2O mixtures. However, those studying the ionisation of such compounds appear to have overlooked the effect of alcohols on acid strength: the pKa of an acid is generally increased in the presence of alcohols. The influence of MeOH concentration on both the pKa_{equil} of *trans*-isohumulone (assessed by potentiometry) and the apparent pKa (assessed by UV spectrophotometry) was examined. Unexpectedly, the concentration of MeOH had little effect on the pKa of *trans*-isohumulone assessed by potentiometry. Table 2.10 shows that in the absence of MeOH, the pKa_{equil} of *trans*-isohumulone was 3.1 while in the presence of 50% (v/v) MeOH a value of 3.2 was obtained. (Clarke [1967] has reported that the pKa of *trans*-isohumulone in 90% MeOH was 3.1.) The influence of MeOH on the apparent pKa assessed by spectrophotometry was marked. In the absence of this solvent a value of 1.30±0.09 was recorded while in the presence of 50%, or 90% MeOH, values of 2.01±0.13 or 3.23±0.12 were obtained.

2.3.5. NMR spectroscopy of undissociated and ionised forms of *trans*-isohumulone in D_2O/MeOD mixture

The structure of *trans*-isohumulone in a mixture of D_2O and MeOD (50:50) and in the same solvent at high pH was examined using NMR spectroscopy. The assignment of the spectra (Tables 2.12 and 2.13) revealed several features of importance to the present study. Firstly, evidence for the ketonic form of *trans*-isohumulone was absent from all spectra. The major species detected under both sets of conditions was enolic. Many of the ^{1}H-NMR and ^{13}C-NMR signals arising from *trans*-isohumulone were little affected by ionisation. Methyl (C-15, C-16), quaternary (C-14), methine (C-13) and methylene (C-12) ^{1}H and/or ^{13}C resonances of the isohexenoyl side chain were almost coincident in acidic and alkaline D_2O/MeOD mixtures. The methyl groups (C-9, C-10) on the isopentenyl side chain also resonated at similar frequency in both solvents. The most significant changes were associated with those carbon atoms which were in conjugation with the ionisable enolic carbon atom (C-3) and the protons attached to such conjugated carbon atoms. The chemical shift of the protonated carbon atom at C-5 was displaced upfield by 1ppm in alkali while that of its attached proton was shifted downfield by 0.44ppm. The ^{1}H-NMR signals arising from the isopentenyl side chain were similar in both solvents. The methylene carbon (C-18) resonance from the isovaleryl side chain moved 5ppm upfield in alkaline D_2O/MeOD while the chemical shift of its attached diastereotopic protons moved upfield by *ca* 0.05ppm. The size of the chemical shift displacement indicates that a change in the electronegativity of the carbonyl carbon on C-17 may have taken place. For example, a different

Table 2.12: NMR spectral data of *trans*-isohumulone in alkaline MeOD/D_2O (1:1)

Position	^{1}H-NMR		^{13}C-NMR	
	δ (ppm)	Coupling Pattern	δ (ppm)	Multiplicity (DEPT)
1	-	-	unassigned	-C-
2	-	-	112.98	-C-
3	-	-	unassigned	-C-
4	-	-	90.6	-C-
5	2.59	d.d J=10.0,5.2	58.5	CH
6	α2.10 β2.52	d.d.d. J=15.6,10.0,10.0 m.	25.44 (25.34 weak tautomer)	CH_2
7	5.14	m.	123.8	CH
8	-	-	133.3	-C-
9	1.64	s.	25.9	CH_3
10	1.49	s.	18.1	CH_3
11	-	-	unassigned	-C-
12	not detected	-	not detected *	CH_2
13	5.21	m.	117.1	CH
14	-	-	135.6	-C-
15	1.70	d. J=1.1	25.88 (25.93 weak tautomer)	CH_3
16	1.56	d. J=1.1	18.3	CH_3
17	-	-	unassigned	-C-
18	α2.62 β2.68	d.d. J=13.6,6.8 d.d. J=13.6,7.2	50.91	CH_2
19	2.08	nonet J=6.8	26.8	CH
20	0.92	d. J=6.8	23.3	CH_3
21	0.90	d. J=6.8	23.2	CH_3

Carbonyl/enolic peaks detected at δ = 199.4, 199.6, 202.8, 211.6ppm.

* Intensity of signal reduced to zero by coupling to deuterium.

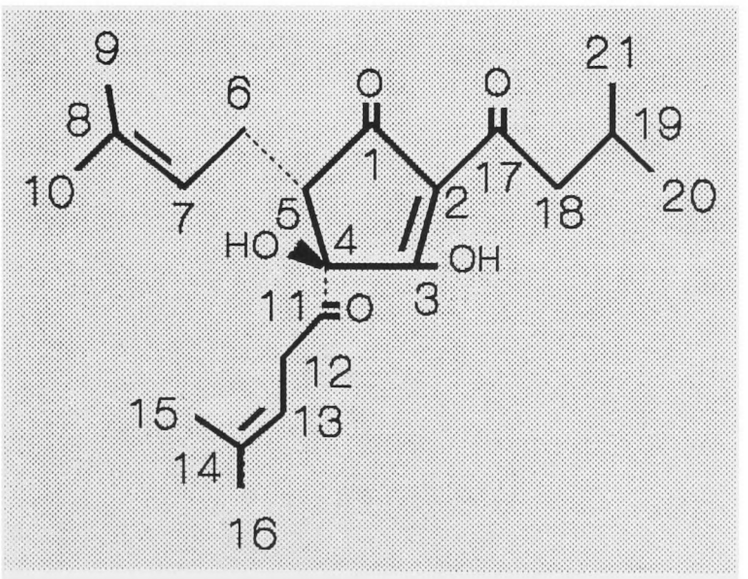
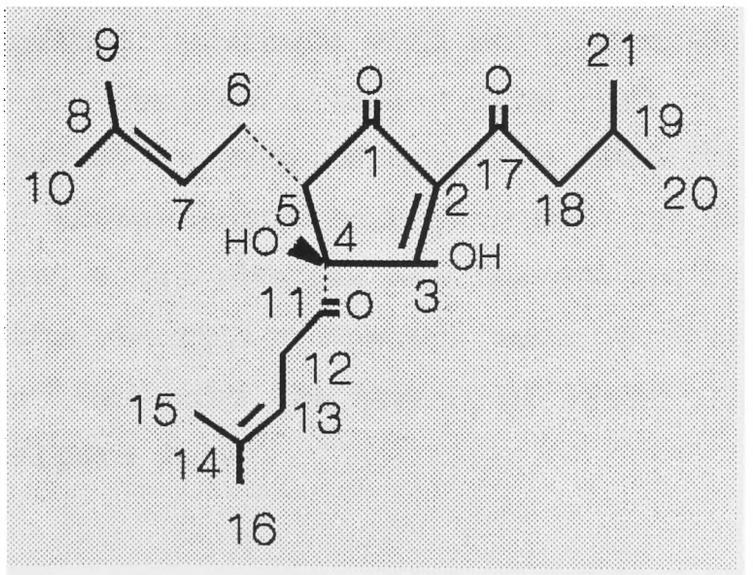


Table 2.13: NMR spectral data of *trans*-isohumulone in acidic MeOD/D_2O (1:1)

Position	^{1}H-NMR		^{13}C-NMR	
	δ (ppm)	Coupling Pattern	δ (ppm)	Multiplicity (DEPT)
1	-	-	unassigned	-C-
2	-	-	112.4	-C-
3	-	-	un xsigned	-C-
4	-	-	90.7	-C-
5	2.95	d.d J=9.5,5.7	57.5	CH
6	α2.26 β2.49	d.d.d. J=15.2,9.5,9.5 m.	24.7	CH_2
7	5.18	m.	122.3	CH
8	-	-	134.7	-C-
9	1.67	s.	25.88 (25.91 weak tautomer)	CH_3
10	1.51	s.	18.0	CH_3
11	-	-	unassigned	-C-
12	α3.41 β3.48	d.d. J=18.4,6.5 d.d. J=18.4,6.5	39.7	CH_2
13	5.23	m.	116.4	CH
14	-	-	136.2	-C-
15	1.72	s.	25.91 (25.88 weak tautomer)	CH_3
16	1.58	s.	18.2	CH_3
17	-	-	unassigned	-C-
18	α2.68 β2.77	d.d. J=13.8,7.0 d.d. J=13.8,7.0	45.9	CH_2
19	2.11	m.J=7.0	27.5	CH
20	0.97	d. J=7.0	22.9	CH_3
21	0.94	d. J=7.0	22.7	CH_3

Carbonyl/enolic resonances detected at δ = 198.5, 198.6, 203.9, 209.9ppm.



enolic tautomer may be favoured in alkali; hydrogen bonding may be more or less favoured; or the carbonyl group may have been covalently hydrated in alkali.

2.3.6. Absorption spectrum of *trans*-isohumulone in piperidine

The absorption spectrum of *trans*-isohumulone in piperidine is shown in Figure 2.13. Piperidine is an anhydrous base and two possibilities exist for the behaviour of a weak acid such as *trans*-isohumulone in this solvent. If *trans*-isohumulone was unable to form covalent hydrates then the spectrum obtained in piperidine should resemble that obtained in aqueous alkaline solvents. However, if the anion of *trans*-isohumulone was able to hydrate in aqueous media then the UV spectra obtained in aqueous alkaline media and piperidine should be different since, in the latter solvent, covalent hydration is not permitted. Since the spectrum obtained differs from that obtained in aqueous solvents at low or high pH, the possibility of covalent hydration of *trans*-isohumulone is clearly indicated.

2.3.7. Complex formation between *trans*-isohumulone and Mn^{2+}

Spectrophotometric analysis revealed that, in MeOH, *trans*-isohumulone interacts with Mn^{2+}. The UV absorption spectrum of the complex differed from that of *trans*-isohumulone in MeOH or alkaline MeOH (Figure 2.14). Titration of *trans*-isohumulone with Mn^{2+} (as $MnCl_2$) indicated that a 1:1 complex was formed. No further changes in the absorption spectra were observed when the amount of Mn^{2+} exceeded the amount of *trans*-isohumulone in the assay (Figure 2.14).

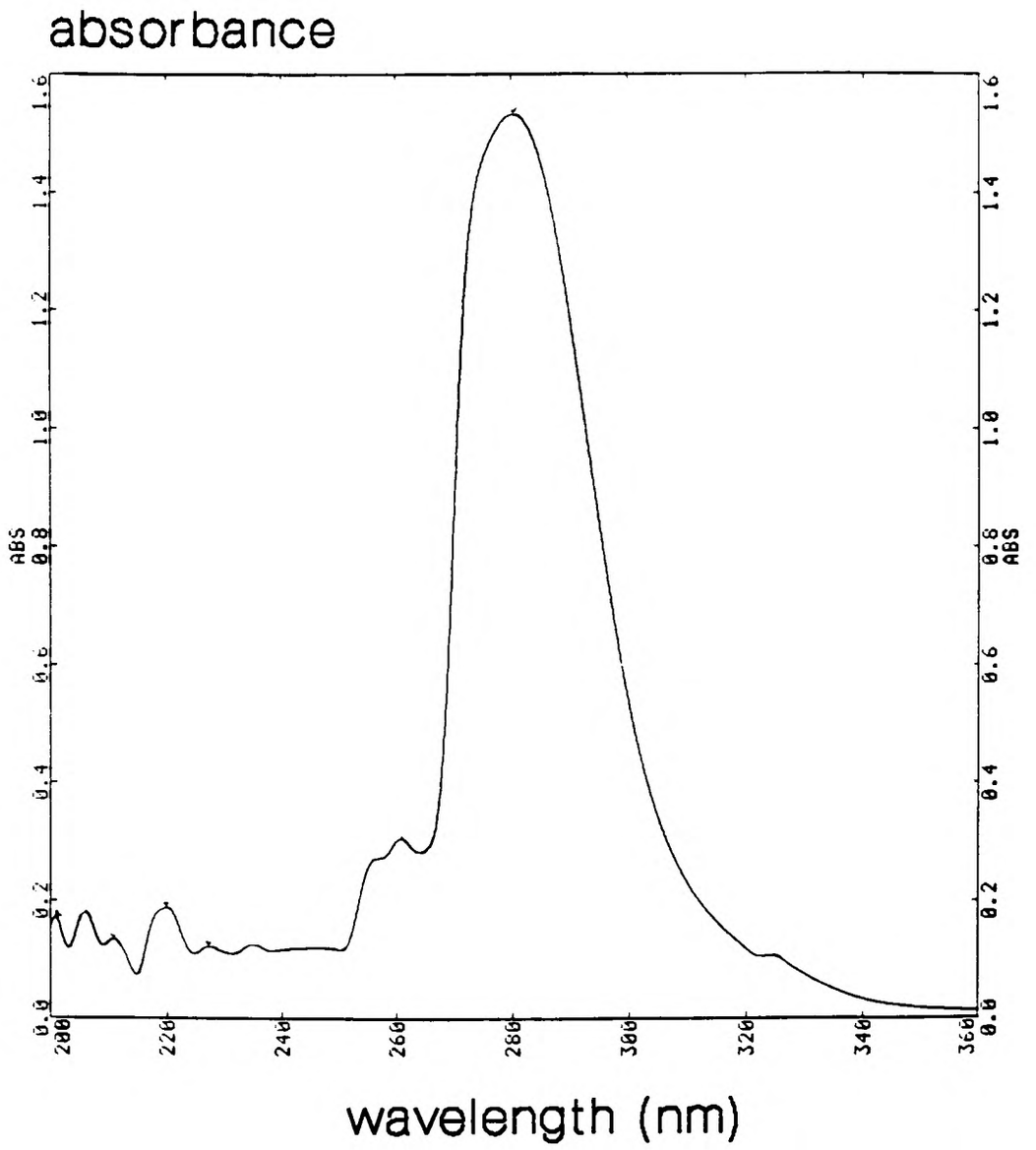


Figure 2.13 UV absorption spectrum of *trans*-isohumulone in piperidine. The spectrum was obtained using quartz cells of 1cm path length at 25°C against a piperidine blank. λ_{max}=281nm; ε=11,860. The portion of the spectrum below 260nm is of no diagnostic value since the solvent absorbed light in this region.

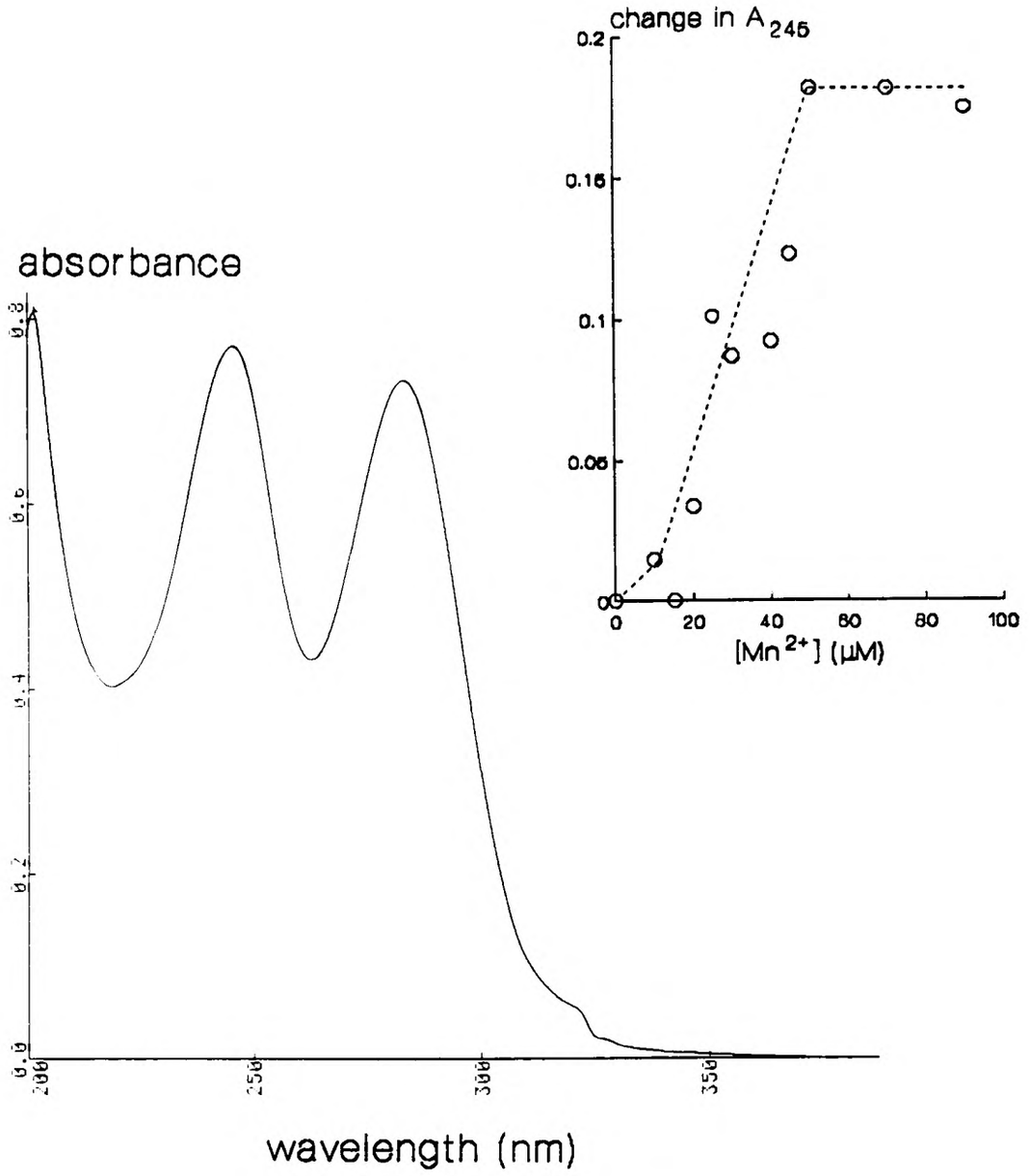


Figure 2.14 UV absorption spectrum of the complex formed between *trans*-isohumulone and Mn^{2+} in MeOH. A complex with identical properties was also formed between *trans*-isohumulone and Mn^{2+} in alkaline MeOH. The inset figure shows the absorbance changes (λ=245nm) which occurred when a solution of *trans*-isohumulone (50μM) was titrated with $MnCl_2$. The data indicates a stoichiometry of 1:1.

2.4. DISCUSSION

2.4.1. Structure of hop compounds and hop-derived compounds

The molecular structure of each of the hop compounds and their derivatives was in agreement with that presented in the literature. UV and IR data were identical to that previously reported (Clarke & Hildebrand 1965; Claus *et al.* 1978). The mass of the molecular ion of colupulone, (-)-humulone, *trans*-isohumulone and dehydrated humulinic acid was easily determined by mass spectrometry since, for the first time, a 'soft' ionisation process employing a thermospray, was used. This technique allowed significant proportions of the parent molecular ion to be detected. In previous MS studies of hop compounds and hop-derived compounds, the parent ion had been detected only in very small quantities (*e.g.* see Brohult *et al.* 1959).

Assignment of NMR data

NMR spectroscopy was first applied to hop compounds and hop-derived compounds more than 40 years ago when ^{1}H-NMR was used to study the structure of α-acids and a derivative called humulinone (Shoolery *et al.* 1960). The technique has since been used to analyse a range of such compounds. Previous NMR studies mostly employed continuous-wave spectrometers of relatively low magnetic field strength. In the present experiments multi-pulse techniques, including a number of two-dimensional methods, were used to probe molecular structure.

In addition to confirming, to a large extent, previous ^{1}H-NMR and ^{13}C-NMR data reported by Shoolery (1960), Alderweireldt & Anteunis (1964), Alderweireldt & Verzele (1964), Alderweireldt *et al.* (1965), Spetsig (1965), Durant (1966), Connett & Elvidge (1969), Collins

et al. (1971), Laws & Elvidge (1971), Borremans *et al.* (1975) and Davis (1977) it has been possible to make several improvements in assignment of the spectra. Thus, the methyl signals from the isohexenoyl and isopentenyl side chains of *trans*-isohumulone, and their counterparts in (-)-humulone, both of which have previously been ambiguously (Burton *et al.* 1964) or incorrectly (Laws & Elvidge 1971) assigned have now been correctly identified. The signals are analogous to those of substituted isobutenes in which the ^{1}H-NMR resonances are separated by *ca* 0.08ppm (Prestsch *et al.* 1989).

The non-equivalence of the methylene protons of the isopentenyl side chain of *trans*-isohumulone and *trans*-isocohumulone and of a number of different methylene groups in similar compounds has not been previously commented upon. The cause of the diastereotropism of some of these methylene groups is not clear.

Dehydrated humulinic acid was predominantly (>99%) enolised in $CDCl_3$ solution. No signals associated with the ketonic form were observed in any of the spectra. The enolisation of the compound in this solvent closely resembled that of each of the other hop compounds and hop-derived compounds as indicated by the chemical shift of the methylene carbon (C-12) adjacent to the carbonyl group on the isovaleryl side chain. The value of 47.93ppm obtained for this group (C-12) in dehydrated humulinic acid was close to that of (-)-humulone (46.50ppm), *trans*-isohumulone (44.44ppm), and *trans*-humulinic acid (46.01ppm). The ^{1}H-NMR data obtained was consistent with that presented by Durant (1966) for dehydrated cohumulinic acid. The ring methylene group at C-4, reported by Durant (1966) as a singlet, gave a doublet (J=1.7Hz) reflecting its coupling to the methine proton on C-6. The chemical shift of the methyl groups (C-14,

C-15), methine group (C-13) and methylene group at C-12 are now reported for the first time. (*n.b.* The report of Durant [1966] on isolation and characterisation of dehydrated humulinic acid was rather misleading since, having described the preparation and characterisation of this compound in some detail, NMR data for dehydrated cohumulinic acid were presented).

With the exception of the carbonyl group on the isovaleryl side chain of humulone and isohumulone and its equivalent in related congeners (Borremans *et al.* 1975) the carbonyl/enolic ^{13}C-NMR signals associated with such compounds have not been previously assigned. Attempts were made to assign such resonances in several ways. NMR lines arising from such carbon atoms were rather broad but could be sharpened by cooling the sample to -50°C during the analysis. COLOC experiments on supercooled *trans*-isohumulone samples were not successful.

A ^{1}H-coupled ^{13}C-NMR experiment, optimised for long-range ^{13}C-^{1}H couplings, allowed the identity of the carbonyl group on the isovaleryl side chain to be confirmed. Previously the identity of this group had been deduced from a comparison of the chemical shifts of the carbonyl groups of related compounds (Borremans *et al.* 1975). The signal from this carbonyl was a triplet of doublets (J_{C-H} = 2.9, 2.5Hz) reflecting its coupling to the methylene protons (C-12) and methine proton (C-13). The carbonyl/enolic carbon atom on C-3 was identified by its lack of ^{13}C-^{1}H coupling. Assignment of the remaining two carbonyl/enolic functions (C-1, C-11) was not possible. Both peaks displayed minor splittings (a doublet would be expected for C-1 and a triplet for C-11) but the peak shape could not be interpreted since the lines were broad, probably as a result of tautomeric equilibria. Further work is required to assign these resonance lines.

Similar experiments were not performed on the other compounds. Although the carbonyl and enolic NMR signals of *trans*-isocohumulone can be assigned by comparison with those of *trans*-isohumulone it was not possible to assign those of the other compounds in this way.

2.4.2. Identification and quantification of contaminants

Many hop compounds and hop-derived compounds are difficult to separate from one-another (Verzele 1986). The HPLC methods reported here allowed samples of (-)-humulone, (-)-cohumulone, *trans*-isohumulone and *trans*-isocohumulone which were free of related congeners (as assessed by HPLC, ^{1}H-NMR and ^{13}C-NMR) to be prepared.

In their usual form, ^{13}C-NMR spectra can not be used to quantitate contaminant congeners since peak areas are distorted by a combination of the NOE and relaxation time differences. Application of the inverse-gated decoupling technique, or use of the relaxation agent chromium acetylacetonate (CrAcAc) allowed contaminant congeners in a (-)-humulone preparation to be quantified. For future investigations, where identification and quantification of contaminant congeners is required, application of CrAcAc is likely to prove most useful since, unlike the inverse-gated decoupling technique, its use does not necessitate an increase in the analysis time.

2.4.3. Ionisation behaviour of hop compounds and hop-derived compounds

A number of methods can be used to determine pKa values (reviewed in Albert & Serjeant 1984) including potentiometric methods; conductimetric methods; methods based on determination of the partition coefficient of the compound in a two-phase solvent system; calorimetric

methods; chromatographic methods; and methods employing Raman spectroscopy, ^{1}H-NMR spectroscopy, ^{13}C-NMR spectroscopy or UV absorption spectrophotometry. pKa values of hop compounds and hop-derived compounds have previously been determined using methods based on potentiometry (Spetsig 1955; Rudin 1960), spectrophotometry (Ono *et al.* 1985) and solvent partition behaviour (Hedlund & Steninger 1948). Reported values for each of the compounds vary widely and were thus considered to be unsatisfactory. For example, values of 3.1-3.8 have been reported for isohumulone (Cook & Harris 1950; Clarke 1967; Westerman *et al.* 1971; Seaton *et al.* 1986); those for humulone range from 4.0-5.5 (Hedlund & Steninger 1948; Cook & Harris 1950; Spetsig 1955; Stevens 1967; Westerman *et al.* 1971; Verzele 1986) while those reported for lupulone range from 5.5-7.8 (Hedlund & Steninger 1948; Spetsig 1955; Westerman *et al.* 1971).

The choice of solvent for such analyses is also questionable. Although reported as pKa values, with the exception of those reported by Spetsig (1955), some of the values (*e.g.* those of Clarke 1967) are in fact PsKa values (Albert & Serjeant 1984) since they were obtained using partly aqueous solvents (usually MeOH/water mixtures). In some cases (Cook & Harris 1950; Stevens 1967; Westerman *et al.* 1971; Seaton *et al.* 1986; Verzele 1986) no indication as to the identity of the solvent employed for the determinations has been given (although some of the values seem to have been taken from uncited references rather than derived experimentally). However, since the solubility product of hop compounds and hop-derived compounds in water is very low, it is likely that many of these values have been obtained by the use of partly aqueous solvents. Notable exceptions to this practice include, (i) the report of Spetsig (1955) in which pKa values for lupulone, humulone and humulinic

acid (probably a mixture of congeners and stereoisomers), determined in water at 25°C, were presented, and (ii) that of Rudin (1960) in which the pKa of isohumulone (mixture of congeners and stereoisomers) was determined in water. In both cases, however, the authors used a saturated, or supersaturated solution of the test compound for the determination. This practice, which is based on a method described by Back & Steenberg (1950) can only yield approximate results, a fact acknowledged by the progenitors of the method. Because the amount of test substance dissolved at any one time is not known, the degree to which it has been neutralised by titrant is also unknown. Without such information it is impossible to derive an accurate pKa. In the present work, pKa values of hop compounds and hop-derived compounds were determined using a potentiometric technique performed in such a way as to maximise precision.

Potentiometric titration of *trans*-isohumulone showed that the pKa value of this compound was *ca* 3.1, while those of (-)-humulone, colupulone and *trans*-humulinic acid were *ca* 5.0, 6.1 and 2.7 respectively. In all cases, however, the values had an unacceptable spread (*e.g.* 2.8-4.0, in the case of *trans*-isohumulone). Control experiments showed that the experimental technique used allowed an acceptably high degree of precision to be obtained.

In order to clarify the position, ionisation constants of hop compounds and hop-derived compounds were measured using a spectrophotometric method. This method is reported to be particularly useful for application to substances of low aqueous solubility (Albert & Serjeant 1984). The pKa of *trans*-isohumulone determined in this way was precise (1.31 ± 0.10) but the value differed considerably from that derived from potentiometric experiments. The lack of agreement between the

spectroscopic behaviour of isohumulone (mixture of *cis*- and *trans*-isomers) and its pKa value determined in water (a saturated solution of isohumulone was titrated) has previously been highlighted by Rudin (1960). In order to find out which values were correct, the solubility of *trans*-isohumulone, (-)-humulone and colupulone was determined at 25°C in aqueous buffers of constant ionic strength. pKa values obtained from solubility data agreed closely with those derived from spectroscopic measurements. Similar pKa values could be calculated using solubility values reported in the literature (Wöllmer 1918; Brohult *et al.* 1955). Measurements of the pKa value of *trans*-isohumulone obtained from solvent partition data were equivocal: partition coefficients for this compound in a two phase system, consisting of aqueous buffer and 2,2,4-trimethylpentane, were too high to allow sufficient accuracy to be obtained. The data obtained using this method, however, indicated that the pKa value of *trans*-isohumulone was <3. Conductimetric measurements indicated that the pKa of *trans*-isohumulone was 3.23 ± 0.17, a value in good agreement with that obtained by potentiometry.

One possible explanation of the discrepancies in pKa was that *trans*-isohumulone participated in other equilibrium processes. Thus, potentiometric and conductimetric determinations indicated the pH range at which ionisation took place, but spectrophotometric and solubility determinations monitored a second equilibrium process. Several possible processes were considered: (i) keto-enol tautomerism, (ii) metal complexation, and (iii) covalent hydration.

As mentioned in Chapter 1, all of the hop compounds and hop-derived compounds considered in this thesis possess a β-triketone grouping. Although such compounds are usually enolised in solution (Forsén *et al.* 1964)

they are in equilibrium with a small amount of ketonic tautomer, the amount being determined by the tautomeric constant (K_t). The tautomeric constants (K_t=[enol]/[keto]) of hop compounds and hop-derived compounds have not been reported. Although in simple aliphatic and alicyclic ketones or aldehydes the equilibrium is greatly in favour of the keto form (Forsén & Nilsson 1970) the enolic form of β,β'-triketones is favoured in aqueous solution. One of the major factors governing this state of equilibrium is stabilisation of the enolic form of such compounds by hydrogen bonding of the enolic proton. Could the anomalous ionisation constants of *trans*-isohumulone and other hop compounds and hop-derived compounds have resulted from keto-enol tautomerism? Supporting this view were the observations that (i) pKa values of ketonic and enolic forms often differ from one another (Perrin *et al.* 1981) and, (ii) the physicochemical behaviour (*e.g.* aqueous solubility, partition coefficient) of keto and enol forms can differ to a substantial degree (Forsén & Nilsson 1970). It seemed possible that in aqueous or alcoholic solution, an equilibrium existed between the ionised enolic, undissociated enolic and undissociated ketonic forms of *trans*-isohumulone (more complex equilibria are also feasible). However, NMR spectra recorded under conditions in which each of the forms was isolated indicated that an enolic tautomer of *trans*-isohumulone was the major species present in both cases. The possibility that keto-enol equilibria were responsible for the anomalous pKa values could therefore be eliminated.

The prospect that metal complexation plays a role in the phenomenon can not be eliminated at present, although the evidence suggests that metal complexation alone can not be responsible. The bulk of the evidence favours the

hypothesis that hop compounds and hop-derived compounds participate in a phenomenon known as covalent hydration.

2.4.4. Covalent hydration

Covalent hydration (reviewed in Albert & Armarego 1965; Perrin 1965; Albert 1967; Ogata & Kawasaki 1970; Albert 1976), one well-documented cause of anomalous pKa values, can potentially occur in molecules which possess one or more double bonds. If a bond is highly polarised it tends to attract a water molecule (or one of the two water ions) to within reacting distance (Albert & Armarego 1965). The water molecules, or ions, then react with the atoms participating in double bond formation to form a covalent hydrate. In alcoholic solution, alcohol rather than water usually adds across the polarised bond. The hydrated forms of weak acids generally have higher pKa values than their anhydrous counterparts. The equilibrium pKa values are not, however, easily predictable (Perrin *et al.* 1981). For example, the observed pKa value of pyruvic acid is 2.49. This value is an equilibrium pKa (pKa_{equil}) arising from the pKa of the anhydrous form of the acid (1.55) and from that of the covalently-hydrated form (2.95) (Albert & Serjeant 1984). In the case of this compound hydration occurs at the carbonyl group and the equilibrium can be depicted as shown in Figure 2.15. Although covalent hydration results in marked changes in the physicochemical and biochemical behaviour of pyruvic acid, the phenomenon is not prominent in any of the major undergraduate biochemistry textbooks. This is perplexing since pyruvic acid is a compound of particular importance in biochemistry. According to Albert (1976), the presence of the covalently-hydrated form of a compound is

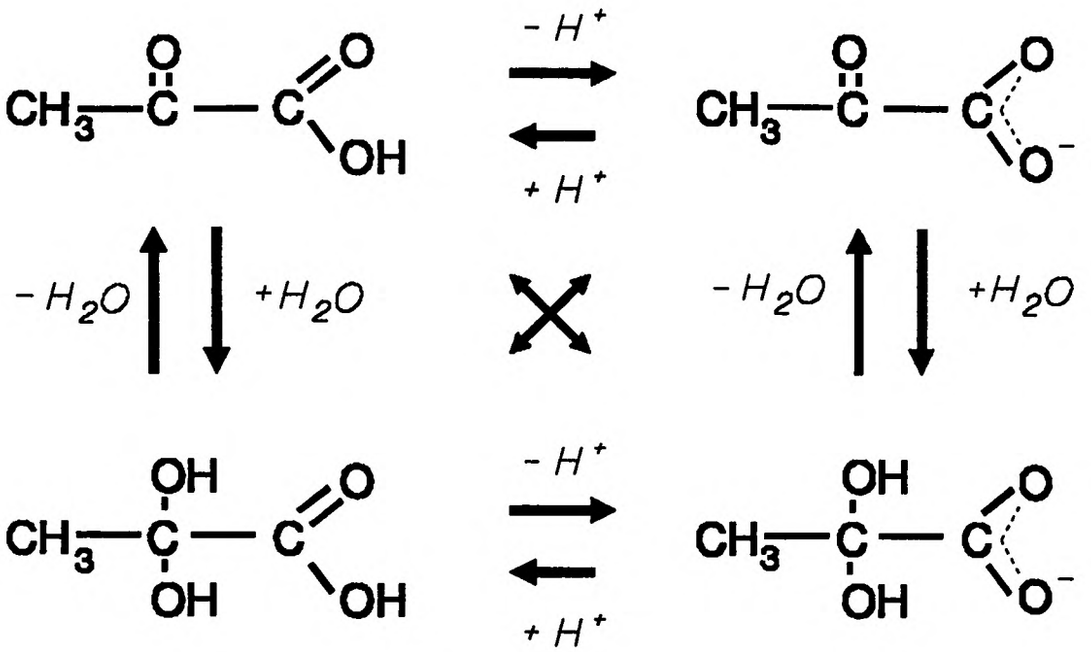


Figure 2.15 Covalent hydration of pyruvic acid. In aqueous solution the compound exists in a state of equilibrium between undissociated hydrated, undissociated dehydrated and ionised forms.

frequently overlooked in analysis of its molecular structure since the hydrated species is seldom subjected to analysis.

There are a number of ways to determine whether a weak acid forms a covalent hydrate. Comparison of pKa values obtained by experiment can be compared to those predicted by calculation (*e.g.* using the methods described by Perrin *et al.* 1981). Sequential potentiometric titration of such compounds in water, using acid then base, can produce a hysteresis curve when the degree of neutralisation is plotted against pH, due to a rate-limiting hydration step. Comparison of the absorption spectrum of the compound in cyclohexane (an aprotic solvent) with that determined in aqueous buffer at low pH allows differences relating to interaction of the test compound with the test solvent to be highlighted (Albert & Armarego 1965). Differences in the spectra can indicate that in aqueous solution, at low pH, the compound is hydrated, though this is not invariably so. A reduced ability to form hydrogen bonds with the solvent could also be responsible for the effect. Also, it is frequently observed that a compound which forms covalent hydrates displays identical light absorption properties in both strong solutions of HCl and cyclohexane (since the availability of water in both solvents is severely limited). Another commonly applied test is to determine the absorption spectrum of the test compound in an anhydrous strong acid such as dichloroacetic acid. In this solvent the spectrum of the dehydrated undissociated molecule should be obtained. Unfortunately, this test cannot be applied to the study of hop compounds and hop-derived compounds since dichloroacetic acid absorbs light at λ=<305nm (the region of the spectrum of interest in the examination of hop compounds and hop-derived compounds). An alternative is to determine the

absorption characteristics of the anhydrous ion of the weak acid by measuring the absorption spectrum of the compound in an anhydrous base such as piperidine. Lack of water molecules results in rapid dehydration of the test compound while the strongly basic nature of the solvent ensures that only the ionised form of the weak acid is present.

Verzele & Dierckens (1969) reported the formation and isolation of an hydrated form of isohumulone. Using countercurrent distribution techniques they isolated and presented ^{1}H-NMR evidence for hydrated *cis*-isohumulone. In the structure presented, water was added across the carbon-carbon double bond of the isopentenyl side chain, but the carbonyl bonds remained dehydrated. They proposed that such compounds were formed during wort boiling and that they probably occurred in beer. They did not, however, discuss the possibility of hydration equilibria.

Several lines of evidence indicate that hop-derived compounds such as *trans*-isohumulone participate in the reversible formation of covalent hydrates. The evidence suggests that hydration occurs at one or more carbonyl groups, but there is no indication that reversible hydration of any C=C bond takes place under any of the conditions used.

The evidence for covalent hydration of hop compounds and hop-derived compounds is as follows. Firstly pKa values differed substantially depending on which measurement method was used. The pKa of *trans*-isohumulone determined by potentiometry was imprecise, a value of *ca* 2.9-3.2 being obtained. There was no evidence of hysteresis when the compound was back-titrated and re-titrated several times: this indicates that the hydration reaction is moderately fast. Conductimetric analysis indicated a pKa value of 3.23 ± 0.17. Measurements made by spectrophotometry and

from solubility measurements were very precise, but considerably lower, values of 1.31±0.10 and 1.28±0.11 being obtained. This behaviour is consistent with the hypothesis that the anhydrous undissociated form of *trans*-isohumulone has a characteristic absorption spectrum which differs from that of the hydrated undissociated form. It is also to be expected that, in aqueous solution, the solubility of the dehydrated form of *trans*-isohumulone would be considerably lower than that of the hydrated form.

It is possible to predict the pKa value of many acids and bases to an accuracy of a few tenths of a pH unit. Several methods are used and have been described in a review (Barlin & Perrin 1966) and a practical handbook (Perrin *et al.* 1981). At a given temperature, the pKa value for the ionisation of a proton from a molecule is related to the change in free energy (ΔG°) by the following equation,

$$\Delta G^\circ = 2.3026RT.pKa \qquad (2.7)$$

where R=gas constant and T=temperature (°K). The pKa values of related compounds can therefore be discussed in terms of factors which affect ΔG°. The change in free energy resulting from insertion of a substituent into a molecule leads to a change in pKa. The observation that such changes are mostly additive provides the basis of the Hammett and Taft equations for prediction of pKa. Methods based on use of the Hammett and Taft equations are the most widely used of pKa prediction techniques (Perrin *et al.* 1981). Calculations of pKa can also be made using methods based on extrapolation from related compounds; by analogy with molecules possessing substituents of a similar nature; and, less successfully, by *ab initio* calculation (Perrin *et al.* 1981).

In the case of hop compounds and hop-derived compounds there was insufficient data available to allow prediction of pKa by extrapolation, analogy or *ab initio* calculation. Since there was also no Hammett and Taft equation available for enolic compounds with a cyclopentadione or cyclohexadione nucleus it was not possible to predict the pKa values of such compounds. To what extent the measured pKa values of such compounds differ from that expected is therefore unknown.

The absorption spectrum of *trans*-isohumulone, in aqueous buffer at low pH (pH >2.5), differed substantially from that obtained in cyclohexane. However, spectra obtained in strong aqueous solutions of HCl resembled those obtained in 2,2,4-trimethylpentane and cyclohexane. If *trans*-isohumulone did not participate in hydration/dehydration equilibria similar spectra should have been obtained in all three media (although shifts of 5-10nm could have been expected to result from the reduction in hydrogen bonding of *trans*-isohumulone molecules with the solvent). In addition, the spectrum obtained for *trans*-isohumulone in piperidine was different from that obtained in any other solvent. It seems likely that the spectrum obtained was that of the anhydrous (non-hydrated) ion of *trans*-isohumulone.

Verzele *et al.* (1965) have reported that, in MeOH, the optical rotation ($[\alpha]_D^{20}$) of *trans*-isohumulone is -7.8°, while in alkaline MeOH a value of +40.4° is obtained. A similar relationship between the pH of the medium and the optical rotation of other hop compounds and hop-derived compounds is reported in the review of Stevens (1967). De Keukeleire and Snatzke (1972) have made more extensive studies of the absolute configuration of *trans*- and *cis*-isohumulone using circular dichroism. However, they did not explore the influence of solvent

composition on stereochemical conformation. It seems unlikely that a change in the optical rotation of *trans*-isohumulone of the magnitude reported by Verzele *et al.* (1965) could result merely from ionisation. Molecular models indicate that while ionisation should have little influence on the stereochemical conformation of *trans*-isohumulone, both keto-enol tautomerism and covalent hydration could be expected to bring about substantial changes in conformation. NMR data indicates that keto-enol tautomerism plays no part.

Further evidence for the involvement of covalent hydration in the solution chemistry of isohumulone comes from calorimetric experiments conducted by Roberts (1976). When isohumulone (2×10^{-4}M) in acetate buffer (pH 4) containing 3.5% (v/v) EtOH was diluted with an equal volume of acetate buffer of the same pH but containing no EtOH, an exothermic reaction of 140kJ/mole isohumulone was recorded. The heat produced was not due to dilution of EtOH, which was balanced out in the reference cell. If such isohumulone solutions were diluted with an equal volume of water, an exothermic reaction of 640kJ/mole isohumulone was observed. In both cases, a small endothermic reaction preceded the exothermic reaction. Roberts concluded that isohumulone was associated with EtOH, and that the small endothermic signal recorded on dilution represented the breakdown of the isohumulone-ethanol complex. The large exothermic reaction that followed was considered to result from *reassociation of isohumulone molecules with water molecules*, but no suggestion was offered as to the way in which such water molecules were combined with isohumulone.

The results obtained in the present work suggest that ionisation of *trans*-isohumulone occurs as described by pKa_{equil}=3.1 but that the hydrated undissociated form is

in equilibrium with a dehydrated undissociated species. This latter species has a characteristic absorption spectrum and is considerably less soluble than either the ionised or hydrated undissociated forms of *trans*-isohumulone. At pH >4 it is postulated that the hydrated ionised enolic form of *trans*-isohumulone predominates. At pH values between 1.6-2.5 the hydrated undissociated enolic form will be the major species present in aqueous solution, while at pH values <1.0 the dehydrated undissociated enolic species predominates. (The pH region >6 has not been studied in detail.) The relative proportions of each species likely to be present in aqueous solution at different pH values (calculated from potentiometric, conductimetric, spectrophotometric and solubility data) are shown in Figure 2.16. Possible molecular structures for each of the different molecular species of *trans*-isohumulone are shown in Figure 2.17.

The phenomenon of covalent hydration has been discussed in detail in the case of *trans*-isohumulone. It is likely that it also occurs, although perhaps to a lesser extent, in other hop compounds and hop-derived compounds.

2.4.5. Technological significance of covalent hydration

The role of covalent hydration in the chemical and biological behaviour of hop compounds and hop-derived compounds is undoubtedly worthy of further study. Questions of substantial technical significance include: (i) What influence does covalent hydration have on the isomerisation of α-acids to iso-α-acids during wort boiling or hop extract preparation? Kuroiwa *et al.* (1961) have shown that the ratio of *cis*-/*trans*-isomers is changed when the concentration of MeOH in a laboratory-

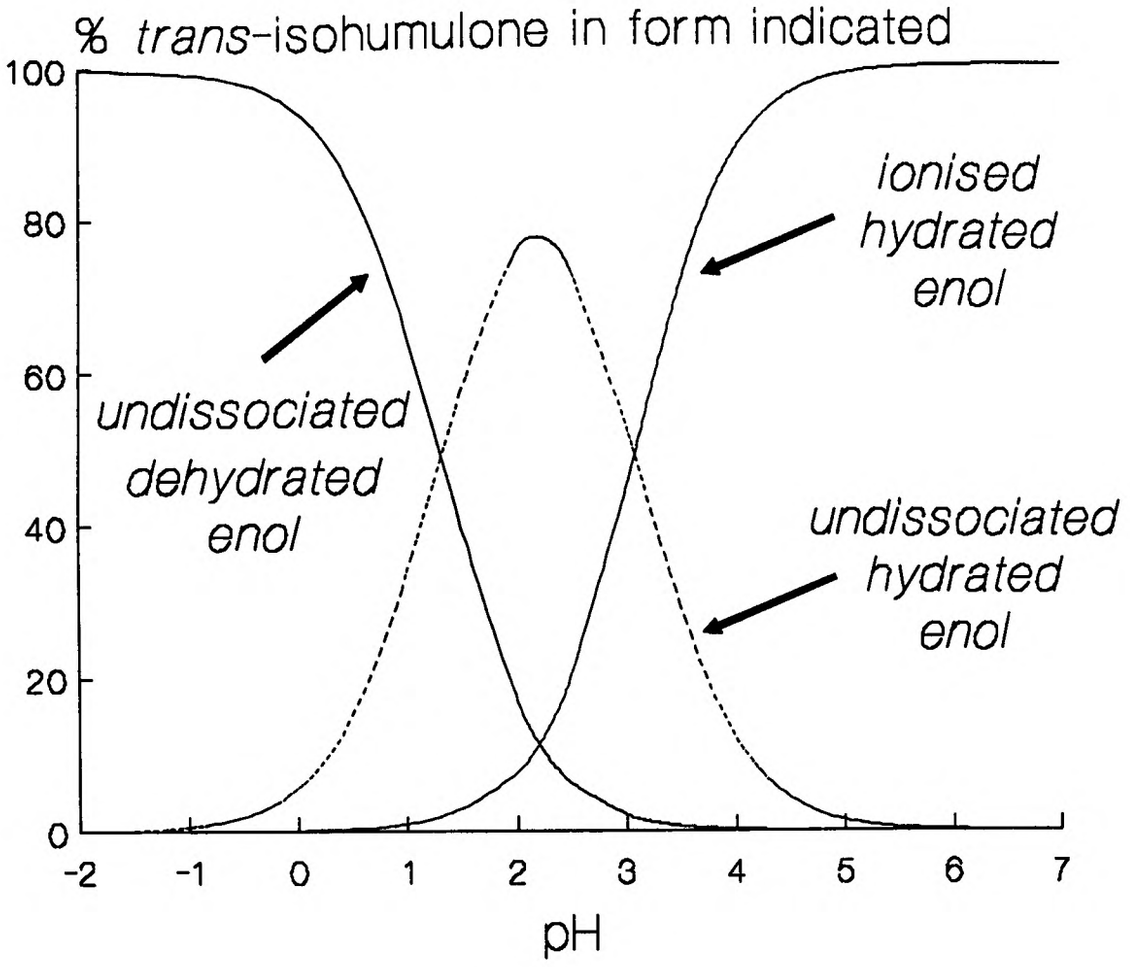


Figure 2.16 Effect of pH on equilibrium between different species of *trans*-isohumulone in aqueous solution. At pH >3.2 the hydrated ionised enol form predominates. At pH 1.4-3.0 the hydrated undissociated enol species predominates while at pH values of <1.3 the dehydrated undissociated enol species predominates. The diagram is based on a pKa_{equil} = 3.1 measured by potentiometry and conductimetry and pKa_{app} = *ca* 1.3 measured by spectrophotometry and solubility determination.

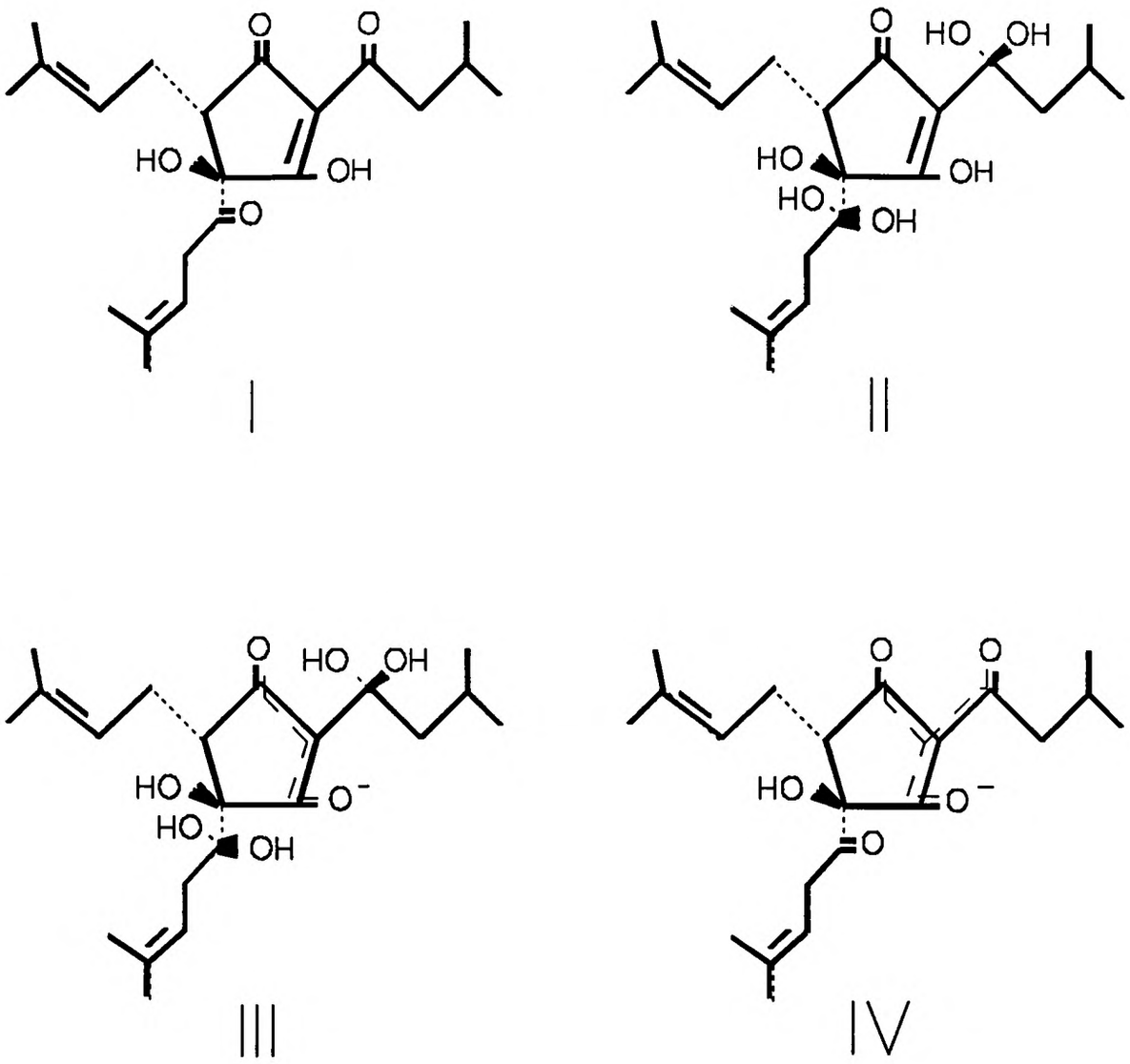


Figure 2.17 Proposed molecular structures of *trans*-isohumulone in different solvents. In strong HCl solution the dehydrated undissociated molecule (I) is the major species present. In aqueous solution (pH 3-11) the undissociated (II) and ionised (III) forms of the hydrated enol predominate (they have identical absorption spectra). In piperidine, the dehydrated ionised form (IV) of *trans*-isohumulone predominates.

scale isomerisation is altered. Do *cis*- and *trans*-isohumulone differ in their ability to hydrate? Can improvements in isomerisation efficiency be made by controlling the rate of hydration, or degree of hydration, of reactants or products? (ii) What effect does covalent hydration have on the ability of iso-α-acids to impart bitter flavour to beer? Is covalent hydration important to bitter taste? Verzele *et al.*(1970) has noted that, when added to beer, iso-α-acids are initially not bitter but develop a bitter flavour after several days. Is covalent hydration involved? (iii) What role does covalent hydration play in the ability of iso-α-acids to stabilise beer foam and enhance foam cling? and (iv) What influence does covalent hydration have on the ability of iso-α-acids to inhibit growth of beer-spoilage lactic acid bacteria?

Answers to such questions may provide practical benefit in the form of novel or improved processes.

2.4.6. Complex formation between *trans*-isohumulone and Mn^{2+}

The ability of hop compounds and hop-derived compounds to complex cations has been exploited in a number of ways. The reaction of hop compounds with metallic salts to give coloured products was reported nearly 80 years ago (Mohr 1913) and the reaction of α-acids with uranyl ions formed the basis of an assay (Guthrie & Philip 1930). Hop compounds and hop-derived compound/cation interactions have been used in purification methods, commercial-scale preparation methods, and conductimetric analysis. Much of the information available has been summarised by Clarke *et al.* (1976). Rudin (1958) found that nickel ions stimulated the ability of isohumulone to promote beer

foam formation and suggested that this provided a basis for the rapid determination of beer isohumulone content. Rudin & Hudson (1958) also commented upon the significance of isohumulone/metal ion interactions in the phenomenon of gushing. The ability of isohumulone to form complexes with a variety of metals (including cadmium, chromium, copper, lead, silver, tin and uranium) was later examined by Hudson & Rudin (1959). In most cases, the stoichiometry of complex formation between isohumulone and metal cations was dependent on the electrovalency of the metal. Elemental analysis, conductimetric titration, and cryoscopic measurement of M_r, indicated that a 1:1 complex was formed between isohumulone and silver, a 1:2 complex between isohumulone and nickel, a 1:3 complex between isohumulone and lanthanum and a 1:4 complex between isohumulone and thorium (Hudson & Rudin 1959).

In the present work the interaction between *trans*-isohumulone and Mn^{2+} was studied, since studies of the antibacterial action of the compound indicated a role for complex formation (section 4.3.4.). (No published reports exist of the interaction between *trans*-isohumulone and Mn^{2+}.) The absorption spectrum of the Mn^{2+}/*trans*-isohumulone complex had maxima at λ=246nm (ε=14520) and 283nm (ε=14320). The form of the spectrum indicates that an enolic species of *trans*-isohumulone was combined with the metal. In MeOH, a 1:1 complex was formed between the species. The behaviour of these moieties is therefore unlike that described by Hudson & Rudin (1959) for the interaction between isohumulone and metal cations since one isohumulone molecule (with an electrovalency of one) combines with one manganese ion (with an electrovalency of two). Further work is required to elucidate the interaction of such compounds with metal cations.

In its ability to complex Mn^{2+} in MeOH, and the changes in light-absorption characteristics which ensue from complex formation, *trans*-isohumulone resembles the divalent cation ionophore A23187. The significance of this will be made apparent in Chapter 4.

CHAPTER 3

Factors affecting antibacterial activity of hop compounds and hop-derived compounds

Discovery consists of seeing what everbody else has seen and thinking what nobody else has thought.

(Albert Szent-Györgyi)

3.1. INTRODUCTION

Prior to undertaking a study of the mechanism of action of an antibacterial compound it is necessary to accumulate information relating to factors which affect its performance. This allows the concentration required to inhibit growth of the test organism to be established and allows the design of future experiments to be optimised. Such studies are valuable from a second viewpoint, however, since they often give clues to the mechanism of action of the antibacterial agent. In this Chapter, the effect of a range of variables on the MIC of several hop compounds and hop-derived compounds was studied. In addition the effect of variations in the preparation of the test inoculum on subsequent sensitivity to such compounds, and the influence of the composition of the growth medium used to assess antibacterial activity were also examined.

3.2. EXPERIMENTAL

3.2.1. Chemicals

All chemicals were of analytical reagent grade and were used without further purification. Hop compounds and hop-derived compounds were prepared as described in section 2.2.3.

3.2.2. Growth media

A modified version of de Man Rogosa Sharpe (MRS) medium (de Man *et al.* 1960) was used. In order to assure its consistency, sufficient quantities of the ingredients were reserved and used as necessary. The composition of this medium (Table 3.1) differs from that originally described in that Tween 80 (polyoxyethylene sorbitan

Table 3.1: Composition of modified de Man Rogosa Sharpe medium (mod. MRS)

Constituent	Concentration (g/l)
peptone	10
lab lemco	10
yeast extract	5
glucose	20
K_2HPO_4	2
Na acetate.$3H_2O$	5
triammonium citrate	2
$MgSO_4.7H_2O$	0.2
$MnSO_4.4H_2O$	0.05
Deionised H_2O	to 1000ml

mono-oleate) was omitted since control experiments showed that it antagonised the antibacterial action of hop compounds and hop-derived compounds (see section 3.3.1.). The pH of the medium was adjusted within the range 4-7 using concentrated HCl prior to autoclaving 200ml quantities at 115°C for 15min. In some experiments, some constituents (*e.g.* $MnCl_2$) were omitted or other materials (*e.g.* NaCl, KCl, α-cyclodextrin, β-cyclodextrin) were added. In these cases, additions were made to autoclaved mod. MRS which was then sterilised by membrane filtration (Minisart NML, Sartorius, Germany) or by autoclaving as described in the appropriate section. For maintenance of the test organism, MRS agar (Oxoid, UK) was employed.

3.2.3. Test organism

Lactobacillus brevis IFO 3960, obtained from the Institute of Fermentation (Osaka, Japan), was maintained on MRS agar (subcultured at 3-month intervals) and stored at 4°C. Organisms for use in assays of minimum inhibitory concentration (MIC) were prepared by transferring a small amount of growth from the surface of an MRS slope culture to 10ml mod. MRS (usually pH 5.2: in some experiments the initial pH of the medium used was varied between 4 and 7). The organisms were incubated, without shaking, at 25°C for 3d.

3.2.4. Assay of minimum inhibitory concentrations

The minimum inhibitory concentrations (MIC) of colupulone, (-)-humulone, *trans*-isohumulone and *trans*-humulinic acid were estimated using a tube dilution technique. Preliminary experiments using doubling dilutions established the approximate MIC in each case. More precise MICs were then obtained using an arithmetic series designed to maintain a constant level of error (as

% coefficient of variation) in each of the tests (Kavanagh 1963). Typically 15 tubes were employed in each series and experiments designed such that inhibition of the test strain was complete after tube 10-12. Stock solutions of the compounds were prepared in MeOH and dilutions made in mod. MRS of the appropriate pH. All solutions were used within 10min of preparation.

The medium containing the test organism (stationary-phase *Lact.brevis* IFO 3960 prepared as described in section 3.2.3.) was diluted (1:100) in sterile deionised water immediately prior to use. A range of concentrations of the test compound in mod. MRS medium was prepared in disposable plastic tubes of 7ml capacity (Sterilin, UK). Tubes containing growth medium (2ml) were inoculated with diluted culture (40µl, containing 4×10^6 organisms). As a precaution against photochemical conversion of the test compounds (Clarke 1967), the tubes were incubated in darkness. Growth was assessed after 48h by measuring the absorbance (λ=560nm, 1cm light path) of samples in disposable plastic cuvettes in a Philips PU 8720 UV/vis spectrophotometer. All tests were performed more than once using independently prepared media and test solutions.

Effect of the ionic composition of the test medium on MIC of hop compounds and hop-derived compounds

The MIC of (-)-humulone, colupulone, *trans*-isohumulone and *trans*-humulinic acid against *Lact.brevis* IFO 3960 was determined over the pH range 4-7. The influence of other cations on antibacterial activity of *trans*-isohumulone was also studied. Monovalent (K^+, Na^+, Cs^+, NH_4^+, Li^+, Rb^+) and divalent (Mg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}) cations were added (0.2M, as chloride salt) to sterile mod. MRS medium. In some experiments the concentration of the cation was varied up to a final concentration of 0.3M. The modified

medium was sterilised by membrane filtration and its pH value determined. MIC measurements were performed as described above.

Effect of (-)-humulone on the MIC of* trans-*isohumulone

The influence of *trans*-isohumulone on the antibacterial activity of (-)-humulone and *vice versa* was studied in mod. MRS (pH 6.4). The MIC of *trans*-isohumulone against *Lact.brevis* IFO 3960 was determined in the presence of 0, 5, 10, 15 and 20μM (-)-humulone (equivalent to 0, 0.19, 0.38, 0.58, and 0.77x MIC). The MIC of (-)-humulone was determined in the presence of 0, 20, 40, 60 and 80μM *trans*-isohumulone (equivalent to 0, 0.125, 0.25, 0.375, and 0.5x MIC). Assays were performed as described above.

Effect of ethanol on the MIC of* trans-*isohumulone

The MIC for *trans*-isohumulone against *Lact.brevis* IFO 3960 was determined in mod. MRS (pH 4.33 and pH 5.2) at different EtOH concentrations. To nine parts mod. MRS one part aqueous EtOH solution (0, 20, 40, 60, 80 or 96% v/v) was added and the medium used for assay of antibacterial activity as described above.

Effect of potential neutralisers on the MIC of* trans-*isohumulone

The effect of a range of potential neutralisers on the antibacterial action of *trans*-isohumulone was studied. Preliminary experiments were carried out using an agar diffusion method (Russell 1981) in which filter paper strips containing the antibiotic and potential neutraliser were each applied to the surface of mod. MRS agar plates seeded with *Lact.brevis* IFO 3960. This method was not ideal since the test compounds ((-)-humulone, *trans*-isohumulone) were not sufficiently soluble in aqueous media to allow satisfactory inhibition

zones to be obtained. Suitable zones could be obtained using suspensions of the compounds, in which the concentration present was in excess of the saturation solubility, but, since it was considered that such a test system was unlikely to yield reproducible results, further tests employed a tube dilution technique. The potential neutralisers (α-cyclodextrin [5mM], β-cyclodextrin [5mM], Tween 80 [5mM], Tween 80:lecithin mixture [3g/l:2g/l], bovine serum albumin [BSA, 1g/l], cholesterol [5mM])) were added to sterile mod. MRS medium (pH 5.2). Each batch of medium was steamed to dissolve the additions, then autoclaved at 121°C/15min. Control batches of media, to which no additions had been made, were treated in an identical way. All of the compounds produced satisfactory solutions, except BSA and cholesterol in which some precipitation of the test materials was evident after autoclaving. In these cases the medium was decanted off the sedimented material using a sterile wide-bore pipette. Alternative sterilisation procedures such as filtration were of limited value since the components were surface-active. Assays of the antibacterial activity of *trans*-isohumulone in these media, using *Lact.brevis* IFO 3960, were performed as described above.

Effect of test incubation conditions on antibacterial activity of trans-isohumulone

Two identical sets of tubes for determination of the MIC of *trans*-isohumulone for *Lact.brevis* IFO 3960 were prepared. One set was incubated aerobically (the usual procedure), the other was incubated in an anaerobic jar (Gas Pak, BBL, UK). Both sets were incubated at 25°C for 2d then the MIC determined as described above.

Influence of initial number of organisms in the assay on MIC of trans-isohumulone

The MIC of *trans*-isohumulone for *Lact.brevis* IFO 3960 in mod. MRS (pH 5.2) was assessed using initial inocula of 1.6×10^3, 8×10^3, 4×10^4, 2×10^5, 1×10^6, and 4×10^6 organisms/ml using the method described above.

Effect of the pH of the medium used to prepare the test inoculum on the MIC of trans-isohumulone

The MIC of *trans*-isohumulone for *Lact.brevis* IFO 3960 was determined in mod. MRS (pH 5.06) as described above with the exception that organisms which had been grown in mod. MRS media of different pH values were used as the inoculum. Inocula were prepared by transferring *Lact.brevis* IFO 3960 from an MRS slope culture to mod. MRS medium of the following pH values: 3.70, 4.19, 4.51, 5.50, 6.00, 6.35. The organisms were grown for 2d at 25°C. At this time, the pH of the cultures had changed to 3.8, 4.2, 4.26, 4.36, 4.59, and 4.54 respectively. Because of the different growth rates of the test organism at each pH, care was taken to ensure that an inoculum of 8×10^4 organisms/ml was used in the tests. A control MIC assay was also set up using cells prepared in mod. MRS (pH 5.06) and grown for the usual incubation period of 3d.

Influence of the age of test inoculum on the MIC of trans-isohumulone

The influence of the age of the organisms used as the test inoculum on the MIC of *trans*-isohumulone was assessed using *Lact.brevis* IFO 3960 grown for 0-14d in mod. MRS (pH 5.2). Cultures were incubated for 0.05, 1, 2, 3, 4, 5, 6, 7, 8, 12, or 14d prior to use. The MICs were then determined as described above.

3.3. RESULTS

Figure 3.1 shows a typical dose response curve for the interaction between *trans*-isohumulone and *Lact.brevis* IFO 3960. Curves of similar form were obtained for each of the test compounds under each of the experimental conditions. A concentration-dependent reduction in growth was induced by each test compound, the minimum inhibitory concentration (MIC) being that which completely inhibited growth of the test organism within 2d. Similar MIC values were obtained when the absorbance values were measured after 1, 2, 3, or 4d incubation.

3.3.1. Factors affecting the MIC of hop compounds and hop-derived compounds

Effect of growth medium pH value on MIC

Since the activity of antimicrobial agents is frequently affected by their degree of ionisation (Simon 1950; Simon & Beevers 1952; Freese *et al.* 1973; Lambert & Smith 1977; Eklund 1983; Albert 1985; Cole & Keenan 1986) it seemed possible that the antibacterial activity of hop compounds and hop-derived compounds could depend upon the pH of the medium in which they were applied. Although data were available in the literature to support this contention (Fernbach & Stoleru 1924; Shimwell 1937b; Walker & Blakebrough 1952), the relationship between pH and antibacterial activity of such compounds had not been previously quantified. (It may be that this is because when the first report of the influence of H^+ activity [pH] on antibacterial activity of hop compounds was published [Fernbach & Stoleru 1924] the concept of pH, as devised by the brewing scientist Sorensen, was relatively new.) Howard (1953) raised the possibility that only the un-ionised form of the compounds displayed antibacterial

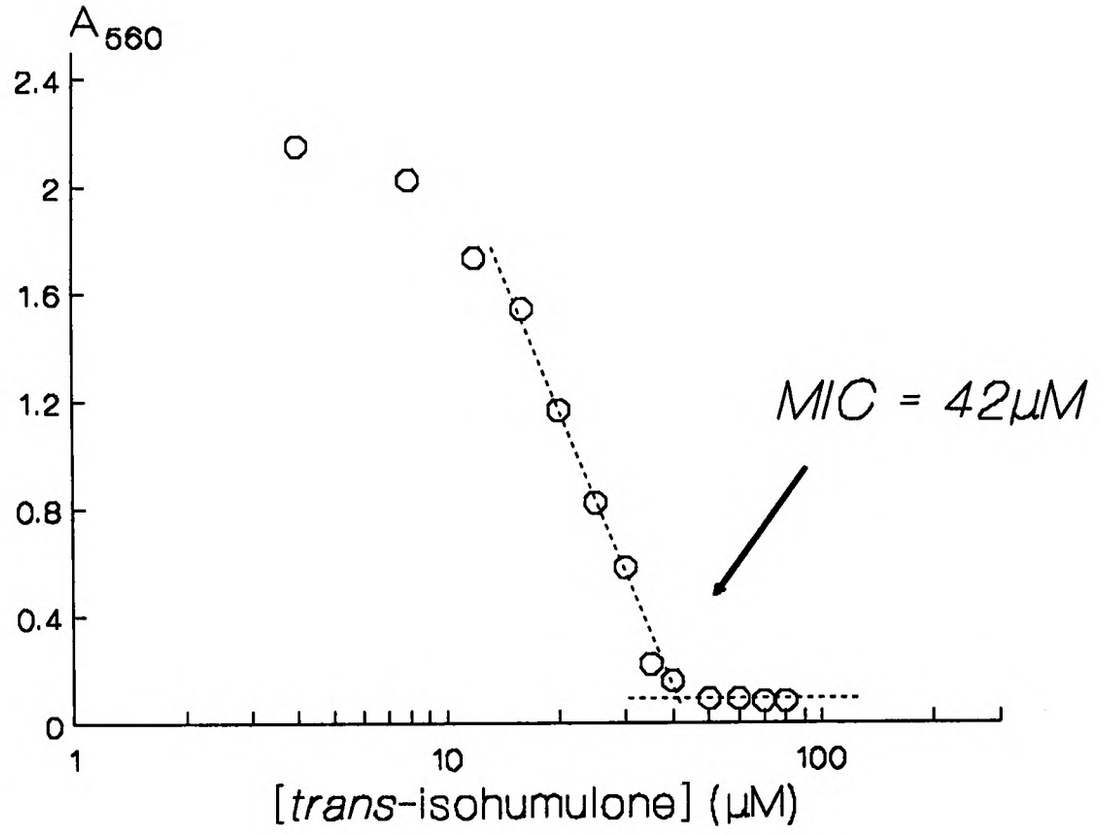


Figure 3.1 Dose response curve for inhibition of *Lactobacillus brevis* IFO 3960 by *trans*-isohumulone. Mod. MRS medium (pH 5.2) containing a range of concentrations of *trans*-isohumulone was inoculated with *Lact.brevis* IFO 3960 (8×10^4 organisms/ml). After 48h incubation at 25°C, growth was assessed spectrophotometrically at λ=560nm in a cell of 1cm path length. Growth of the test organism was progressively inhibited by *trans*-isohumulone. The minimum inhibitory concentration (MIC) was 42μM.

activity, but no experiments have been previously reported in connection with this perceptive suggestion.

The influence of the growth medium pH on antibacterial activity of a range of hop compounds and their derivatives was studied using *Lact.brevis* IFO 3960 as test organism. Figure 3.2 shows that adequate growth of the organism was obtained in 2d if the initial pH of the medium was between 4 and 7. No significant growth occurred during this time outside this pH range. The antibacterial activity of colupulone, (-)-humulone, *trans*-isohumulone, and *trans*-humulinic acid was dependent on the pH of the medium (Figure 3.3). The change in MIC with respect to change in pH was greatest in the case of *trans*-isohumulone and *trans*-humulinic acid, less for (-)-humulone and only slight for colupulone.

In aqueous solution weak acids exist in a state of equilibrium between their undissociated and ionised forms. The state of this equlibrium is related to the equilibrium constant (*Ka*) of the compound and the H^+ activity of the solution by the following expression,

$$Ka = \frac{[H^+]\,[A^-]}{[HA]} \qquad (3.1)$$

where A^- and *HA* are the ionised and undissociated forms of the weak acid respectively. (Although *concentrations* are shown, it is the *activities* of each species that controls the equilibrium.) To simplify the arithmetic, both Ka values and H^+ activities are usually expressed as their negative logarithms, pKa and pH, respectively. Provided that both the pKa of the test compound and the pH of the test solution are known it is possible to

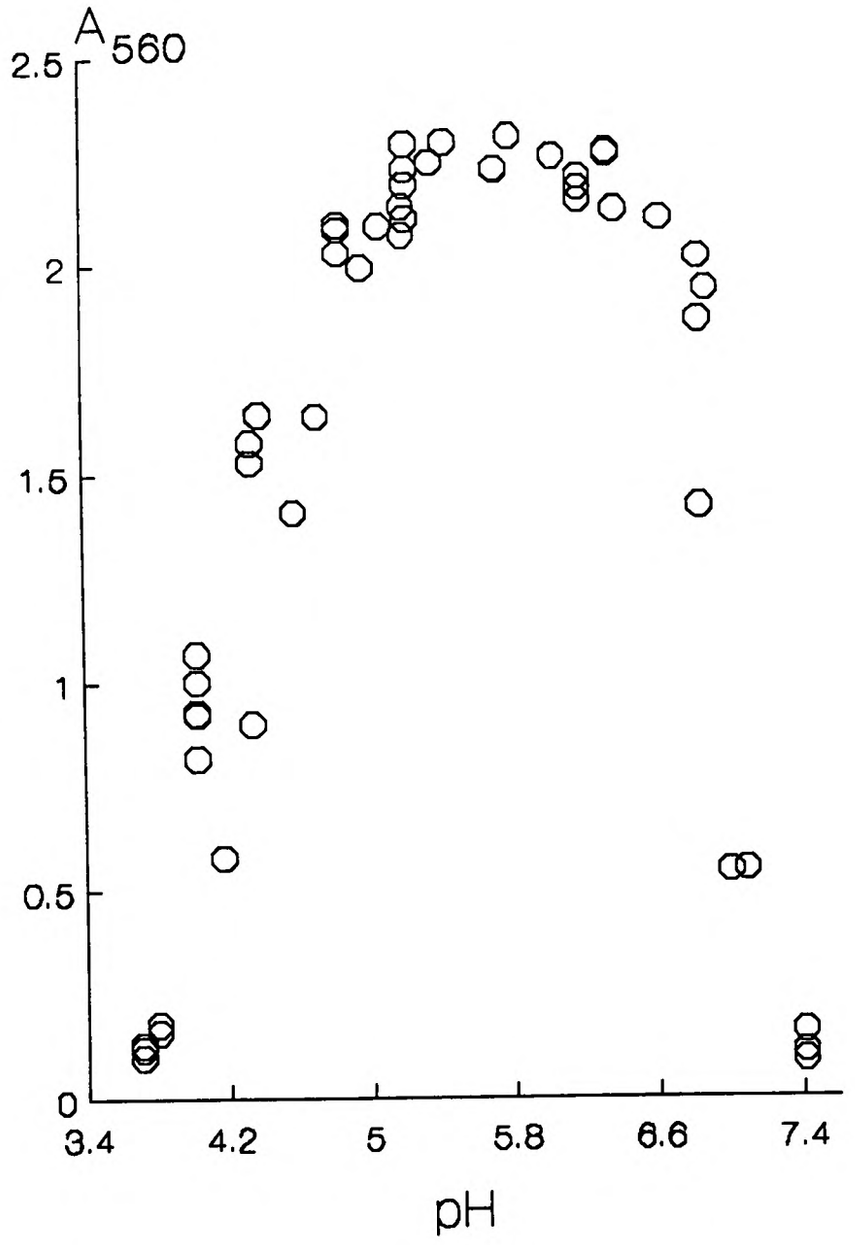


Figure 3.2 Effect of the initial pH of the medium on growth of *Lactobacillus brevis* IFO 3960. Mod. MRS medium, adjusted to a range of pH values, was inoculated with *Lact.brevis* IFO 3960 (8×10^4 organisms/ml). After 48h incubation at 25°C, growth was assessed spectrophotometrically at λ=560nm in a cell of 1cm path length. Growth of the test organism occurred within the 48h test period if the initial pH of the growth medium was between 4 and 7.

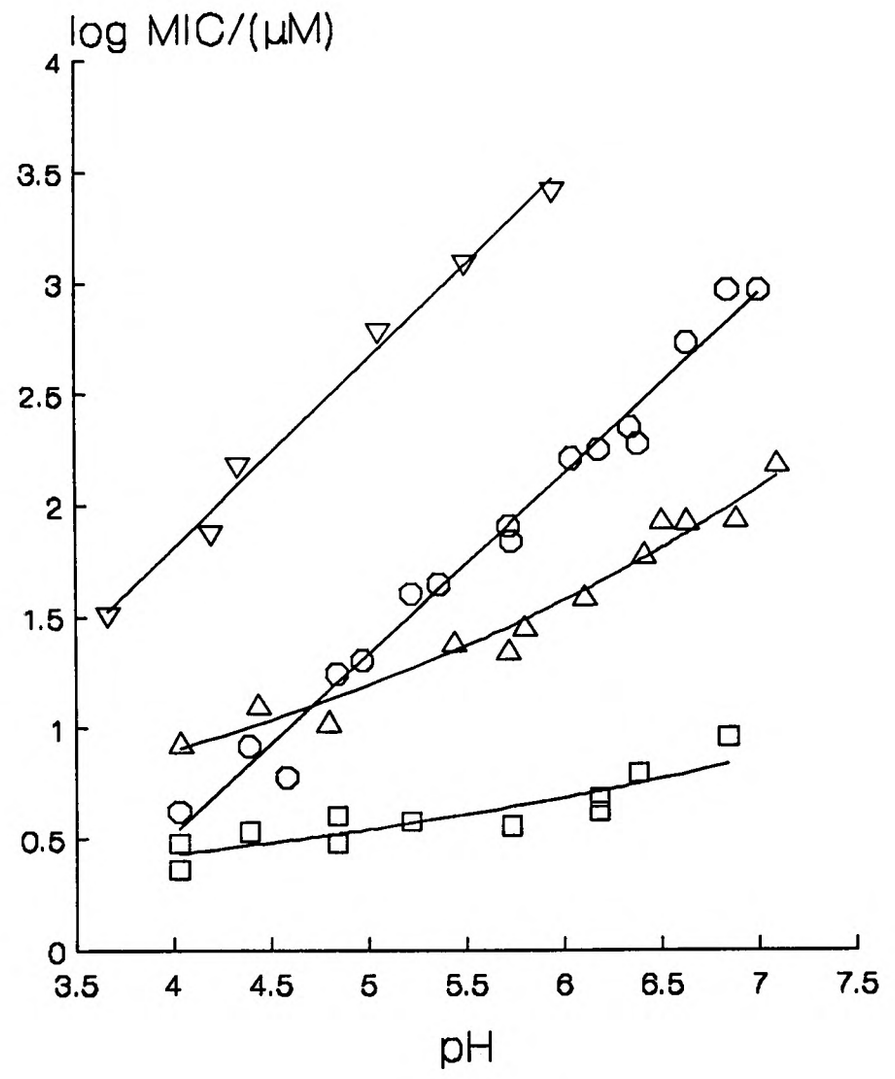


Figure 3.3 Effect of the initial pH of the medium on antibacterial activity of hop compounds and hop-derived compounds. Minimum inhibitory concentrations (MIC) for colupulone (□), (-)-humulone (△), *trans*-isohumulone (○) and *trans*-humulinic acid (▽) were assessed using *Lactobacillus brevis* IFO 3960 (8×10^4 organisms/ml) in mod. MRS at 25°C.

calculate the activities of the various species using the following expression,

$$[HA] = \frac{[H^+] + [HA]}{10^{pH-pKa} + 1} \qquad (3.2)$$

The ionisation behaviour of any individual hop compound and hop-derived compound cannot be described by a single pKa since more than one equilibrium process dictates the ionisation behaviour. However, pKa_{equil} values were obtained experimentally (section 2.3.4.) and used to approximate the proportion of the compounds present in both the undissociated and ionised forms at a range of pH values (Figure 3.4).

For example, for *trans*-isohumulone, which has pKa_{equil}=3.1, application of equation (3.2) revealed the proportion of the material which would be in the undissociated form at various pH values in the range 3-8 (Figure 3.4). Although it exists substantially in the ionised form at all pH values within this range the amount of the undissociated form, although small, varies substantially. When the amount of the undissociated form of each compound required to inhibit bacterial growth at each pH was calculated, a relatively constant amount of each was found to be required (Figure 3.5). This indicates that undissociated molecules were responsible for antibacterial activity and that ionised molecules had negligible activity. The number of molecules of each compound required for inhibition differed. Approximately 0.1-0.4μM of the undissociated form of *trans*-isohumulone was sufficient to inhibit growth of the test organism while *ca* 1.7-3.0μM, 1.0-8.0μM and 1.4-3.3μM of the undissociated forms of colupulone, (-)-humulone and *trans*-humulinic acid respectively were each required. MIC_{undiss} values obtained at pH 5.2 are shown in Table 3.2.

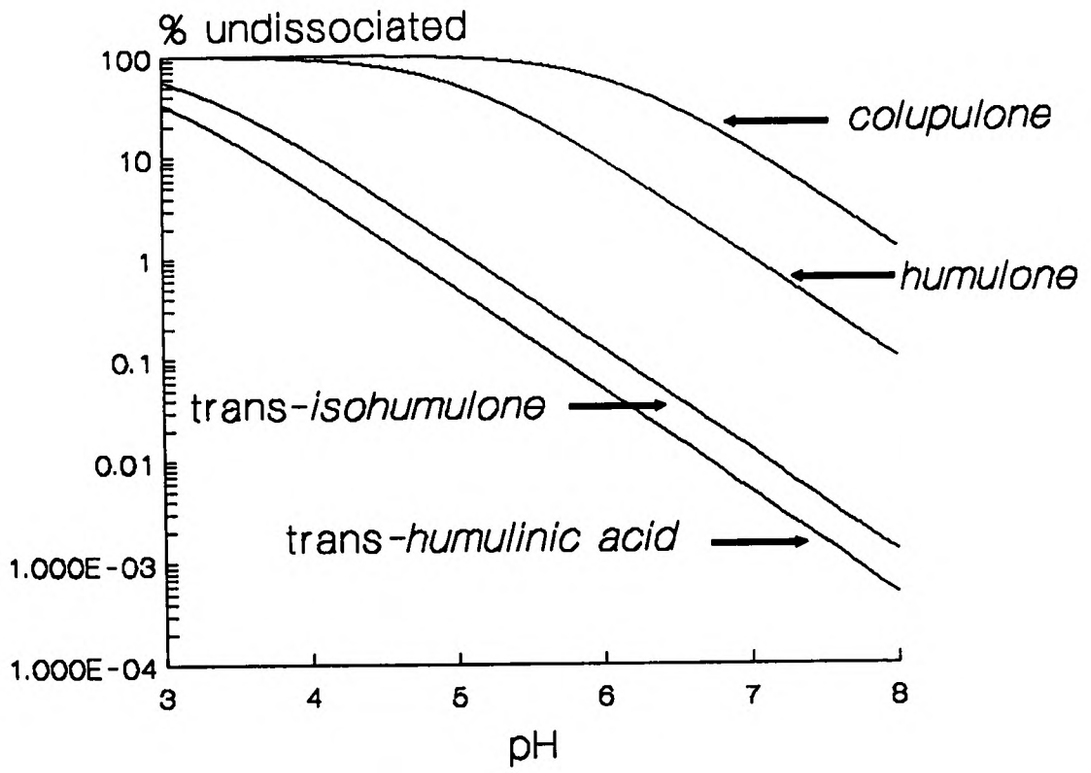


Figure 3.4 Relationship between the pH of the medium and the proportion of each compound present in the undissociated form. The undissociated proportion of colupulone, (-)-humulone, *trans*-isohumulone and *trans*-humulinic acid at each pH was calculated using equilibrium pKa values of 6.1 (colupulone), 5.0 ((-)-humulone), 3.1 (*trans*-isohumulone) and 2.7 (*trans*-humulinic acid). The values shown are approximate since the compounds participate in other equilibrium processes.

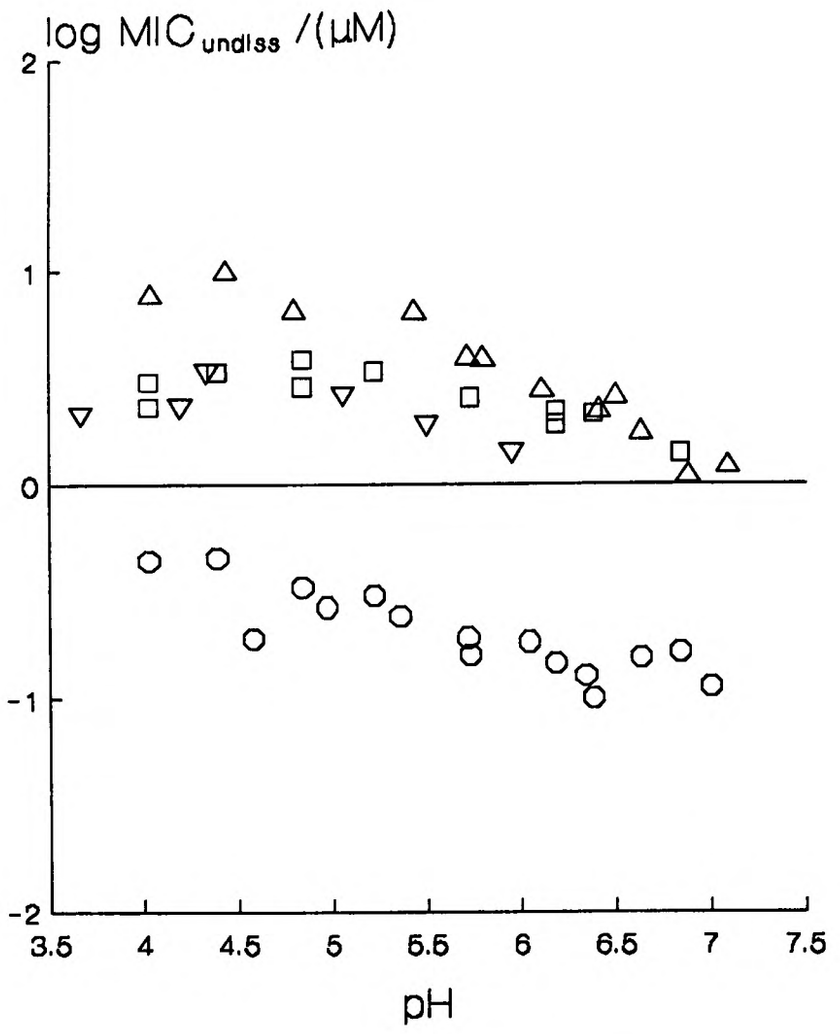


Figure 3.5 Relationship between the initial pH of the growth medium and the concentration of the undissociated form (MIC_{undiss}) of hop compounds and hop-derived compounds required to inhibit growth of *Lactobacillus brevis* IFO 3960. Data calculated from that shown in Figure 3.3 using equilibrium pKa values of 6.1 (colupulone, □), 5.0 ((-)-humulone, △), 3.1 (*trans*-isohumulone, ○) and 2.7 (*trans*-humulinic acid, ▽).

Table 3.2: Minimum inhibitory concentration of the undissociated form (MIC_{undiss}) of various hop compounds and hop-derived compounds.

Test compound	**MIC_{undiss} (μM)[1]**
colupulone	3.4
(-)-humulone	6.4
trans-isohumulone	0.3
trans-humulinic acid	2.6

1. MIC determined in mod. MRS at pH 5.2 using an inoculum of 8 x 10^4 organisms/ml. Concentration of undissociated form calculated using the following equilibrium pKa values: colupulone (6.1); (-)-humulone (5.0); *trans*-isohumulone (3.1); *trans*-humulinic acid (2.7).

Effect of cations other than H^+ on MIC of trans-isohumulone

The concentration of metal ions in the growth medium can affect the efficacy of antibacterial agents. For example divalent cations, such as Mg^{2+} and Ca^{2+} affect the susceptibility of *Pseudomonas* spp. to tetracycline (Houang *et al.* 1983). The sensitivity of *Bacillus subtilis* (Best & Durham 1964) and *Escherichia coli* (Russell & Thomas 1966) to vancomycin is markedly reduced by Mg^{2+}. The antibacterial activity of gentamicin is affected by the ionic strength of the medium, variation in activity usually being due to changes in the medium NaCl content (Houang *et al.* 1983). Such effects can be due to (i) direct interaction between metal ions and antibiotic resulting in the formation of a less-active, or more-active, complex; (ii) competition between ions and antibiotic for sites on the bacterial cell surface resulting in a diminution in antibacterial activity; or (iii) indirect effects caused by alterations in the sensitivity of the test organism to the antibiotic resulting from an effect of the cation on the physiology of the test organism.

Ions such as Na^+ and K^+ were found to have no deleterious effect on growth of *Lact.brevis* IFO 3960 in the absence of *trans*-isohumulone, although Na^+ caused the cells to clump. They had, however, a significant effect on the antibacterial activity of *trans*-isohumulone causing a decrease in the amount of *trans*-isohumulone required to inhibit growth. In preliminary experiments, the MIC of *trans*-isohumulone against *Lact.brevis* IFO 3960 (at pH 5.1) was reduced from 32μM in the absence of added K^+ to 16μM in the presence of 0.2M added K^+. In the case of Na^+ the MIC for *trans*-isohumulone was reduced from 32μM to 20μM in the presence of 0.2M added Na^+.

The experiments were repeated using a wider range of cations. As was found to be the case for Na^+ and K^+, the growth yield of control cell suspensions was not affected by the presence of 0.2M NH_4^+, Rb^+, or Li^+ but growth was completely inhibited by 0.2M Cs^+. Addition of each of the salts caused a change in the pH of the medium. To allow a comparison to be made of the antibacterial activity of *trans*-isohumulone in the presence of each of the salts, the amount of undissociated *trans*-isohumulone present at the MIC was calculated. The results in Table 3.3 show that the increase in antibacterial activity of *trans*-isohumulone elicited by such salts ranged from about a 1.2-fold improvement (in the case of Li^+) to a 3-fold improvement (in the case of Rb^+). Such stimulation was concentration dependent (Figure 3.6). Potassium ions stimulated antibacterial activity of *trans*-isohumulone at each of the concentrations tested, the shape of the response curve suggesting that at very low K^+ concentrations, *trans*-isohumulone had little antibacterial activity. This was later confirmed by more detailed experiments on the mechanism of action of *trans*-isohumulone (see section 4.3.4.).

Calcium ions reduced the antibacterial activity of *trans*-isohumulone to about 3/4, but the effect of Mg^{2+} was more pronounced, a reduction in activity of greater than 4-fold being recorded (Table 3.3). The effect of this latter ion was particularly interesting since, in the absence of *trans*-isohumulone, no growth of *Lact.brevis* IFO 3960 took place over the 2d incubation period. However, addition of *trans*-isohumulone in the range 3.75-11.25μM caused a concentration-dependent stimulation of growth. Above this concentration, growth was progressively inhibited by *trans*-isohumulone with an MIC of 56μM (Figure 3.7). No growth of the organism occurred

Table 3.3: Effect of added cations on minimum inhibitory concentration (MIC) of *trans*-isohumulone for *Lactobacillus brevis* IFO 3960

Addition[1]	**pH of medium**[2]	**MIC (μM)**[3]	**MIC_{undiss} (nM)**[4]
none	5.18	32	264
K^+	5.33	16	94
Rb^+	5.38	16	84
NH_4^+	5.28	19	125
Na^+	5.20	18	142
Li^+	5.13	23	213
Ca^{2+}	4.86	21	359
Mg^{2+}	4.77	56	1172

1. 0.2M added as the chloride. Prior to additions being made, the medium contained (mM) K^+ (41), Rb^+ (<0.03), NH_4^+ (28), Na^+ (84), Li^+ (<0.03), Ca^{2+} (<0.03), Mg^{2+} (0.75) (determined by ion-exchange chromatography).
2. Measured immediately before inoculation.
3. Determined using *Lactobacillus brevis* IFO 3960 as test organism with an inoculum of 8×10^4 organisms/ml.
4. Calculated using $pKa_{equil} = 3$.

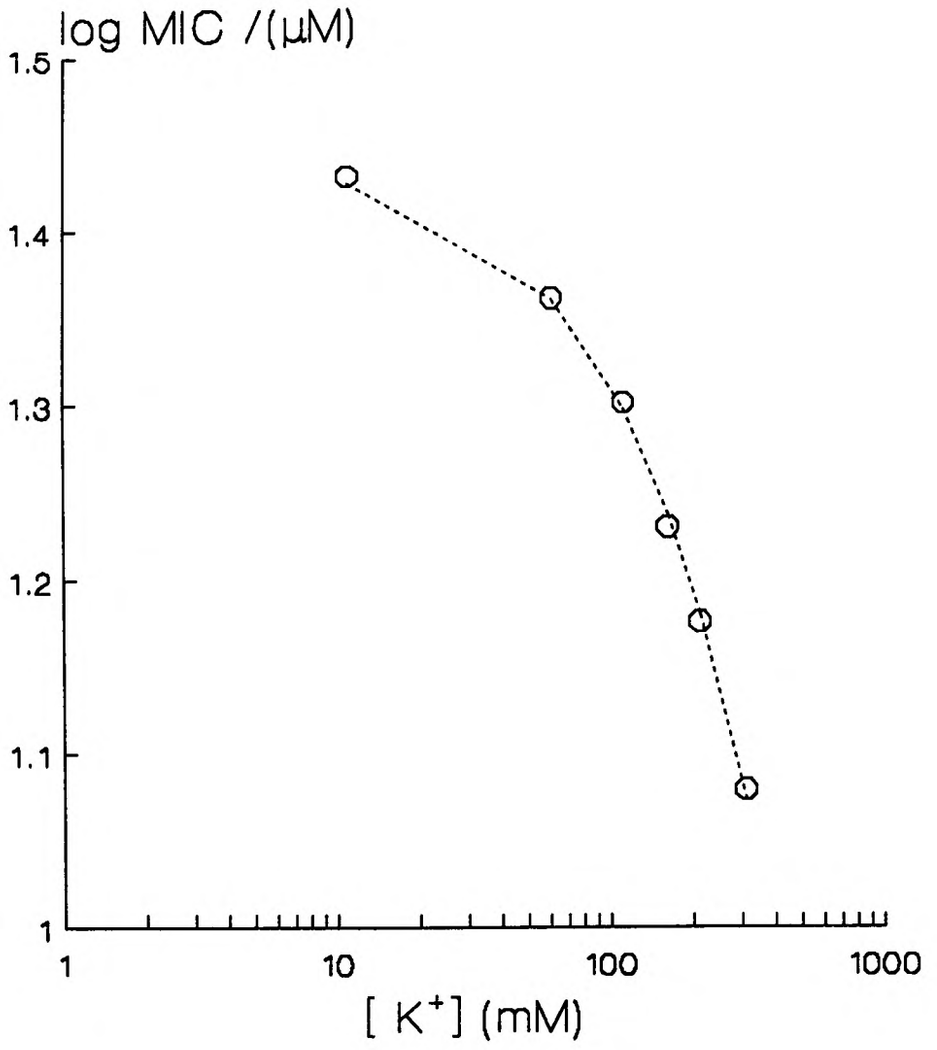


Figure 3.6 Relationship between the K^+ concentration of the medium and antibacterial activity of *trans*-isohumulone. The minimum inhibitory concentration (MIC) of *trans*-isohumulone was determined in mod. MRS at various [K^+] (added as KCl) using *Lactobacillus brevis* IFO 3960 (8 x 10^4 organisms/ml). Antibacterial activity of *trans*-isohumulone was potentiated by K^+.

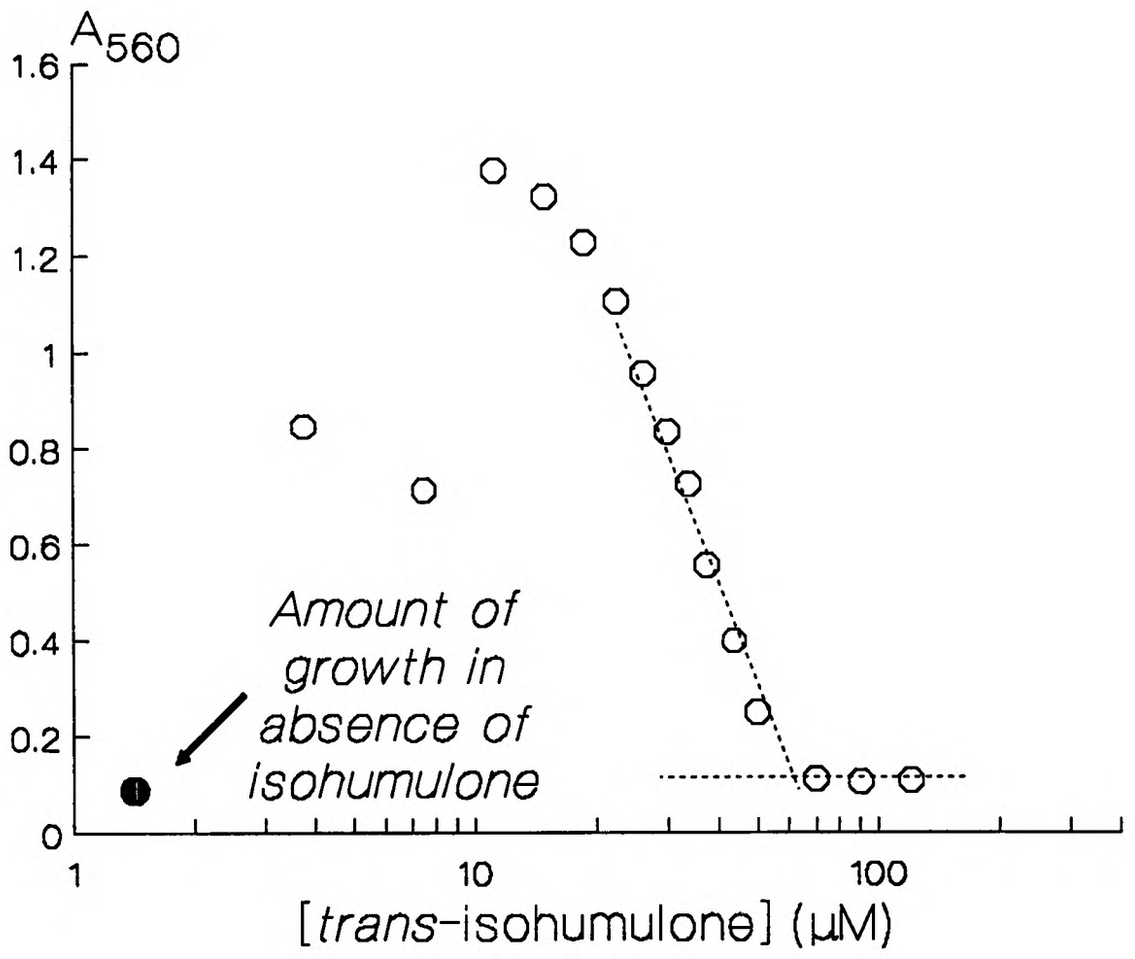


Figure 3.7 Rescue of Mg^{2+}-inhibited cells of *Lactobacillus brevis* IFO 3960 by *trans*-isohumulone. Mod. MRS medium (pH 4.77) containing $MgCl_2$ (0.2M) and a range of concentrations of *trans*-isohumulone was inoculated with *Lact.brevis* IFO 3960 (8×10^4 organisms/ml). After 48h incubation at 25°C, growth was assessed spectrophotometrically at λ=560nm in a cell of 1cm path length. At low concentration, *trans*-isohumulone stimulated growth of the test organism. At higher concentrations growth was progressively inhibited.

either in the presence or absence of *trans*-isohumulone, when 0.2M Co^{2+} or Ni^{2+} was present.

Lactic acid bacteria are unusual in that they require significant quantities of manganese for growth (MacLeod & Snell 1947). Aside from its function as an essential cofactor for several reactions, this metal also plays an important role in protecting the cells from the toxic effects of oxygen. In this respect it substitutes, either wholly or in part, for the protective action of superoxide dismutase. Intracellular Mn^{2+} concentrations can reach 25mM (Archibald & Fridowich 1981a, 1981b). Figure 3.8 shows the relationship between the Mn^{2+} content of the medium and the MIC of *trans*-isohumulone for *Lact.brevis* IFO 3960. Mn^{2+} antagonised the antibacterial action of *trans*-isohumulone. The concentration of *trans*-isohumulone required to inhibit growth of *Lact.brevis* IFO 3960 changed from 18μM to 64μM as the manganese content of the growth medium was increased from 5μM to 0.2M.

Effect of (-)-humulone on antibacterial activity of trans-isohumulone

Table 3.4 shows that the MIC of *trans*-isohumulone was not affected by (-)-humulone. Similarly, the antibacterial activity of (-)-humulone was not affected by *trans*-isohumulone (Table 3.5). These results suggest that significant competition between (-)-humulone and *trans*-isohumulone molecules for sites on the bacterial cell surface does not take place, a conclusion supported by studies on the uptake of *trans*-isohumulone by sensitive bacteria (see section 4.3.3.).

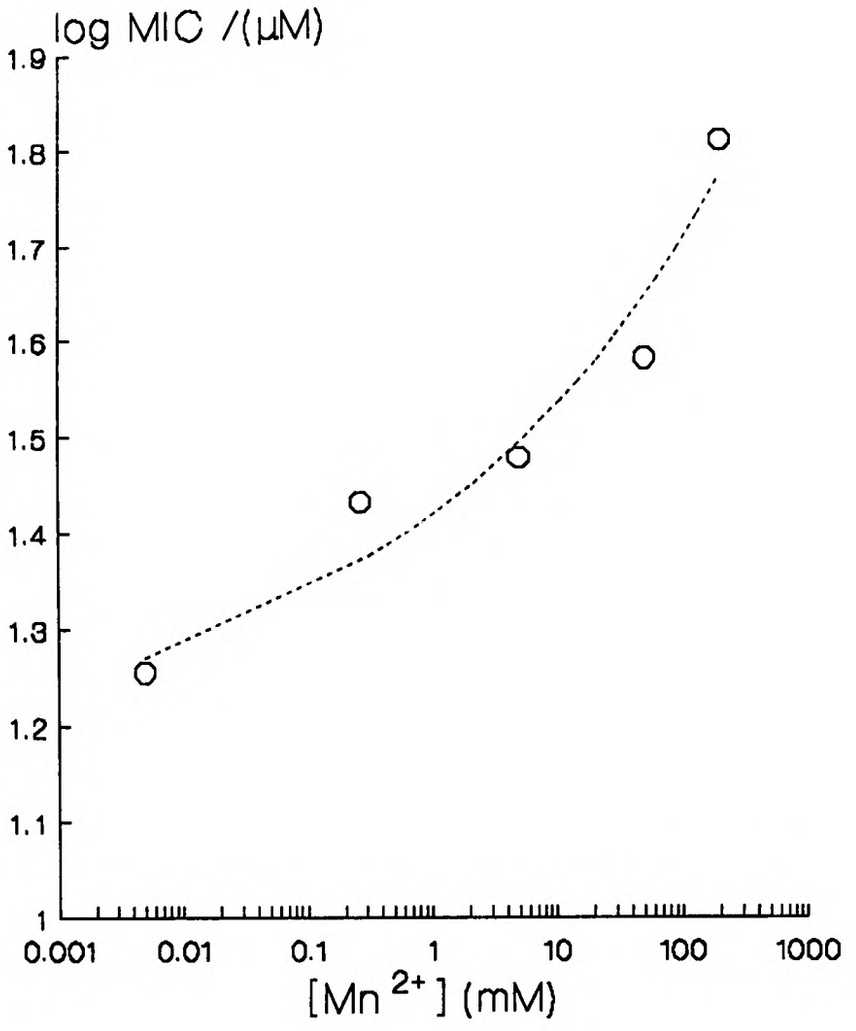


Figure 3.8 Relationship between the Mn^{2+} concentration of the growth medium and antibacterial activity of *trans*-isohumulone. The minimum inhibitory concentration (MIC) of *trans*-isohumulone was determined in mod. MRS at various [Mn^{2+}] (added as $MnCl_2$) using *Lactobacillus brevis* IFO 3960 (8 x 10^4 organisms/ml). Antibacterial activity of *trans*-isohumulone was antagonised by Mn^{2+}.

Table 3.4: Effect of (-)-humulone on antibacterial activity of *trans*-isohumulone.

[(-)-humulone] (μM)[1]	MIC *trans*-isohumulone (μM)[2]
0	160
5	160
10	160
15	140
20	140

1. MIC (-)-humulone determined under these conditions was 26μM.
2. Determined in mod. MRS (pH 6.4) using an inoculum of 8 x 10^4 *Lactobacillus brevis* IFO 3960/ml.

Table 3.5: Effect of *trans*-isohumulone on antibacterial activity of (-)-humulone.

[*trans*-isohumulone] (μM)[1]	MIC (-)-humulone (μM)[2]
0	26
20	27
40	26
60	26
80	23

1. MIC *trans*-isohumulone determined under these conditions was 160μM.
2. Determined in mod. MRS (pH 6.4) using an inoculum of 8 x 10^4 *Lactobacillus brevis* IFO 3960/ml.

Effect of ethanol on the antibacterial activity of trans-isohumulone

Table 3.6 shows that EtOH did not potentiate the antibacterial action of *trans*-isohumulone at either pH 4.33 or pH 5.2.

Effect of potential neutralisers on antibacterial activity of trans-isohumulone

The effect of a range of compounds on the antibacterial activity of *trans*-isohumulone was studied in mod. MRS at pH 5.2. Antibacterial activity was antagonised by Tween 80, lecithin solubilised in Tween (a widely recommended neutralising solution [Russell *et al.* 1979]), and β-cyclodextrin (Table 3.7). In the case of lipid materials (Tween 80:lecithin mixture or Tween 80), antagonism of antibacterial activity was probably the result of mixed micelle formation. The antagonistic effect which β-cyclodextrin displayed is of greater interest since it is an example of neutralisation brought about by a phenomenon known as inclusion complex formation (see section 3.4.5.).

Effect of incubation conditions employed in the test on antibacterial activity of trans-isohumulone

The MIC of *trans*-isohumulone for *Lact.brevis* IFO 3960 was determined in mod. MRS (pH 5.12) under both aerobic and anaerobic conditions. An MIC of 32μM was found under both sets of conditions.

Influence of the original number of organisms in the assay on antibacterial activity of trans-isohumulone

Hamilton (1968) has drawn attention to the fact that many membrane-active antibacterial compounds tend to be adsorbed onto the surface of sensitive cells in reasonably large amounts, and that their efficacy is

Table 3.6: Effect of ethanol on antibacterial activity of *trans*-isohumulone

Ethanol concentration (% v/v)	MIC (μM)[1]	
	@ pH 4.33	@ pH 5.2
0	10.5	40
2	11.2	46
4	9.5	44
6	12.0	43
8	n.d.	n.d.[3]
10	n.d.	52[2]

1. Determined in mod. MRS at the stated pH value using an inoculum of 8 x 10^4 *Lactobacillus brevis* IFO 3960/ml.
2. Determined after a 7d incubation period
3. n.d. = not determined

Table 3.7: Effect of potential neutralisers on antibacterial activity of *trans*-isohumulone

Potential neutraliser	Concentration of neutraliser	MIC (μM)[1]
none	-	35
Tween 80	5mM	65
Tween 80:lecithin	3g/l:2g/l	70
α-cyclodextrin	5mM	40
β-cyclodextrin	5mM	82
BSA[2]	1g/l	41
cholesterol	5mM	35

1. MIC determined in mod. MRS at pH 5.2 using an inoculum of 8 x 10^4 *Lactobacillus brevis* IFO 3960/ml.
2. Bovine serum albumin.

directly related to the amount absorbed. Most biochemical or enzymic activities, he pointed out, are assayed using suspensions which contain at least 5 x 10^9 organisms/ml, whereas bacteriostatic activities are generally assayed with inocula of 10^4-10^7 organisms/ml. At different cell densities the same concentration of antibacterial compound in the suspension might result in very different concentrations of the compound being accumulated on, or in, sensitive organisms. Consequently, different degrees of inhibition of particular cell functions may result from the action of such antibiotics at different cellular concentrations. Hamilton has suggested that, when seeking to explain bacteriostasis or death in terms of the loss of a particular cell function, it is extremely important to do so only by comparison of the activities of cell populations in the presence of identical *cellular* concentrations of the antibacterial agent.

The response of some bacteria to certain antibiotics can be related to the number of organisms present in the assay for a different reason. For example, in the case of the inhibition of *E.coli* by penicillin, the MIC is strongly dependent on the number of cells in the assay. This is because some strains of bacteria possess a β-lactamase enzyme which catalyses hydrolysis of penicillin to an inactive breakdown product. When sensitivity tests are carried out using a small inoculum (*ca* 10^2 organisms), the total amount of β-lactamase enzyme available is insufficient to cause significant destruction of penicillin. As a result, the organism is inhibited by a relatively small concentration of penicillin. However, if the test is performed using a larger inoculum (*ca* 10^6-10^7 organisms), the culture appears to be virtually insensitive to the antibiotic. This is because the amount of β-lactamase available from

this larger number of cells is sufficient to hydrolyse most of the penicillin in a reasonably short period. Once the penicillin molecules have been destroyed, growth of the organisms can continue. Neither of these potential problems was encountered in assays of the antibacterial activity of *trans*-isohumulone using *Lact.brevis* IFO 3960 as test organism.

Table 3.8 shows that the concentration of *trans*-isohumulone required to inhibit growth of *Lact.brevis* IFO 3960 did not vary to a great extent when the number of cells in the assay was varied in the range 1.6×10^5/ml - 4×10^6/ml. This indicates that the number of molecules bound to each cell must be small, since increasing the number of organisms in this range did not limit the availability of the antibacterial agent. In addition, inactivation of *trans*-isohumulone by *Lact.brevis* IFO 3960 does not seem to determine the sensitivity of the organism to this compound.

Effect of the pH of the medium used for preparation of the test inoculum on the MIC of* trans-*isohumulone

In many cases the pH at which organisms are grown prior to challenge with an antibacterial agent has little effect on their subsequent sensitivity (Farwell & Brown 1971). In some cases however, growth medium pH can influence cell behaviour in subsequent sensitivity tests. For example, the resistance of cariogenic lactic acid bacteria to fluoride ions is enhanced when the organisms are grown at low pH values prior to fluoride challenge (Hamilton *et al.* 1985). The results in Table 3.9 show that the pH at which the organisms were grown prior to testing did not affect the concentration of *trans*-isohumulone required to inhibit growth of *Lact.brevis* IFO 3960.

Table 3.8: Effect of the number of organisms in the assay on the minimum inhibitory concentration (MIC) of *trans*-isohumulone

Initial no. organisms in assay (/ml)	**MIC (μM)[1]**
1.6×10^3	38
8×10^3	41
4×10^4	40
2×10^5	41
1×10^6	42
4×10^6	45

1. MIC determined in mod. MRS at pH 5.2 using *Lactobacillus brevis* IFO 3960.

Table 3.9: Effect of initial pH of growth medium used for preparation of the test inoculum on the MIC of *trans*-isohumulone

Initial pH of mod. MRS used for preparation of inoculum	**MIC (μM)[1]**
3.70	28
4.19	30
4.52	29
5.06	28
5.50	28
6.00	30
6.35	30

1. Determined in mod. MRS (pH 5.06) using an inoculum of 8 x 10^4 *Lactobacillus brevis* IFO 3960/ml.

Effect of the age of the inoculum on antibacterial activity of* trans-*isohumulone

Sometimes, the response of bacteria to inhibitory substances varies with their phase of growth. The greater resistance of bacterial spores to antibacterial agents, in comparison to that of vegetative cells, is well documented (Russell 1971) and similar effects are known for the variable sensitivity to antimicrobial agents of cells in their vegetative phase of growth. For example, log-phase *Saccharomyces cerevisiae* are significantly more sensitive to the toxic action of ethanol than are stationary-phase cells (Cartwright *et al.* 1986).

Table 3.10 shows that the antibacterial activity of *trans*-isohumulone was not influenced by the age of the organisms. Similar values were obtained regardless of whether the inoculum consisted of lag-phase (0.05d), exponential-phase (1d), late exponential-phase (2d), early (3d) or late (4,5,6d) stationary-phase organisms, or organisms in various stages of the decline phase of growth (7,8,12,14d).

3.4. DISCUSSION

The quantitative expression of antibacterial activity by many compounds is influenced by (i) the composition of the growth medium used to evaluate antibacterial activity (Houang *et al.* 1983) and (ii) the history of the inoculum employed for the tests. The influence of both these variables on the antibacterial activity of *trans*-isohumulone has been investigated.

3.4.1. Influence of growth medium composition on MIC of *trans*-isohumulone

All known hop resin materials and their derivatives contain a β-triketone grouping and are, therefore, weak

Table 3.10: Effect of age of the organisms used as inoculum on the MIC of *trans*-isohumulone

age of organisms[1] (d)	pH of spent culture medium[2]	MIC (μM)[3]
0.05	5.12	24
1	4.55	24
2	4.16	27
3	4.00	26
4	3.96	26
5	3.97	24
6	3.97	23
7	3.96	22
8	3.95	22
12	3.91	24
14	3.85	22

1. Number of days for which mod. MRS culture of *Lactobacillus brevis* IFO 3960 was incubated at 25°C prior to use.
2. pH of medium immediately prior to use of the inoculum.
3. Determined in mod. MRS (pH 5.01) using an inoculum of 8×10^4 *Lact.brevis* IFO 3960/ml.

acids. As discussed in section 2.3.4., various factors contribute to the acid strength of these compounds and their pKa values thus vary over a wide range. The results obtained clearly show that an undissociated form of each of the compounds was required for antibacterial activity. The ionised form of each of the compounds appears to have little activity. Previous workers have come to the erroneous conclusion that the antibacterial and antifungal activities of different hop compounds and hop-derived compounds vary substantially (Chapman 1925; Walker 1925; Hastings *et al.* 1926; Michener *et al.* 1948; Hough *et al.* 1957; Teuber & Schmalreck 1973; Schmalreck *et al.* 1975; Mizobuchi & Sato 1985). For example, Teuber & Schmalreck (1973) found that, against *Bacillus subtilis* 168, the antibacterial activity of lupulone was twice that of humulone, 25 times that of isohumulone (mixture of *trans*- and *cis*- isomers) and 250 times that of humulinic acid (mixture of *trans*- and *cis*- isomers). Against *Staphylococcus aureus*, Mizobuchi & Sato (1985) found the antibacterial activity of lupulone to be twice that of humulone, eight times that of isohumulone and 64 times that of humulinic acid. Against the fungus *Trichophyton mentagrophytes* only humulone was active, precluding a comparison of antifungal activities (Mizobuchi & Sato 1985). Most of the variation in the antimicrobial activity of hop compounds and hop-derived compounds reported here and by other workers can be attributed to differences in the degree to which the compounds ionised under the test conditions. When differences in ionisation were corrected for, either by calculation or by carrying out the assays at a pH value low enough to minimise their ionisation, the antibacterial activity of colupulone, (-)-humulone, *trans*-isohumulone, and *trans*-humulinic acid displayed little variation. Of the compounds tested,

trans-isohumulone was the most active, being significantly more active in its undissociated form than any of the others. Similar calculations also allowed data reported by other workers to be rationalised (results not shown).

The influence of cations other than H^+ on the antibacterial properties of *trans*-isohumulone was variable. All monovalent cations tested stimulated activity but the effect was small compared to that caused by interaction between *trans*-isohumulone and H^+. Thus, variation in the metal ion content of microbiological growth media should not significantly affect the reproducibility of assays of the MIC of this compound. Differences in the ability of ions to stimulate antibacterial activity may relate to their effect on bacterial physiology, or to their influence on the interaction between *trans*-isohumulone and the bacterial cell surface.

Comparisons of the efficacy with which different cations stimulate antibacterial activity were impeded by the fact that, although the final concentration of each cation was known (ion-exchange chromatography indicated the concentrations given in Table 3.3), the activity of each ion was unknown. The activity of the ion (the free, effective, thermodynamic concentration) takes into account the attraction which ions exert on one another as well as the incomplete hydration of ions in solutions which are too concentrated. At a given concentration, the activity of the different cations tested may have varied to a significant degree. (Experiments reported in detail in section 4.3.4 indicate that such monovalent cations play a direct role in the antibacterial action of hop compounds and hop-derived compounds: in their absence, such compounds possess no antibacterial activity.)

Divalent cations did not stimulate antibacterial activity of *trans*-isohumulone suggesting that neither the chloride ion nor ionic strength effects were responsible for the enhancement observed with monovalent cations. In contrast to the effect of monovalent cations, Ca^{2+} reduced the antibacterial efficacy of *trans*-isohumulone to about 3/4. Unlike Ca^{2+}, which had no effect on the growth yield of control cultures, Mg^{2+} strongly inhibited growth of *Lact.brevis* IFO 3960. *Trans*-isohumulone could protect the organisms from the toxic effects of Mg^{2+}. It seems unlikely that this could be brought about by direct interaction between *trans*-isohumulone and Mg^{2+}, since the amount of *trans*-isohumulone involved (molar ratio Mg^{2+}/*trans*-isohumulone *ca* 18,000 - 50,000) was so small. An effect of *trans*-isohumulone on the response of the cells to Mg^{2+}-toxicity is therefore more likely. Of all divalent cations tested the effect of Mn^{2+} was most marked. This ion antagonised the antibacterial action of *trans*-isohumulone. Later work, reported in section 4.3.4, has demonstrated that this is because *trans*-isohumulone has the ability to carry Mn^{2+} across the cell membranes of sensitive bacteria. The antagonism observed thus resembles that between nigericin and K^+, or monensin and Na^+ (Nicholls 1982), rather than being the result of either precipitation of *trans*-isohumulone by Mn^{2+} or a direct effect of Mn^{2+} on the test organism.

3.4.2. Incubation conditions

Sometimes the response of a microorganism to an antibacterial agent is affected by the oxygen content of the medium. For example, cells of *Staphylococcus aureus* are up to 20x less sensitive to aminoglycoside antibiotics when incubated in anaerobic rather than aerobic conditions (Reynolds *et al.* 1976). *S.aureus* and *Escherichia coli* are up to 100x less sensitive to

sulphamethoxazole and trimethoprim in anaerobic conditions than they are in aerobic conditions (Virtanen 1974). The antibacterial activity of *trans*-isohumulone, however, was the same regardless of whether the organisms were exposed to the compound in aerobic or anaerobic conditions. Thus, variation in the oxygen tension of the medium does not lead to variation in assay results.

3.4.3. Influence of (-)-humulone on antibacterial activity of *trans*-isohumulone

At pH 6.5, the antibacterial activity of (-)-humulone is about six times greater than that of *trans*-isohumulone (Figure 3.3). This can be explained on the basis of, (i) differences in the intrinsic antibacterial activity of the (undissociated) (-)-humulone or *trans*-isohumulone molecules and, (ii) differences in the degree to which each of the compounds ionise at this pH. At pH 6.5, (-)-humulone is about 97% ionised (3% undissociated) while *trans*-isohumulone is about 99.96% ionised (0.04% undissociated). The MIC_{undiss} of (-)-humulone is about 20x greater than that of *trans*-isohumulone. To inhibit bacterial growth, antimicrobial compounds must first come in contact with a sensitive cell component. If this component is the cell membrane, or is located in the cytoplasm, the antibiotic must pass through the cell wall to reach its target. Frequently binding of the compound to the cell wall precedes transport of the antobiotic. The binding process is often ionic and therefore influenced by the degree to which the antibacterial agent and the cell wall component ionise. For example, polymixin, which acts on bacterial cell membranes, binds reversibly to the surface of sensitive bacterial cells (Newton 1956). Competition between polymixin molecules and divalent cations such as Mg^{2+} can reduce the extent to

which polymixin binds to the cell with a consequent reduction in antibacterial activity (Newton 1954).

Since the extent to which (-)-humulone and *trans*-isohumulone ionise in solution at pH 6.5 differs by a factor of 75, it seemed possible that, if ionic binding played a role in the interaction of such compounds with the bacterial cell surface, competition could occur between *trans*-isohumulone and (-)-humulone ions resulting in a change in the antibacterial activity of one or both of the compounds. Clearly this does not occur.

3.4.4. Effect of ethanol

Ethanol did not affect the antibacterial activity of *trans*-isohumulone. This finding has practical significance since, as stated in section 1.1.2, the major use of hop-derived compounds is in beer production.

Several factors limit growth of bacteria in beer. These include, (i) the scarcity of nutrient material, (ii) the low pH, (iii) the presence of EtOH, (iv) the presence of hop-derived compounds, and (in some beers, in some countries), (v) the presence of preservatives.

Up until now, there has been no information available relating to the effect of EtOH on the sensitivity of beer-spoilage lactic acid bacteria to compounds derived from hops. This is a particularly important area since a small, but significant, proportion of the world beer market now consists of low-(<1%) and non-(<0.05%) alcoholic beers. The present findings indicate that it should be possible to model the bacteriological stability of beers, including low-alcohol and non-alcoholic beers, without recourse to complex multivariate analysis. Future work in this area could prove to be of significant economic benefit.

3.4.5. Neutralising agents

When studying the action of antibacterial agents, or when isolating microorganisms from an antibiotic-containing medium, it is often necessary to arrest antibacterial activity and this can be achieved in several ways (reviewed in Russell *et al.* 1979): (i) by dilution of the compound to a sub-inhibitory concentration; (ii) by modifying the growth conditions such that the agent becomes less active or; (iii) by adding a neutralising agent to complex the antibacterial compound.

Inactivation of *trans*-isohumulone can be achieved by altering the pH of the treated cell suspension since only undissociated molecules have antibacterial activity. The observation that dilution of *trans*-isohumulone-treated cells into fresh growth medium allows resumption of bacterial growth (section 4.3.1.) indicates that the material is weakly-bound to the cells. Where appropriate, dilution of the cell suspension (preferably into a medium of higher pH) will diminish antibacterial activity.

For some applications, the use of a chemical inactivator may be more appropriate (*e.g.* in studies of the mode of action of hop compounds and hop-derived compounds). Tween 80 is widely used to inactivate antimicrobial activity of organic acids such as benzoic and sorbic acids (Russell *et al.* 1979). Mixtures of Tween 80 and lecithin are similarly used to inactivate quaternary ammonium compounds (Russell *et al.* 1979). Both Tween 80 and Tween 80:lecithin combinations antagonised the antibacterial action of *trans*-isohumulone. Inactivation most likely resulted from a reduction in the concentration of free undissociated isohumulone molecules in solution brought about by the fact that some isohumulone molecules became trapped in micelles of the neutralising agent(s). Such inactivation was not unexpected. Sacks and Humphreys

(1951), following up on a preliminary observation of Chin *et al.* (1949), had previously shown that phospholipids present in serum reduced the *in vivo* efficiency with which the β-acid lupulone inhibited growth of *Mycobacterium tuberculosis*.

Inactivation of the antibacterial activity of *trans*-isohumulone by cyclodextrins is particularly interesting. Cyclic carbohydrates, such as α-cyclodextrin and β-cyclodextrin are obtained by the action of cyclodextrin transferases on starch (for review see Bender & Komiyama 1978). α-Cyclodextrin is composed of six glucose units; β-cyclodextrin is composed of seven glucose units. The glucose molecules are arranged such that, while the exterior of the cyclodextrin molecule is hydrophilic, on account of the presence of a large number of polar hydroxyl groups, the interior is non-polar.

A cyclodextrin molecule can be considered as an empty doughnut-shaped capsule with a hydrophilic exterior and a hydrophobic inner cavity. They have the ability to form inclusion complexes with a large number of organic compounds. Such 'guest' compounds become partially or completely trapped within the hydrophobic cavity of the cyclodextrin molecule. Whether complex formation takes place depends on a number of factors including the size, shape and polarity of the guest compound, and the concentration and type of cyclodextrin. In general, other factors permitting, guest compounds with a high oil/water partition coefficient have greater affinity for the non-polar cyclodextrin cavity. The molecule can only be accomodated, however, if it is of an appropriate size (not too big, not too small) to fit into the cyclodextrin cavity (α-cyclodextrin possesses a cavity of internal diameter *ca* 0.45nm while the cavity of β-cyclodextrin has an internal diameter of *ca* 0.7nm). α-Cyclodextrin had little influence on the antibacterial activity of

trans-isohumulone, but β-cyclodextrin reduced its activity as effectively as did Tween 80 and the Tween 80:lecithin combination. It seems likely that inactivation of *trans*-isohumulone activity was the result of the formation of a complex between β-cyclodextrin and *trans*-isohumulone.

The use of cyclodextrins as inactivating agents offers several advantages over more commonly-used procedures. For example, lecithin (2%) solubilised with Lubrol W (3%) is a recommended (Anon 1960) inactivator for quaternary ammonium compounds. However, this inactivator can be lethal to some bacteria (Laycock & Mulley 1970). Also, although high concentrations of Tweens and other detergents antagonise the action of parabens and quaternary ammonium compounds, low concentrations can potentiate their action (Allwood 1973). Such problems could probably be avoided by the use of cyclodextrins since they are not surface-active and do not influence microbial growth (Simpson 1991). In addition, the ability to selectively inactivate a specific antimicrobial agent through the use of an appropriate type of cyclodextrin offers intriguing possibilities.

3.4.6. Significance of inoculum history on subsequent sensitivity of *Lact.brevis* IFO 3960 to *trans*-isohumulone

Variation in the procedure used to prepare an organism for use in assay of the antibacterial efficacy can affect assay precision. In the present experiments it was shown that neither the pH at which the cells were grown nor their phase of growth immediately prior to testing influenced their subsequent sensitivity to

trans-isohumulone. This helps to explain, at least in part, the consistency of the results obtained.

3.4.7. Practical significance

These results demonstrate the important contribution which hop compounds and hop-derived compounds make to the biological stability of beer. As little as 4μM (1.45mg/l) *trans*-isohumulone inhibited growth of *Lact.brevis* IFO 3960 in mod. MRS at pH 4.0. Beer typically contains 69μM of iso-α-acids and has a pH value of 3.8-4.2. The conclusion of Teuber (1970) that isohumulone plays little part in beer stability is thus clearly erroneous. It is significant that Teuber's conclusion was based on tests carried out under conditions which were much less acidic than those of beer (the pH of the medium employed was not stated but was probably in the range 6.5-7.0). Under such conditions a much greater quantity of isohumulone is required for inhibition of bacterial growth since the proportion of undissociated molecules present decreases with increasing pH. Indeed, it should be possible to increase the intrinsic antibacterial activity of beer without affecting the intensity and quality of the bitter flavour which hop-derived materials impart. For example, careful control of beer pH could be used to optimise the microbiological stability of beer (Simpson & Hammond 1991) since the bitterness of hop-derived compounds is not markedly affected by pH (Meilgaard 1960).

The work described in this Chapter has also highlighted several pertinent features concerning the antibacterial action of hop compounds and hop-derived compounds. An undissociated form of each compound was the active antibacterial moiety. The antibacterial activity of the undissociated form of the compounds was

similar, indicating that antibacterial activity was not dependent on possession of a five-membered or six-membered ring or possession of specific side chains. The structural feature common to each was a β-triketone group. Antibacterial activity was influenced by the metal content of the medium, suggesting that interactions between metal ions and the cell or test compounds are important. The lack of variation observed in the sensitivity of *Lact.brevis* IFO 3960 to any of the compounds suggests that resistant mutants do not arise at high frequency.

CHAPTER 4

The mechanism by which *trans*-isohumulone inhibits growth of *Lactobacillus brevis* IFO 3960

Death opens new doors.

(John Mansfield)

4.1. INTRODUCTION

The mechanism by which hop compounds and hop-derived compounds inhibit growth of lactic acid bacteria has previously received little attention. This is surprising as lactic acid bacteria are the *only* Gram-positive bacteria capable of growth in beer, and beer is the *only* beverage which is preserved with such materials. The first attempt to elucidate the mechanism of growth inhibition was made by Teuber & Schmalreck (1973). They showed that lupulone, humulone, isohumulone and humulinic acid induced 'primary membrane leakage' in *Bacillus subtilis* 168. Interference with membrane function led to complete inhibition of active transport of α-methyl glucoside and several amino acids into whole bacteria and isolated membrane vesicles within 5min at the minimum inhibitory concentration (MIC). They later examined the antimicrobial activity of various natural and synthetic hop compounds and hop-derived compounds (Schmalreck *et al.* 1975). The data presented supported their view that the cell membrane was the target for such materials in *B.subtilis*, but the mechanism by which membrane function was affected was not studied.

Until now, however, similar studies have not been made on the interaction between hop compounds and hop-derived compounds and lactic acid bacteria. This is of importance since the organism studied by Teuber's group (*B.subtilis*) generates adenosine 5'-triphosphate (ATP) by oxidative phosphorylation, in contrast to lactic acid bacteria which exclusively employ substrate level phosphorylation (Harold 1986). Since the most likely target site was the plasma membrane this distinction is particularly relevant.

4.2. EXPERIMENTAL

4.2.1. Chemicals

All chemicals were at least of analytical reagent grade, and used without further purification. Hop compounds and hop-derived compounds were prepared, and their identity and purity assessed, as described in section 2.2.3. D-[U-^{14}C]-sorbitol (specific activity 12GBq/mmol), methyl(α-D-[U-^{14}C])-glucopyranoside (α-methyl glucoside) (specific activity 5.33GBq/mmol), L-[U-^{14}C]-leucine (specific activity 11.4GBq/mmol) and tetra[^{3}H]phenylphosphonium bromide ([^{3}H]-TPP) (specific activity 851GBq/mmol) were obtained from Amersham International, UK. Salicylic acid-carboxy-^{14}C (specific activity 0.43GBq/mmol) was obtained from Sigma, USA.

4.2.2. Buffers

Hepes/EDTA buffer (for use in assay of adenosine 5′-triphosphate [ATP]) was prepared as follows. Hepes (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid) (50mM), containing ethylenediamine tetra-acetic acid (EDTA, 2mM) was adjusted to pH 7.75 with solid NaOH. Care was taken to avoid contamination of the buffer with ATP from laboratory equipment, perspiration *etc.*

Sodium 3,3′-dimethylglutarate (NaDMG) buffer was used in most experiments involving the use of live bacterial cell suspensions. This buffer, prepared by titration of 0.1M NaOH with solid 3,3′-dimethylglutaric acid to the desired pH (Lambert & Smith 1977) within the range 4-7, was used when it was necessary to maintain a constant concentration of metal cations over the pH range employed. Dimethylglutarate was chosen as the buffer anion for several reasons: (i) it provided buffering capacity over the wide pH range used, (ii) it did not form precipitates with any of the hop compounds and hop-

derived compounds or with monovalent or divalent metal cations, (iii) it did not absorb light in the region of λ=260nm, (iv) it did not have an adverse effect on the physiology of *Lactobacillus brevis* IFO 3960.

This latter criterion was demonstrated in two ways. Firstly, dimethylglutarate was shown to have little inhibitory effect on growth of the organism in mod. MRS (although other lactic acid bacteria were inhibited by the compound to varying degrees). Secondly, a comparison of intracellular ATP concentrations of cells suspended in NaDMG buffer (pH 5.2) containing glucose [10g/l] or other media (deionised water, mod. MRS, phthalate buffer containing glucose [10g/l], phosphate buffer containing glucose [10g/l], acetate buffer containing glucose (10g/l): all media adjusted to pH 5.2) revealed that the cellular ATP content of the organism was not adversely affected by NaDMG buffer over several hours exposure.

All other buffers were prepared using standard methods (Dawson *et al.* 1987). Details of their composition are given in each experimental section.

4.2.3. Growth media

A modified version of de Man Rogosa Sharpe (MRS) medium (de Man *et al.* 1960) was used for most experiments: the original formulation of MRS medium (containing agar) was used for maintenance of the test organism (full details of these media are given in section 3.2.2.). MRS agar (Oxoid, UK) was used to perform viable counts.

4.2.4. Test organism

Lact.brevis IFO 3960, obtained from the Institute of Fermentation, Osaka, Japan, was maintained on MRS agar (subcultured at 3-month intervals) and stored at 4°C.

4.2.5. Assessment of growth

Growth was assessed as follows: (i) the absorbance of cell suspensions in mod. MRS medium was measured in disposable plastic cuvettes of 1cm light path at λ=560nm; (ii) the number of viable organisms was estimated by plating serial dilutions of the sample onto MRS agar. Sterile deionised water was used as diluent. Control experiments employing *Lact.brevis* IFO 3960 showed that the viability of the organism was not affected by this diluent. The number of colonies which developed on the surface of MRS agar plates after 5d incubation under aerobic, and occasionally anaerobic, conditions at 25°C was estimated; (iii) the concentration of microbial ATP in the culture fluid was estimated as described in section 4.2.7.; (iv) The dry weight (dry wt.) of stationary-phase cultures was also measured using a gravimetric method. Where appropriate, cell concentrations have been expressed in terms of dry wt. using the relationship,

$$1\text{mg dry wt.} = 2 \times 10^8 \text{ organisms.}$$

4.2.6. Growth conditions

Standardised cell suspensions of *Lact.brevis* IFO 3960 were prepared in the following way. Attemperated growth medium (mod. MRS medium, pH 5.2, 10ml) was inoculated with a small amount of growth from an MRS slope culture and incubated without shaking at 25°C for 72h. Fresh medium (150ml in a 250ml Erlenmeyer flask, attemperated to 25°C prior to use) was inoculated with the culture (1.5ml) then incubated with shaking (50rpm, 25°C). Organisms were harvested or sampled as described below. For experiments which required the use of organisms in a standard physiological condition, such cultures were harvested after 16.5h growth. At this time A_{560}=1.0, the

number of viable organisms was 8 x 10^7/ml, the dry wt. of the suspension was 0.4mg/ml and the pH of the growth medium was 5.1. After washing, the organisms were suspended in an appropriate physiological buffer (as specified in the relevant experimental section).

The procedure employed for preparation of standardised cell suspensions was reproducible. The absorbance (λ=560nm) of replicate cultures prepared using these methods had a standard error $<\pm 5\%$.

4.2.7. Assay methods

The assay methods used, the principle on which each was based and the limit of detection achieved in each of the assays is given in Table 4.1. The individual test methods are described in detail below.

Anion and cation analysis

Anion and cation concentrations were estimated by ion-exchange chromatography using a Dionex 4000i advanced chromatography system. Monovalent cations (K^+, NH_4^+) were assayed using an eluant (flow rate 1ml/min) which contained HCl (12mM) and diaminopropionic acid monohydrochloride (0.5mM). The ion-exchange columns (CG3, guard: GS3, analytical; equipped with a cation micromembrane suppressor) were regenerated using tetrabutylammonium hydroxide solution (40mM). Samples were diluted 1:50 prior to assay. Divalent cations (Mg^{2+}, Ca^{2+}) were assayed using an eluant (flow rate 1ml/min) which contained HCl (40mM) and diaminopropionic acid monohydrochloride (4mM). The ion-exchange column (CG3, equipped with a cation micromembrane suppressor) was regenerated with tetrabutylammonium hydroxide (40mM). Samples were diluted 1:5 prior to assay.

Anions (Cl^-, NO_3^-, Br^-, PO_4^{3-}, SO_4^{2-}) were assayed using an eluant (flow rate 1.8ml/min) which contained Na_2CO_3

Table 4.1: Assay methods: Principles and limits of detection

Analyte	Principle of method	Limit of detection
Anions/Cations (K^+, NH_4^+, Mg^{2+}, Ca^{2+}, Cl^-, PO_4^{3-}, SO_4^{2-})	Ion-exchange chromatography	1-5 x 10^{-5}M
Inorganic phosphate	Colorimetric assay based on reaction with molybdate	1 x 10^{-5}M
Adenosine 5'-triphosphate	Firefly luciferase reaction	2 x 10^{-12}M
Trans-isohumulone	Solvent partition extraction followed by spectrophotometry	5 x 10^{-6}M
Protein	Protein-induced spectral changes in dye solution	0.1g/l (bovine gamma-globulin)
^{3}H-labelled compounds	Liquid scintillation counting	*ca* 2Bq
^{14}C-labelled compounds	Liquid scintillation counting	*ca* 1Bq

(0.9mM) and $NaHCO_3$ (0.9mM). The ion-exchange columns (AG4A, guard: AS4A, analytical; equipped with anion micromembrane suppressors) were regenerated with H_2SO_4 (25mM). Samples were diluted to varying degrees for analysis. It was not possible to assay phosphate under these conditions as phosphate and dimethylglutarate ions co-eluted in this analysis system.

Extraction and analysis of adenosine 5'-triphosphate

Adenosine 5'-triphosphate (ATP) was measured in cell extracts and extracellular fluids using the firefly luciferase assay which relies upon the ATP-dependent oxidative decarboxylation of D-luciferin. The reaction, catalysed by firefly luciferase, produces light. For assay of ATP, the reaction conditions can be manipulated such that the rate at which light is produced is directly proportional to the concentration of ATP in the reaction. Lyophilised luciferase reagent (LUMIT PM, Lumac bv, the Netherlands) was reconstituted according to the manufacturer's instructions. Adenosine 5'-triphosphate (disodium salt) was obtained from Boehringer Mannheim (UK, Prod. no. 127531) and dissolved in sterile deionised water to a concentration of 0.1M. Solutions of ATP were stored frozen (at -20°C) in separate plastic tubes until required then diluted to a final concentration of 0.2μM. This solution contained 2pmol ATP in each 10μl volume and was used as an internal standard. In most cases extraction of ATP from cells was performed using trichloroacetic acid (TCA) (Lundin 1984) as follows. Cell suspension (1ml) and TCA solution (1ml, 5% w/v, 4°C) were mixed in a sterile disposable polythene bijou. The extract was diluted in Hepes/EDTA buffer (pH 7.75) then assayed. Such measurements provided an estimate of the total (intracellular and extracellular) ATP concentration. In addition, analyses were made of cell

extracts which had been prepared using cationic detergents. This technique was particularly useful when the number of viable organisms in the culture, or the amount of ATP present in the organisms, was small. Due to commercial considerations, this technique will not be described in detail here, but a discussion of its performance and the preparation of such reagents can be found in the literature (Simpson & Hammond 1989a, 1989b). Cell extracts prepared by these methods were assayed as follows. TCA extracts were diluted (typically 1:40) in Hepes/EDTA buffer. A portion (100µl) of diluted extract was then transferred to a clean disposable luminometer cuvette and firefly luciferase reagent (100µl) added. Light emission, measured in relative light units (RLU), was monitored with a Lumac M2010A biocounter using a 10sec integration period then ATP (10µl, 2pmol) was added to the cuvette. Light emission was then recorded once again. Cell extracts prepared using cationic detergents were prepared for analysis by addition of a 'neutralising reagent' (Simpson & Hammond 1989b) (50µl) to the sample (50µl) in a clean disposable luminometer cuvette then analysed as described above.

The concentration of ATP in the extracellular medium was estimated from analysis of membrane-filtered growth medium. The ATP content (pmol/ml) of the fluids was calculated using the following expression

$$[ATP] = \frac{RLU_{sample} - RLU_{reagent\ blank}}{2\,(RLU_{sample+standard} - RLU_{sample})} \times D \qquad (4.1)$$

where RLU_{sample}=light output from sample reaction, $RLU_{reagent\ blank}$=light output from reaction with reagent and buffer, $RLU_{sample\ +\ standard}$=light output from reaction with reagent, sample and added standard and D=dilution factor. The content of intracellular ATP was estimated by subtracting the ATP content of the extract from that of

the suspending fluid (after making a correction for dilution of the sample).

Most of the ATP analysis data is presented in units of pmol/ml culture but in some cases the data was converted to cellular concentrations. For this purpose, the intracellular volume of the bacterial cells was determined, under identical conditions to those in which the ATP content was assayed, using a radiolabel technique (section 4.2.13.). Typically a value of 1.5fl/cell was obtained for *Lact.brevis* IFO 3960.

***Estimation of* trans-*isohumulone* concentrations**

Samples were analysed using a method based on that described by Brenner *et al.* (1956) in which the compound was assayed by spectrophotometry after isolation by solvent partition extraction. Solutions for analysis (9.75ml) were transferred to glass McCartney bottles of 20ml capacity and acidified by addition of concentrated HCl (0.25ml); 2,2,4-trimethylpentane (10ml) was then added and the tubes shaken on a wrist-action shaker at *ca* 20Hz for 10min. The organic solvent layer was decanted and its absorbance at λ=280nm (in quartz cells of 1cm path length) measured against a 2,2,4-trimethylpentane blank. Control experiments measured the contribution to the absorbance of any interfering compounds which may have been extracted from the microorganisms during the solvent partition process. No interference from this source was evident. Additional control experiments estimated the degree to which *trans*-isohumulone was recovered from the cell supernatant by this methodology. In all cases recovery exceeded 95%. As an additional precaution, absorption spectra (λ=200-500nm) of representative samples were recorded to ensure that the compound was recovered from the test system unchanged. This was invariably the case.

Assay of protein

A dye-binding assay (Bradford 1976; Spector 1978), based on the protein-induced spectral changes which occur in acidic solutions of Coomassie Brilliant Blue G-250, was employed. One volume of a commercially-available reagent (Bio-Rad protein assay reagent, Bio-Rad Laboratories GmbH, Germany) was diluted with four parts of sterile deionised water and the mixture filtered through Whatman No.1 filter paper prior to use. Dilute reagent was stored at 4°C and used within two weeks. The assay was performed by mixing sample (100µl) and assay reagent (5ml) in a glass test tube then incubating the mixture at 30°C for *ca* 20min (the timing of the assay incubation was not critical). The absorbance of the solution at λ=595nm, in plastic disposable cuvettes of 1cm path length, was then determined against a water blank. The protein used to prepare standard curves (spanning the range 0-1g/l), for the purpose of calibrating the assay, was bovine gamma-globulin (Sigma, UK).

Plasma membranes were prepared for assay by mixing a volume of the membrane preparation with an equal volume of 0.1M NaOH. The mixture was boiled for 5min, cooled, then its pH adjusted by addition of one volume of 0.1M HCl. Samples (100µl) were assayed as described above.

Assay of inorganic phosphate

Inorganic phosphate was assayed as described by Serrano (1978). Samples (1ml) were mixed with 2ml of a solution which contained H_2SO_4 (2% v/v), ammonium molybdate (5g/l) and sodium dodecyl sulphate (5g/l)[a]. Ascorbic acid (20µl, 100g/l) was then added and the colour allowed to develop over 5min at 30°C. The absorbance of the

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[a]This reagent also stopped the ATPase reaction in assays of ATPase activity.

solution in disposable plastic cuvettes of 1cm path length was measured at λ=750nm against a water blank. The amount of phosphate in the sample was calculated by reference to a standard curve. The standard curve was prepared using KH_2PO_4 (dried at 105°C, 3h prior to use) to give a range of standard phosphate solutions (0-500μM).

Liquid scintillation counting

Samples were assayed for radioactivity using a TRI-CARB 460C automatic scintillation counter (Packard Instrument Company Inc., USA). Counting was performed for 5min in all cases. Spectral windows (channel a/b) of 0-19 and 2-19 for ^{3}H or 0-156 and 4-156 for ^{14}C were selected as appropriate. Quench corrections were automatically performed by the instrument by means of a computer program. All samples were externally standardised by means of a ^{226}radium source placed adjacent to the scintillation vial. Values were automatically corrected by the instrument on the basis of the response to this external standard. Control experiments confirmed that the counting corrections performed by the scintillation counter were valid.

Scanning electron microscopy

Cells of *Lact.brevis* IFO 3960, exposed to a range of concentrations of *trans*-isohumulone, were examined by scanning electron microscopy. This technique was also used to obtain precise estimates of the dimensions of growing organisms exposed to different concentrations of *trans*-isohumulone. The cryogenic technique employed for preparation and observation of the test samples minimises the incidence of artifacts associated with examination of such materials (Echlin 1986). A Philips scanning electron microscope (SEM 505) equipped with a cryostage (Oxford Instruments, UK) was employed.

Samples were prepared for analysis in the following way. Organisms were filtered through polycarbonate membrane filters (0.22μm pore size, Nucleopore, USA), washed twice with sterile water, then blotted dry from beneath with velin tissue. The filters were then attached to carbon or aluminium stubs (3mm thick) using a mixture which contained equal parts of colloidal graphite and Tissue Tek (Mill Laboratories, USA). The stubs were plunge-frozen in liquid nitrogen slush and transferred under vacuum to the cryosystem sample unit attached to the electron microscope. Samples were treated to remove surface water by controlled sublimation of the water at -80°C, transferred to the outer compartment of the cryochamber, then sputter-coated with gold at -180°C. Finally, they were transferred back into the main compartment of the cryochamber and observed at -188°C.

4.2.8. Effect of *trans*-isohumulone on exponentially growing cells of *Lact.brevis* IFO 3960

The effect of *trans*-isohumulone on exponentially growing cells of *Lact.brevis* IFO 3960 in mod. MRS broth (pH 5.2) at 25°C was monitored by measurement of absorbance, viable cell count (on MRS agar) and intracellular ATP. When the number of organisms in the culture reached 4×10^7/ml (A_{560}=0.5) *trans*-isohumulone (0, 8, 16, 24, 32, 40, 80 or 120μM) was added as a methanolic solution (0.75ml/150ml) (control cultures received methanol alone). Growth was monitored before and after addition of *trans*-isohumulone. Cell morphology was monitored by bright-field, phase-contrast and dark-field microscopy. Accurate measurements of the size of control and isohumulone-treated cells were made by scanning electron microscopy. One hundred each of control and isohumulone-

treated cells were measured as described in section 4.2.7. Leakage of ATP was assessed by analysis of cell-free (membrane-filtered) growth medium.

4.2.9. Effect of *trans*-isohumulone on non-growing cells of *Lact.brevis* IFO 3960

A concentrated standardised cell suspension of *Lact.brevis* IFO 3960 was prepared as described in section 4.2.6. The buffer used to resuspend the organisms (0.1M NaDMG, pH 5.2) contained glucose (10g/l). A series of solutions of *trans*-isohumulone was prepared in the same buffer such that, when the solutions were mixed with organism suspension (one part organism suspension, nine parts buffer) the concentration of *trans*-isohumulone in the organism suspension ranged from 0-400μM. After adding the suspension to the buffered solutions in plastic universal tubes of 50ml capacity, the tube contents were incubated at 25°C, with shaking (50rpm) for 60min. Each suspension was then analysed as follows:
(i) viable count was determined on MRS agar;
(ii) intracellular ATP content was measured using the firefly luciferase reaction; (iii) cell morphology was examined by bright-field, phase-contrast and dark-field microscopy of unstained preparations at a magnification of 400x. In addition, the absorbance of each suspension was measured at λ=560nm in cells of 1cm path length against a water blank. To assess whether the plasma membrane of *Lact.brevis* IFO 3960 was damaged by *trans*-isohumulone, cell-free (membrane-filtered) supernatant fluids were analysed for intracellular materials, which could have leaked from the organisms. Extracellular ATP was measured using the firefly luciferase reaction. Anions and cations (including K^+) were measured by ion-exchange chromatography. Material

absorbing light at λ=260nm was measured by spectrophotometry in quartz cells of 1cm path length after removal of residual *trans*-isohumulone by solvent partition extraction of the acidified (2.5% v/v HCl) extract with 2,2,4-trimethylpentane. Control experiments were performed to determine the influence of the solvent partition process on the recovery of 260nm material. No significant losses occurred.

4.2.10. Measurement of the uptake of *trans*-isohumulone by non-growing cells of *Lact.brevis* IFO 3960

Standardised cell suspensions were grown in mod. MRS as described in section 4.2.6. until the organisms reached the late logarithmic phase of growth (A_{560}=1.0, 16.5h). They were harvested by centrifugation (3000 x *g*, 10min, 4°C), washed once with sterile deionised water (4°C) then resuspended in NaDMG buffer (0.1M, pH 6.5 or pH 4.5) at 25°C to A_{560}=1.0 (equivalent to 8 x 10^7 organisms/ml). Suspensions prepared in this way were used to measure, (i) the rate of uptake of *trans*-isohumulone by cell suspensions, (ii) the pattern of uptake of *trans*-isohumulone by cell suspensions (adsorption isotherms).

Rate of uptake of* trans-*isohumulone

Trans-isohumulone (1ml, 60mM in abs. MeOH) was added to standardised cell suspension (0.1M NaDMG, pH 6.5, 200ml) at 25°C to give a final concentration (after allowing for dilution by cell material) of 0.273mM (*ca* MIC for growth in mod. MRS medium at this pH). At various times (0-30min) after addition of the test compound, samples (15ml) were filtered through disposable membrane filters. In one experiment the pH of the buffer used was 4.5.

Since *trans*-isohumulone is 100x less soluble at pH 4.5 than at pH 6.5 the concentration used at this pH was 30μM (*ca* 3x MIC: *n.b.* The test compound was not applied at the MIC in this experiment since the amount of compound remaining in solution could not be measured precisely at this low concentration). The amount of *trans*-isohumulone remaining in solution in each experiment was estimated as described in section 4.2.7.

***Pattern of* trans-*isohumulone* uptake**

Trans-isohumulone (0-150μl, 50mM in abs. MeOH) was added to NaDMG buffer (0.1M, pH 6.5) to give a range of concentrations (0-2mM). Such solutions were then mixed with double-strength standardised organism suspensions (A_{560}=2.0) to expose the organisms to 0,100,200,300,400, 600,800 or 1000μM *trans*-isohumulone. After 10min exposure, the cells and test compound were separated by filtration and the amount of *trans*-isohumulone remaining in solution estimated as described in section 4.2.7.

4.2.11. Effect of hop compounds, hop-derived compounds and selected ionophores on the passive proton permeability of *Lact.brevis* IFO 3960

The effect of *trans*-isohumulone and selected ionophores (carbonyl cyanide *m*-chlorophenylhydrazone [CCCP], valinomycin) on the passive proton permeability of *Lact.brevis* IFO 3960 was studied using a titrimetric technique (Mitchell 1961b; Mitchell & Moyle 1967; Harold & Baarda 1968b). Organisms were prepared using the procedure described in section 4.2.6. and harvested by centrifugation (3000 x *g*; 4°C; 30min) when they had entered the late logarithmic phase of growth (A_{560}=1.0). The pellet was washed twice with cold (4°C) deionised

water and stored at 4°C until used. The organisms were resuspended to a concentration of 20mg wet wt./ml (*ca* 1 x 10^9 organisms/ml) in an unbuffered medium containing KCl (150mM) and $MgCl_2$ (2mM) at 24°C then transferred to a pyrex beaker (25ml capacity) and sparged with O_2-free N_2 gas. The suspension pH, monitored continuously throughout the experiment using a pH meter connected to a chart recorder (LKB 2210, Pharmacia Ltd., UK), was adjusted to 6.5 using NaOH, then a pH gradient imposed on the organisms by addition of HCl (10mM; 200µl/20ml cell suspension). A solution of the test antibiotic (CCCP, valinomycin, *trans*-isohumulone or colupulone in MeOH) was then added. Further antibiotic additions were made as appropriate. Occasionally, deionised water was used as the suspending medium and the pH of the suspension was not adjusted with NaOH. In some experiments the flux of ions other than H^+ was monitored by addition of monovalent and divalent cations (added as chlorides) to the suspension. Ions tested included Na^+, Ca^{2+}, Mg^{2+}, and Mn^{2+}. Attempts were also made to identify the cations involved in the antibacterial action of *trans*-isohumulone by analysing the suspending medium, before and after treatment with *trans*-isohumulone, for the presence of Na^+, K^+, NH_4^+, Ca^{2+} and Mg^{2+}.

As discussed in section 1.2.2. ionophores, which catalyse transport of ions across biological membranes, fall into two classes. True ionophores, which move freely through the plasma membrane, and *quasi*-ionophores, which form a stationary pore. The activity of true ionophores is sensitive to changes in temperature, unlike that of *quasi*-ionophores. The type of ionophoric activity exhibited by *trans*-isohumulone was characterised by comparing the results of titrimetric experiments performed at 24°C and at a range of temperatures from

0-24°C. The suspending medium employed for such experiments contained KCl (150mM) and $MgCl_2$ (2mM).

4.2.12. Effect of *trans*-isohumulone on activity of the membrane-bound ATPase of *Lact.brevis* IFO 3960

The proton-translocating ATPase of *Lact.brevis* IFO 3960 was isolated using a procedure based on that described by Bender *et al.* (1986). Organisms, grown in mod. MRS (pH 5.2; 1 litre) at 25°C to late stationary phase, were harvested by centrifugation (3000 x *g*, 15min, 4°C) then washed twice with sterile deionised water. The pellet was suspended in prewarmed (30°C) buffer (200ml; tris [75mM], $MgCl_2$ [2mM], adjusted to pH 7.5 with concentrated HCl, containing sucrose [0.4M], lysozyme [2.5g/l], and mutanolysin [10,000 units/l]) then incubated, with gentle shaking at 30°C for 2h. The efficiency of protoplast formation was assessed by adding a portion (100µl) of the suspension to salt solution (9ml, 0.8M NaCl) and examining the organisms using phase-contrast microscopy. After 3h, when the conversion of cells to protoplasts was almost complete (>99%), the protoplasts were harvested by centrifugation (9000 x *g*, 20min, 4°C) then resuspended in membrane buffer (50ml; tris [50mM], $MgCl_2$ [10mM], 6-amino-*n*-hexanoic acid [40mM] and 4-aminobenzamide [6mM] adjusted to pH of 7.5 with HCl). The protoplasts were lysed by addition of NaCl (final concn. *ca* 0.8M). DNase (Type I, Sigma, DN-25) and RNase (Type XII-A, Sigma, R5500) (0.01mg/ml each) were added to hydrolyse DNA and RNA respectively. After 45min incubation at 25°C (with gentle shaking), to complete hydrolysis of DNA and RNA, the membranes were pelleted by ultracentrifugation (35,000 x *g*, 30min, 4°C) and washed twice with membrane

buffer (30ml each wash). The pellet was stored in plastic tubes at -20°C until required.

ATPase activity, in the presence and absence of *trans*-isohumulone, was assessed by measuring the release of inorganic phosphate from ATP. The reaction mixture consisted of 3,3'-dimethylglutarate buffer (16ml, 50mM, pH 4.5) containing $MgSO_4$ (10mM), glycerol (10% v/v) and sufficient plasma membrane material to give a final concentration of protein in the assay of 54mg/l. Prior to initiation of the reaction, the mixture was warmed to 30°C then split into two equal portions. Methanol (40µl) was added to one of the portions. A solution of *trans*-isohumulone in MeOH (40µl, 12mM) was added to the other. In the latter case the final concentration of *trans*-isohumulone in the reaction mixture was 60µM (which was *ca* 10x the concentration required to inhibit growth of *Lact.brevis* IFO 3960 in mod. MRS, pH 4.5). The reaction was initiated 10min after addition of *trans*-isohumulone or MeOH by addition of ATP (160µl, 0.3M) to a final concentration of 6mM. Samples (1ml) were withdrawn from the reaction mixture immediately and at intervals up to, and including, 30min. The reaction was stopped by mixing the sample with 2ml phosphate assay reagent and the phosphate content of the sample determined as described in section 4.2.7. The protein content of the membrane preparation was determined as described in section 4.2.7.

4.2.13. Measurement of intracellular pH and membrane potential of *Lact.brevis* IFO 3960 in the presence and absence of hop compounds and hop-derived compounds

Methods for measurement of (pH_{int}) and the transmembrane pH gradient (ΔpH) can be divided into two classes

(Rottenberg 1979; Padan *et al.* 1981), namely (i) ion distribution methods (*e.g.* radiolabel methods) and (ii) methods which rely on intracellularly-localised indicators of pH_{int} (*e.g.* ^{31}P NMR spectroscopy, fluorescence spectroscopy). Methods for determination of the membrane potential ($\Delta\psi$) include (i) determination of the transmembrane distribution of ions using ion-selective electrodes, liquid scintillation counting or fluorimetry (Rottenberg 1979; Padan *et al.* 1981). (ii) Measurements using molecules which penetrate into the cell membrane and respond to changes in the local membrane electrical field elicited by changes in the membrane potential. Examples of molecules which exhibit such an electrochromic effect include intrinsic membrane components, such as chlorophylls and carotenoids (Kashket 1985), and extrinsic synthetic molecules, such as the charge-shift probe DI-4-ANEPPS (Gross & Loew 1989). (iii) The membrane potential can also be measured by direct insertion of microelectrodes into the cell interior (Padan *et al.* 1981). While this method can be applied to certain eukaryotic cells and to specially-prepared 'giant' cells of *Escherichia coli*, it is not generally applicable to measurement of the membrane potential of bacteria.

In the present work, $\Delta\psi$ was determined from the equilibrium distribution of TPP^+, while ΔpH was measured using methods based on (i) the equilibrium transmembrane distribution of [^{14}C]-salicylic acid, (ii) ^{31}P NMR spectroscopy, and (iii) the pH-dependent fluorescence of fluorescein. Where possible, the effect of *trans*-isohumulone on these bioenergetic parameters in *Lact.brevis* IFO 3960 was also studied.

Radiolabel method (measurement of ΔpH and $\Delta\psi$)

The intracellular pH and membrane potential of *Lact.brevis* IFO 3960 were determined from the distribution, between cells and suspending medium, of [^{14}C]-salicylic acid and tetra[^{3}H]phenylphosphonium bromide ([^{3}H]-TPP) respectively (Rottenberg 1979). The basis of the method for measurement of pH_{int} and ΔpH is that plasma membranes of bacteria allow free passage of the undissociated form (HA) of certain weak acids such as salicylic acid (Conway & Downey 1950). In contrast, since it carries a negative charge, the ionised form (A^-) of such weak acids is unable to diffuse across the membrane. (An indispensible condition for use of such weak acids in ΔpH determination is that the cell should not possess a transport system for the acid used). When a permeable acid has reached an equilibrium distribution $HA_{in}=HA_{out}$ (HA_{in}=intracellular activity of undissociated acid; HA_{out}=extracellular activity of undissociated acid). Since the acid dissociates on both sides of the membrane (and assuming the pKa is not changed)

$$Ka = \frac{H^+_{in}\ A_{in}}{HA_{in}} = \frac{H^+_{out}\ A^-_{out}}{HA_{out}} \qquad (4.2)$$

where A^-_{in} and A^-_{out} are the intracellular and extracellular activities of the ionised form of the weak acid respectively and H^-_{in} and H^+_{out} the respective H^+ activities. It follows that, at equilibrium,

$$pH_{int}-pH_{out} = \Delta pH = \log\frac{A_{in}}{A_{out}} \qquad (4.3)$$

If pH_{out}>pKa+1, most of the weak acid is ionised on both sides of the membrane and measurement of the distribution ratio of the acid allows calculation of pH_{int} and ΔpH.

TPP belongs to a group of substances which have been synthesised with the specific objective of using them for measurement of $\Delta\psi$. The ability of π-bonded electron orbitals to delocalise charge and enhance lipid solubility has been exploited in the design of this molecule which can move across lipid bilayer membranes even though it carries a positive charge (Nicholls 1982). When a potential difference ($\Delta\psi$) exists across a membrane, a permeable ion (such as TPP^+) moves in response to this potential until electrochemical equilibrium is reached. At equilibrium,

$$\Delta\bar{\mu}_{A^+} = RT\ln(A_{in}/A_{out}) + zF\Delta\psi = 0 \qquad (4.4)$$

where $\Delta\bar{\mu}_{A+}$ is the electrochemical potential difference of the test ion. Hence,

$$\Delta\psi = -\frac{RT}{zF}\ln(A_{in}/A_{out}) \approx -\frac{RT}{zF}\ln(C_{in}/C_{out}) \qquad (4.5)$$

(A, z, C are the activity, charge and concentration of the ion, respectively). Thus, determination of the concentration ratio of the test ion at equilibrium allows calculation of $\Delta\psi$.

Provided its concentration is low (<100μM), TPP^+ has no effect on $\Delta\psi$ (Padan *et al.* 1981). Because of its charge and high lipid solubility, TPP^+ binds to cell components so, to estimate the internal and external activities, corrections must be made for the presence of bound TPP^+ (Kashket 1985; Lolkema *et al.* 1982).

Two steps are involved in measurement of ΔpH and $\Delta\psi$ using radiolabelled materials. Firstly, the intracellular volume of the organism suspension must be determined. This can be done using a freely permeant probe (^3H_2O), to estimate the combined intracellular and extracellular volumes, and a non-permeant probe ([^{14}C]-α-methyl glucoside) to determine the extracellular, or excluded, volume of the organism suspension. The

intracellular volume can be estimated from the difference between the two values. [^{14}C]-Sorbitol is widely used as a non-permeant probe for lactic acid bacteria (McDonald *et al.* 1990), but the present experiments showed that this compound was not suitable for use on *Lact.brevis* IFO 3960, since it was actively transported by this organism. The second step in such measurements is to determine the transmembrane distribution of [^{14}C]-salicylic acid (for pH_{int} measurements) and [^{3}H]-TPP^{+} (for $\Delta\psi$ measurements). To calculate pH_{int} and ΔpH the extracellular pH must also be measured using a pH meter.

Measurements of pH_{int} and $\Delta\psi$ were performed as follows. Organisms (*Lact.brevis* IFO 3960 grown 3d in mod. MRS, pH 5.2) were chilled to 4°C for 60min, harvested by centrifugation (3000 x *g*, 10min, 4°C) then washed twice with cold (4°C) buffer (0.1M NaDMG, pH 5.2, containing glucose [10g/l]). They were then suspended in fresh buffer at 25°C to a cell density of *ca* 8 x 10^{8}/ml. Antibiotics (*trans*-isohumulone, CCCP or CCCP and valinomycin, as methanolic solutions) were added to the cell suspensions (control suspensions received MeOH alone) then the organisms were incubated at 25°C for 60min. The organism suspension (1ml) was transferred to each of four plastic disposable tubes (Nunc, 50mm x 13mm, Inter Med, Denmark). To each of the tubes, $^{3}H_2O$ (3.7-7.4 x 10^{4}Bq), [^{14}C]-α-methyl glucoside (1.5-4.4 x 10^{4}Bq), [^{14}C]-salicylic acid (1.5-4.4 x 10^{4}Bq, final concn. 35-230μM) or [^{3}H]-TPP^{+} (3 x 10^{4}Bq, final concn. 35nM) was added. After 5min, cells were separated from the suspending fluid by centrifugation through silicone oil. Cell suspension (200μl) was pipetted on top of a volume (200μl) of silicone oil (4 parts AR 200, 1 part AR 20, Wacker Chemicals Ltd., Walton-on Thames, UK) contained in a polypropylene Eppendorf tube of 400μl capacity (Alpha Laboratories, Eastleigh, UK). The tube was then

centrifuged (*ca* 12,000 x *g*, 3min) in an MSE Micro Centaur centrifuge at room temperature. The organisms passed through the layer of oil to form a pellet at the bottom of the tube while the supernatant fluid remained in the upper half of the centrifuge tube. The whole tube was frozen (at -20°C) for a minimum of 2h then cut with a blunt knife through the silicone oil layer at a point just above the cell pellet. Both cell pellet and supernatant (complete with the portions of the Eppendorf tubes in which they were contained) were each transferred to plastic scintillation tubes (disposable polythene minivials [17 x 54mm] of 6ml capacity, Packard Instrument Co. Inc. USA) containing 4ml scintillation fluid (Pico-Fluor 15 scintillation solution, Packard Instrument Co. Inc., USA). The contents of each of the portions of the Eppendorf tubes were thoroughly mixed with the scintillant by vigorous shaking. The amount of radioactivity in each was then determined by liquid scintillation counting as described in section 4.2.7.

Control experiments were carried out to estimate the degree to which [^{14}C]-salicylic acid bound to de-energised cells. Distribution values were corrected for this bound material before calculation of intracellular pH. In addition, due to the proclivity of [^{3}H]-TPP^{+} to bind to bacterial cells (Lolkema *et al.* 1982), a correction was made to the [^{3}H]-TPP^{+} data to allow calculation of the true distribution ratios. This was achieved in both cases by measuring the distribution ratio of [^{14}C]-salicylic acid or [^{3}H]-TPP^{+} in cells which had been de-energised for 60min at 25°C (in 0.1M NaDMG buffer, pH 5.2 containing 10g/l glucose) with CCCP (30μM) and valinomycin (0.45μM) (Rottenberg 1979). Typically, in control cell suspensions of *Lact.brevis* IFO 3960 with $\Delta pH=0.52$ and $\Delta\psi=-55mV$, approximately 10% of the

[^{14}C]-salicylic acid and 20% of the [^{3}H]-TPP^{+} associated with the cell pellet was bound to cell materials. In all cases, it was assumed that $^{3}H_2O$ and [^{14}C]-α-methyl glucoside did not bind to cell components. Total and excluded volumes were calculated using the expression,

$$V\ (\mu l) = \frac{counts\ in\ pellet}{counts\ in\ supernatant}\ x\ 200\mu l \qquad (4.6)$$

The intracellular volume of the cell suspension was estimated by subtracting the value for the excluded volume from the total pellet volume. Intracellular pH was calculated using the expression,

$$pH_{int} = pKa + \log\left[\frac{sal_{pellet}}{sal_{super}} x \frac{vol_{super}}{vol_{int}} (10^{pH-pKa}+1) - 1\right] \qquad (4.7)$$

where sal_{pellet} and sal_{super}=radioactive counts from [^{14}C]-salicylic acid in pellet and supernatant respectively; vol_{super}=volume (μl) in which cells were suspended; vol_{int}=volume (μl) of cytoplasm associated with the total cell pellet, pH_{int} the intracellular pH value and *pKa* the negative logarithm of the acid dissociation constant (Ka) of salicylic acid (a value of 2.98 [Albert & Serjeant 1984)] was used).

The membrane potential (Δψ, in mV) was calculated using the Nernst equation,

$$\Delta\psi = 2.3\ \frac{RT}{mF}\ \log\frac{[TPP^{+}]_{int}}{[TPP^{+}]_{ext}} \qquad (4.8)$$

(at 25°C, 2.3RT/F = 59mV).

The proton-motive force (Δp, in mV) was estimated using the expression,

$$\Delta p = \Delta\psi - \frac{2.3RT}{F}\Delta pH \qquad (4.9)$$

Nuclear magnetic resonance spectroscopy method

An unsuccessful attempt was made to measure the intracellular pH of *Lact.brevis* IFO 3960 using ^{31}P NMR spectroscopy. Suspensions of *Lact.brevis* IFO 3960 were prepared for analysis using the methods described in section 4.2.6. with the exception that a higher number of organisms (*ca* 10^{10}-10^{11}/ml) was employed. To find out which phosphorylated compounds were available for *in vivo* ^{31}P NMR assay, cell extracts prepared with trichloro-acetic acid (TCA) were analysed. Extracts were prepared by mixing one part organism suspension (as used in the whole-cell ^{31}P NMR experiments) with one part TCA solution (10% w/v) at room temperature.

^{31}P NMR spectra of live organism suspensions and of extracts were acquired under the following conditions using a JEOL JNM-GX270 FT NMR spectrometer: Acquisition time 0.535sec; Pulse delay 0.3sec; Broadening factor 1Hz; Number of data points 8192. The number of scans varied between 158 and 4454 depending on the material being analysed (corresponding to an assay time of between 2.2 and 62min). Proton decoupling was not applied since the coupling constants for ^{1}H-^{31}P spin systems are generally small (0-20Hz) (Williams & Fleming 1989) in comparison with the line widths obtained in such experiments.

Due to difficulties encountered with line broadening, spectra were also recorded using organisms and extracts of *Lact.brevis* IFO 3960 which had been subcultured three times in 'low manganese' media (mod. MRS, pH 5.2 from which $MnCl_2$ had been omitted). For the same reason and to serve as control experiments, ^{31}P NMR spectra were collected of inorganic phosphate or of added adenosine triphosphate (*ca* 5-10mM each) in, (i) mod. MRS; (ii) mod. MRS plus EDTA; (iii) mod. MRS without added manganese and; (iv) NaDMG buffer.

Fluorescein diacetate method

A qualitative estimate of the effect of *trans*-isohumulone on the intracellular pH of *Lact.brevis* IFO 3960, and a number of other lactic acid bacteria, was made using a method based on that described by Tsien (1989). This method made use of the ability of live cells to hydrolyse fluorescein diacetate to fluorescein and acetate (Rotman & Papermaster 1966). Fluorescein is a weak acid which, in its ionised form, is fluorescent. The undissociated form of this compound, however, has negligible fluorescent properties. Since the pKa value of fluorescein lies between 6.4 and 6.5 (Tsien 1989) loading of microbial cells with fluorescein (as its acetate ester) should result in the production of highly fluorescent organisms provided that (i) the intracellular pH is 6 or above, (ii) a pH gradient exists across the cell membrane to facilitate accumulation of the fluorescein ester and allow retention of the fluorescent hydrolysis product.

Fluorescein diacetate (FDA) was dissolved in acetone (5g/l) and stored in the dark at -20°C until used. Organisms were stained in various media (NaDMG buffer, pH 5.2; Hepes/EDTA buffer, pH 7.75; mod. MRS medium, pH 5.2 & 7.0) by addition of FDA to a final concentration of 50mg/l, incubated at 25°C, then examined at intervals up to and including 3h using a Nikon Optiphot research microscope equipped with an episcopic-fluorescence attachment (Nikon, Japan) which provided incident blue illumination (λ=495nm) from a mercury lamp. In addition, organisms which had, first of all, been suspended in sodium acetate solution (5g/l) for 20, 60 and 120min (Molzahn & Portno 1975) were tested using the same method. Control experiments were performed in all cases using an unidentified strain of yeast isolated from spoiled beer. Fluorescence of the yeast cells was

observed in all cases, though, as reported by Molzahn & Portno (1975) fluorescence was enhanced by prior treatment of the cells with sodium acetate solution.

4.2.14. Effect of *trans*-isohumulone on uptake and efflux of [^{14}C]-L-leucine by *Lact.brevis* IFO 3960

The effect of *trans*-isohumulone on the ability of *Lact.brevis* IFO 3960 to accumulate [^{14}C]-L-leucine was studied under conditions in which protein sythesis was inhibited. In brief, experiments consisted of mixing a standardised suspension of organisms with a small amount of the radiolabelled compound and, after separating the organisms from the suspending fluid at different times, estimating the amount of radioactivity remaining in solution and accumulated by the cells using liquid scintillation counting. Initial experiments made use of a membrane filtration technique (Freese *et al.* 1973; Maloney *et al.* 1975) to effect separation of the organisms from the suspending medium. This method was later discarded in favour of the silicone oil centrifugation method (Rottenberg 1979) which gave more reproducible results. Preliminary experiments revealed that, of three substances tested ([^{14}C]-α-methyl glucoside, [^{14}C]-D-xylose and [^{14}C]-L-leucine), only [^{14}C]-L-leucine was taken up by *Lact.brevis* IFO 3960 to any significant extent under the test conditions (appropriate control experiments were performed to discriminate material accumulated in intracellular pools from that incorporated into cell material).

The effect of *trans*-isohumulone and selected ionophores on uptake of [^{14}C]-L-leucine was examined as follows. Organisms (*Lact.brevis* IFO 3960 grown 3d at 25°C in mod. MRS, pH 5.2) were chilled to 4°C, harvested

by centrifugation (3000 x *g*, 4°C, 10min) then washed twice with cold (4°C) buffer (0.1M NaDMG containing glucose [10g/l], pH 5.2). They were then resuspended in fresh buffer at 25°C (*ca* 8 x 10^8/ml) and incubated for 60min, without shaking, at 25°C. The buffer used to resuspend the organisms contained chloramphenicol (50mg/l) to prevent incorporation of [^{14}C]-L-leucine into cellular protein (Maloney *et al*. 1975). Portions of the suspension (1ml) were transferred to plastic disposable tubes and [^{14}C]-L-leucine (1.85 x 10^4Bq, final concn. 1.62μM) added. The organisms were separated from the suspending fluid by silicone oil centrifugation and the radioactivity in the cell pellet and supernatant determined as described in section 4.2.7.

In addition, the effect of ionophores (CCCP, valinomycin) and *trans*-isohumulone on the ability of *Lact.brevis* IFO 3960 to retain preloaded [^{14}C]-L-leucine was studied. In these experiments, cells were grown and harvested as described in section 4.2.6. and loaded with [^{14}C]-L-leucine for 20min. Immediately prior to the end of the 20min incubation, the suspension was dispensed into five separate plastic tubes (1.2ml/tube). At 20min, samples (200μl) were withdrawn from each of the tubes and centrifuged through silicone oil. Simultaneously, the following additions (6μl) were made to each of the tubes, (i) MeOH, (ii) *trans*-isohumulone (8mM), (iii) *trans*-isohumulone (80mM), (iv) CCCP (60mM), (v) valinomycin (90μM), CCCP (6mM). Samples (200μl) were taken from each of the tubes at 5,10, 20 and 30min, centrifuged through silicone oil and the distribution of radioactivity determined as described above.

4.3. RESULTS

4.3.1. Effect of *trans*-isohumulone on exponentially growing cells of *Lact.brevis* IFO 3960

Addition of *trans*-isohumulone to exponentially growing cells of *Lact.brevis* IFO 3960 immediately reduced their growth rate. The extent of the reduction depended on the concentration of *trans*-isohumulone added (Figure 4.1). The minimum inhibitory concentration (MIC) was 42μM. If isohumulone-treated organisms were recovered by centrifugation and added to fresh medium at 25°C, growth resumed after a lag phase of about 7h (Figure 4.2). The lag phase of untreated organisms was about 1h. A study was made of the effect of higher concentrations of *trans*-isohumulone on growing organisms. In these experiments, *trans*-isohumulone was applied to the suspension at 1x, 2x and 3x MIC. As expected, growth was inhibited in all three cases. However, prolonged examination of the cultures revealed that, at the 'MIC', some growth occurred (Figure 4.3). Thus, to a small extent, the MIC was dependent on the incubation time.

A difference in the behaviour of organisms exposed to *trans*-isohumulone for short (<24h) and long (>24h) periods was observed. In the initial stages (<24h) of contact, the viability of *Lact.brevis* IFO 3960, as assessed by counts on MRS agar, remained constant regardless of the concentration of *trans*-isohumulone to which the organisms were exposed (Figure 4.3). No aggregation of the organisms occurred, as demonstrated by a constant A_{560}/viable count ratio and confirmed by microscopy. The absorbance of cultures treated with 2x and 3x MIC, however, declined with increasing contact time (Figure 4.4). No leakage of ATP took place,

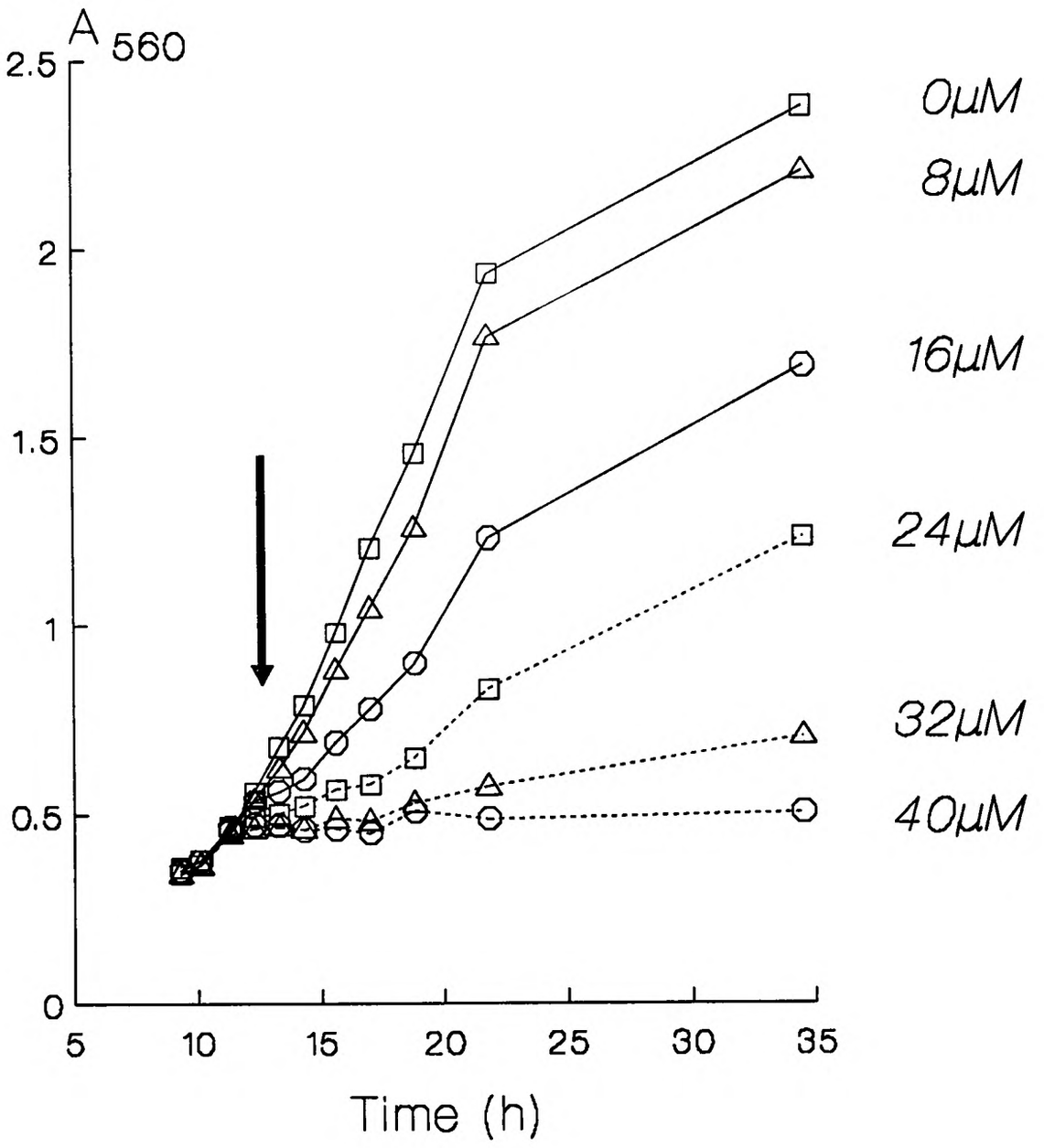


Figure 4.1 Effect of *trans*-isohumulone on growth of *Lactobacillus brevis* IFO 3960. Organisms were grown in mod. MRS (pH 5.2) at 25°C with shaking. *Trans*-isohumulone was added at the point indicated to a final concentration (μM) as shown. Growth was monitored by absorbance at λ=560nm.

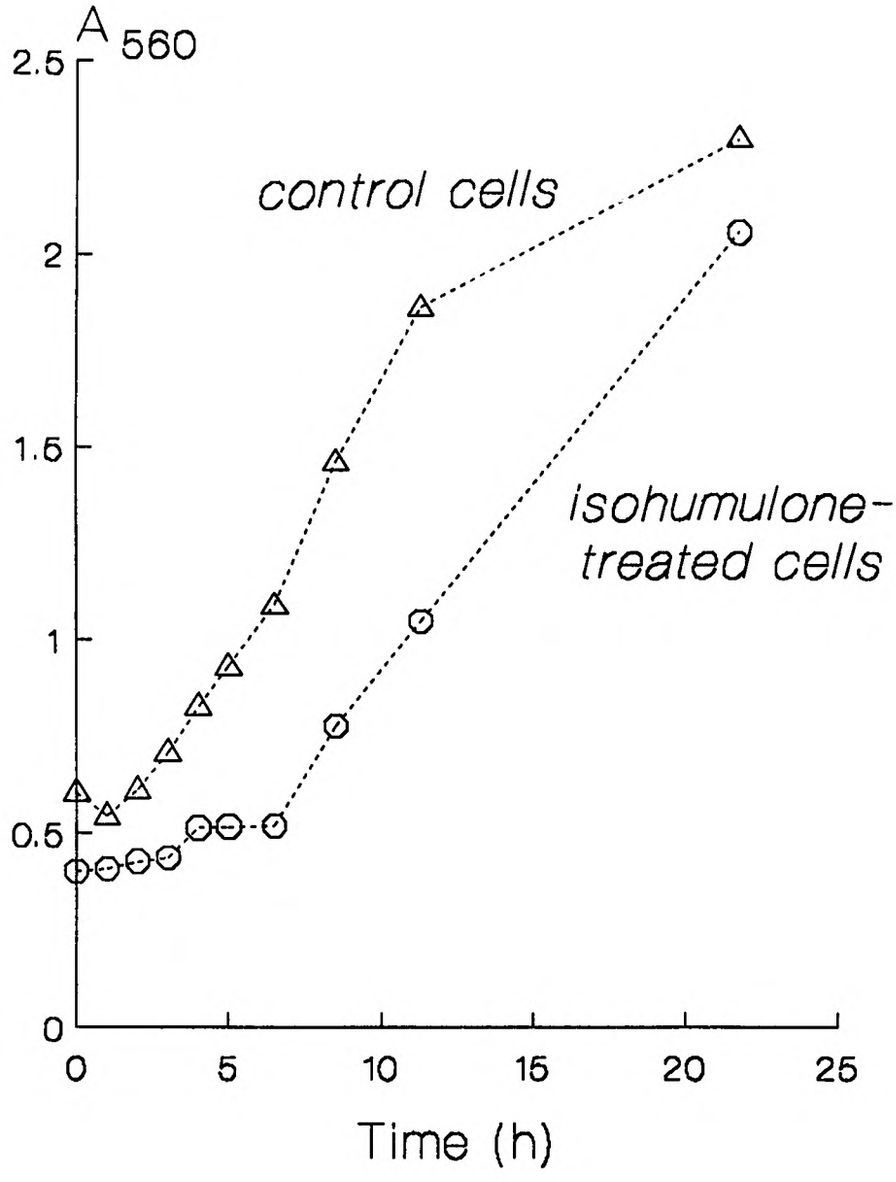


Figure 4.2 Recovery of *Lactobacillus brevis* IFO 3960 from exposure to *trans*-isohumulone. Organisms were exposed to *trans*-isohumulone (40μM) in mod. MRS (pH 5.2) as shown in Figure 4.1. After 23h exposure they were harvested by centrifugation (3000 x *g*, 15min, 25°C) then resuspended in fresh, prewarmed (25°C) growth medium. Control organisms were harvested when the A_{560} reached 0.5. Growth was monitored by absorbance at λ=560nm.

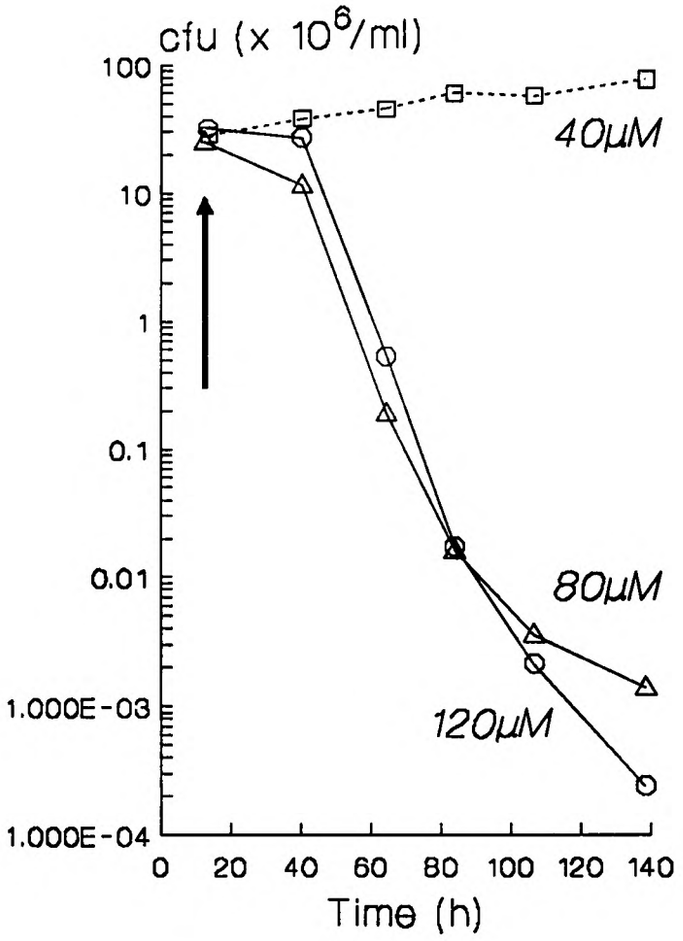


Figure 4.3 Effect of *trans*-isohumulone on viability of *Lactobacillus brevis* IFO 3960. Organisms were grown in mod. MRS (pH 5.2) at 25°C with shaking (50rpm). *Trans*-isohumulone was added to exponentially-growing organisms at the point indicated. Immediately before addition of *trans*-isohumulone, the culture contained 3 x 10^7 organisms/ml. Viability was monitored by plating on MRS agar. Each point represents the mean of three determinations. At the minimum inhibitory concentration (MIC, 40μM) *trans*-isohumulone exerted a bacteriostatic action. Prolonged contact between organisms and *trans*-isohumulone at concentrations >MIC (80, 120μM) resulted in death.

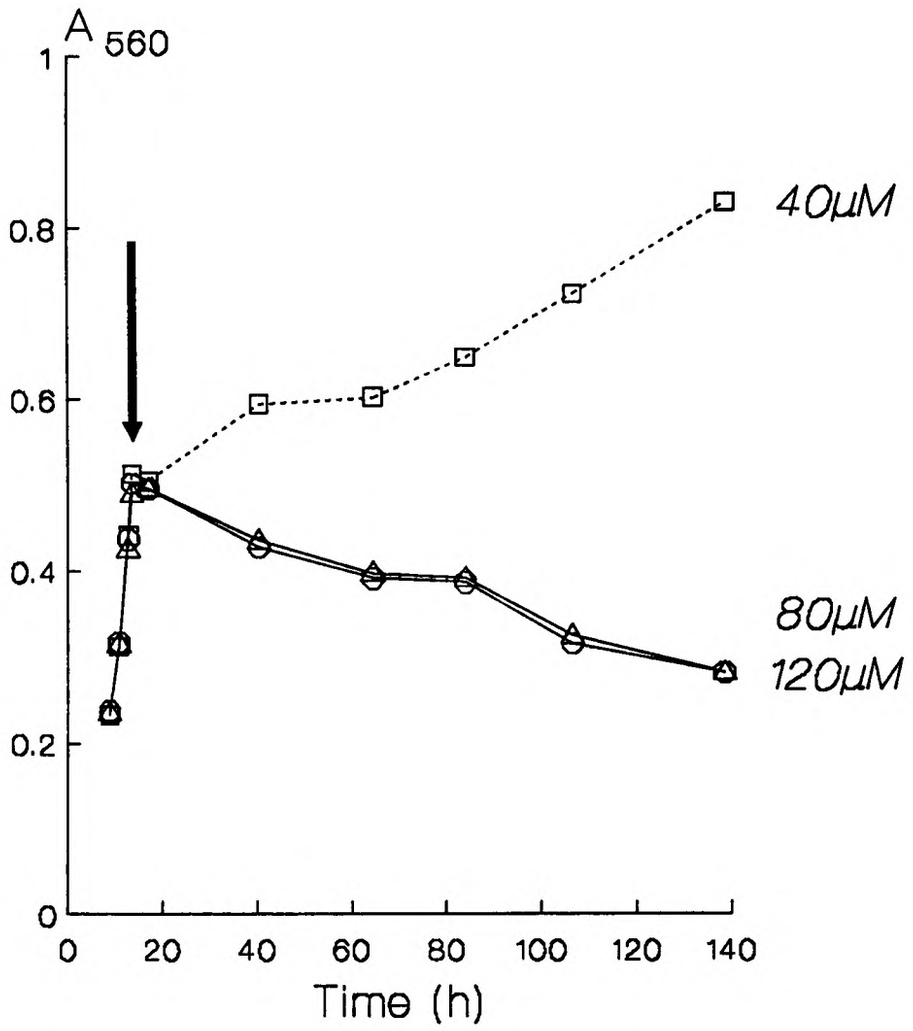


Figure 4.4 Effect of *trans*-isohumulone on absorbance at λ=560nm (A_{560}) of *Lactobacillus brevis* IFO 3960. Organisms were grown and additions of *trans*-isohumulone made as described in Figure 4.3.

indicating that the integrity of the cell membrane was not impaired by *trans*-isohumulone.

Effect on intracellular ATP

Analysis of intracellular ATP pool levels showed that the metabolism of *Lact.brevis* IFO 3960 was affected by *trans*-isohumulone. Figure 4.5 shows that immediately upon addition of the compound at the MIC, the cellular ATP concentration was significantly affected. Initially, this took the form of a decrease in the ATP pool level but was soon followed by an increase of *ca* 3-fold. The increase took place over a period of almost 4h. After this time, the ATP concentration decreased until 65h when the cells began to grow at a very slow rate, resulting in a doubling of the ATP pool over the following 70h (Figure 4.6). Organisms treated with 2x and 3x MIC responded in a different way from those treated with the MIC of *trans*-isohumulone. In such cases, the ATP pool level was briefly increased then reduced. After 20h or so, the intracellular ATP concentration of treated organisms declined with increasing contact time (Figures 4.5, 4.6). As was the case for the time-dependent reduction in the A_{560} of organisms treated with 2x and 3x MIC *trans*-isohumulone (Figure 4.4), the effect of both concentrations of *trans*-isohumulone on the ATP pool of treated cells was similar (results not shown).

In this connection, it is helpful to define the term 'pool' as applied to ATP since its meaning in this context differs from that normally inferred with respect to the concept of the 'nutrient pool'. In the case of intracellular pools of compounds such as amino acids, sugars, vitamins, or metal ions, an equilibrium exists between the concentrations of the nutrient on either side of the plasma membrane. At any one time, a higher concentration of the nutrient may be maintained in the

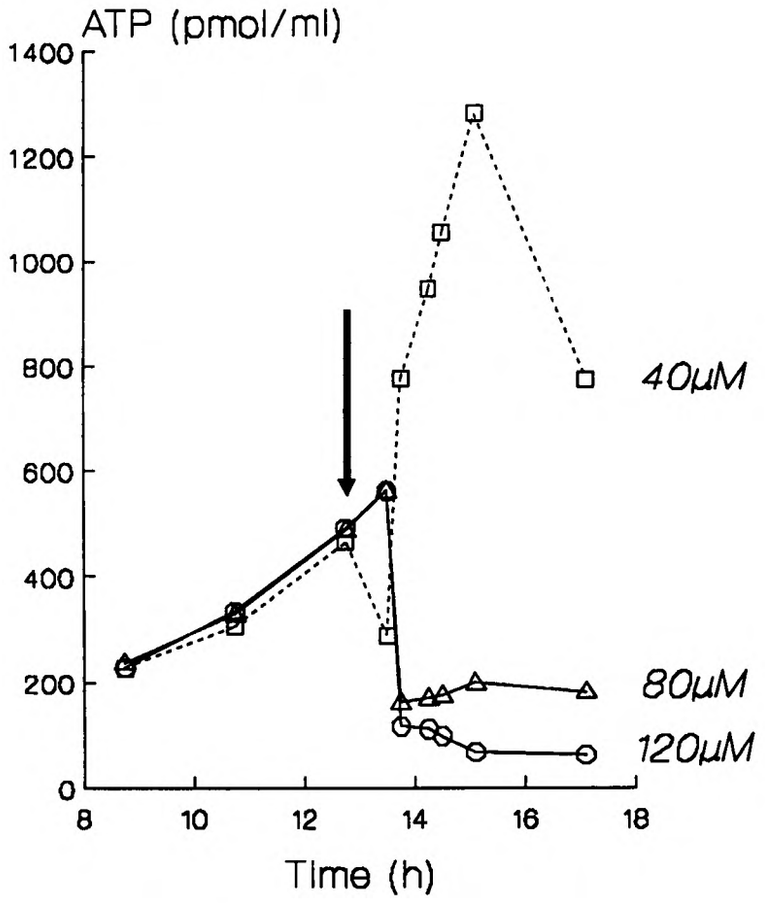


Figure 4.5 Effect of *trans*-isohumulone on intracellular ATP content of *Lactobacillus brevis* IFO 3960. Organisms were grown and *trans*-isohumulone added as described in Figure 4.3. Immediately before addition of *trans*-isohumulone to the suspension, the culture had an ATP content of 600pmol/ml. Initially, *trans*-isohumulone at the minimum inhibitory concentration (MIC) caused an increase in the intracellular ATP content. Higher concentrations (2x, 3x MIC) caused an immediate reduction in ATP content. ATP content was determined using the firefly luciferase reaction. Each point represents the mean of three determinations.

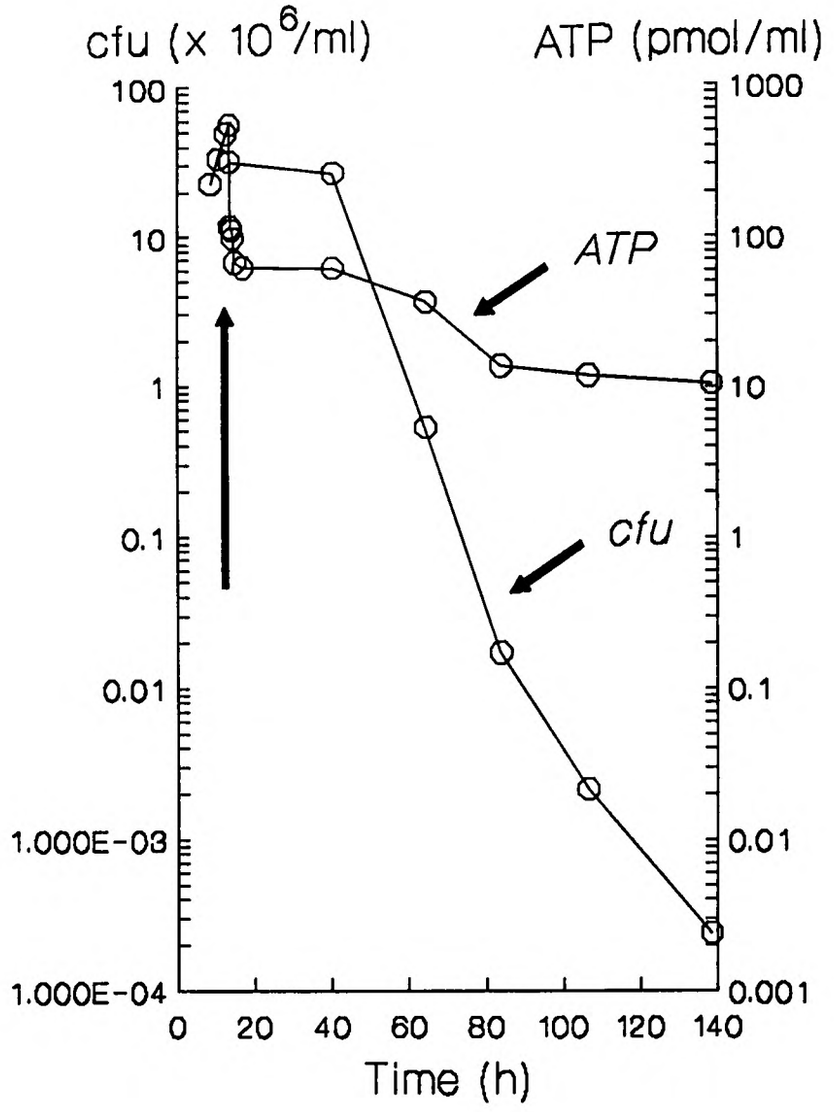


Figure 4.6 Discrepancy between death rates of organisms treated with *trans*-isohumulone assessed by plate count and by ATP analysis. The data shows that the death rate of organisms treated with 120μM (3x MIC) *trans*-isohumulone assessed by the two methods differs substantially suggesting the possible formation of 'viable but non-culturable' cells. Each point represents the mean of three determinations.

intracellular matrix than that in the extracellular medium as a result of the activities of membrane-bound transport proteins. In addition, the chemical stability of such accumulated nutrients in the intracellular environment is relatively high. Loss of coupling between energy generation and the ability to transport nutrients across the cell membrane usually results in leakage of the unmetabolised compound back into the growth medium. Material leaks from the cell via specific transport proteins, moving down its transmembrane concentration gradient. In the case of ATP no such equilibrium exists between molecules on either side of the cell membrane since bacteria have no means of transporting this compound. Thus, although a (potential) chemical gradient of ATP exists across bacterial membrane, it cannot be utilised to do work. Also, since ATP is a hydrophilic molecule which at physiological pH carries both positive and negative charges, the (intact) plasma membrane imposes an insurmountable barrier to its movement. Furthermore, the lifetime of an individual molecule of ATP in the intracellular matrix is very short. It has been estimated that every molecule of ATP in the cell 'pool' is synthesised and consumed every 0.3sec (Karl 1980).

The results obtained show that, (i) the physical integrity of the plasma membrane of *Lact.brevis* IFO 3960 was not manifestly disturbed by *trans*-isohumulone and, (ii) the energy-generating metabolism, or the means by which such energy is utilised, was disturbed, but not completely inhibited, by the compound.

Effect on viable counts

The greatest difference between cells exposed to *trans*-isohumulone for short and long periods was in their ability to grow on MRS agar after challenge with the

compound. Figure 4.6 shows that the initial phase of bacteriostasis changed to a period of bactericidal activity after *ca* 24h. Similar survivor curves were obtained regardless of whether the survivors were incubated in aerobic or anaerobic conditions (results not shown). The rate of death was first order with respect to time but, interestingly, independent of *trans*-isohumulone concentration. The same death rate was observed when the compound was applied at 2x and 3x MIC. This can be explained by assuming that death (or, specifically, loss of the ability to form colonies on MRS agar) was a secondary consequence of a loss of ΔpH. *Trans*-isohumulone, when applied at the MIC, or above, completely dissipated ΔpH in non-growing cells (section 4.3.5.). If it is assumed that, when applied at 2x and 3x MIC, *trans*-isohumulone elicited a similar dissipation of ΔpH in growing organisms, then death can be regarded as a secondary consequence of loss of the permeability barrier to protons. The 'lethal' response observed could result from exposure of intracellular sites (enzymes, ribosomes, RNA, DNA) to low pH values. Alternatively, loss of the ability to maintain ΔpH may result in an inability to accumulate essential ions such as K^+ (many inorganic ions are accumulated in response to $\Delta\psi$). Inhibition of protein synthesis could cause treated cells to lose viability as a result of an inability to replace essential proteins.

When the rates of death, as assessed by loss of ability to form colonies on MRS agar and reduction in the intracellular ATP pool level were compared, a notable discrepancy was evident (Table 4.2). As death proceeded, the intracellular ATP content apparently increased, resulting in ATP contents of 10amol - 43,000amol/organism. Several possible explanations of this behaviour exist. The apparent increases could have

Table 4.2: Intracellular ATP content of *Lactobacillus brevis* IFO 3960 treated with different concentrations of *trans*-isohumulone

Time (h) after addition of *trans*-isohumulone	**ATP content (amol/organism)**		
	40μM *trans*-isohumulone	**80μM *trans*-isohumulone**	**120μM *trans*-isohumulone**
0.01	10	22	17
26.9	12	7.5	2.3
50.9	5.8	91	140
70.4	5.7	770	790
92.9	9.0	2900	470
124.9	8.7	7500	43000

been caused by contamination of the test system with a microorganism which was unable to grow on MRS agar. However, the experiment was repeated on three more occasions, identical results being obtained in each case. The attention given to axenic technique and the microscopic observation of the cultures carried out also makes this possibility remote. A second possibility is that the ATP content of organisms stressed by *trans*-isohumulone was, indeed, considerably increased. However, this is preposterous since the highest ATP content measured (43fmol/organism) corresponds to an intracellular *concentration* of 28.7M (equivalent to over 14,000g/l). A third possibility is that formation of discrete ATP-containing structures of external diameter >2μm accompanies bactericidal activity of *trans*-isohumulone. Such structures could derive from autolytic processes and could consist of plasma membrane fragments, resembling liposomes, in which significant quantities of ATP have been trapped. There do not appear to be any reports of the formation of such structures in dying bacterial cell suspensions. In addition, although light microscopy and electron microscopy revealed the presence of amorphous material of effective diameter >2μm in cultures in which significant lysis had taken place, there was insufficient material to account for the excessive levels of ATP detected. The most likely explanation of this phenomenon is that prolonged exposure of *Lact.brevis* IFO 3960 to *trans*-isohumulone results in the formation of organisms which, although metabolically active, were incapable of growth on agar media. Formation of such cells occurs in the natural environment and they have been termed viable but non-culturable cells. They are found in potable water (McFeters *et al.* 1986), river and lake water (Roszak *et al.* 1984; Colwell *et al.* 1985) and some foodstuffs. In addition, exposure

of bacteria to certain antibiotics (*e.g.* nalidixic acid) induces this state.

It is plausible that prolonged exposure of sensitive intracellular sites (ribosomes or nucleic acid) to low pH (due to loss of ΔpH, see section 4.3.5.) renders the cell unable to reproduce on transfer to fresh medium, although they may remain metabolically active and contain significant levels of ATP. In the case of viable but non-culturable cells of *Salmonella enteritidis* the ability to grow on agar media can be restored by addition of nutrients to the cell suspension prior to plating (Roszak *et al.* 1984). Furthermore, viable but non-culturable *Vibrio cholerae* retain pathogenicity after apparent 'die-off' (Colwell *et al.* 1985).

This phenomenon has practical implications but requires more study before any conclusions can be drawn.

4.3.2. Effect of *trans*-isohumulone on non-growing cells of *Lact.brevis* IFO 3960

Cells of *Lact.brevis* IFO 3960 suspended in NaDMG buffer (0.1M, pH 5.2) were exposed to a range of concentrations of *trans*-isohumulone for 60min. After this time, the number of viable cells, as determined by plating of diluted suspensions of treated and untreated cells on MRS agar, was found to be unaffected (Figure 4.7) showing that, as found in the case of growing organisms, *trans*-isohumulone was not bactericidal to non-growing organisms over this time period. In addition, the morphology of isohumulone-treated and control organisms did not differ as assessed by microscopy and measurement of the absorbance of test suspensions (results not shown).

The intracellular concentration of ATP was, however, affected by *trans*-isohumulone (Figure 4.8). Control

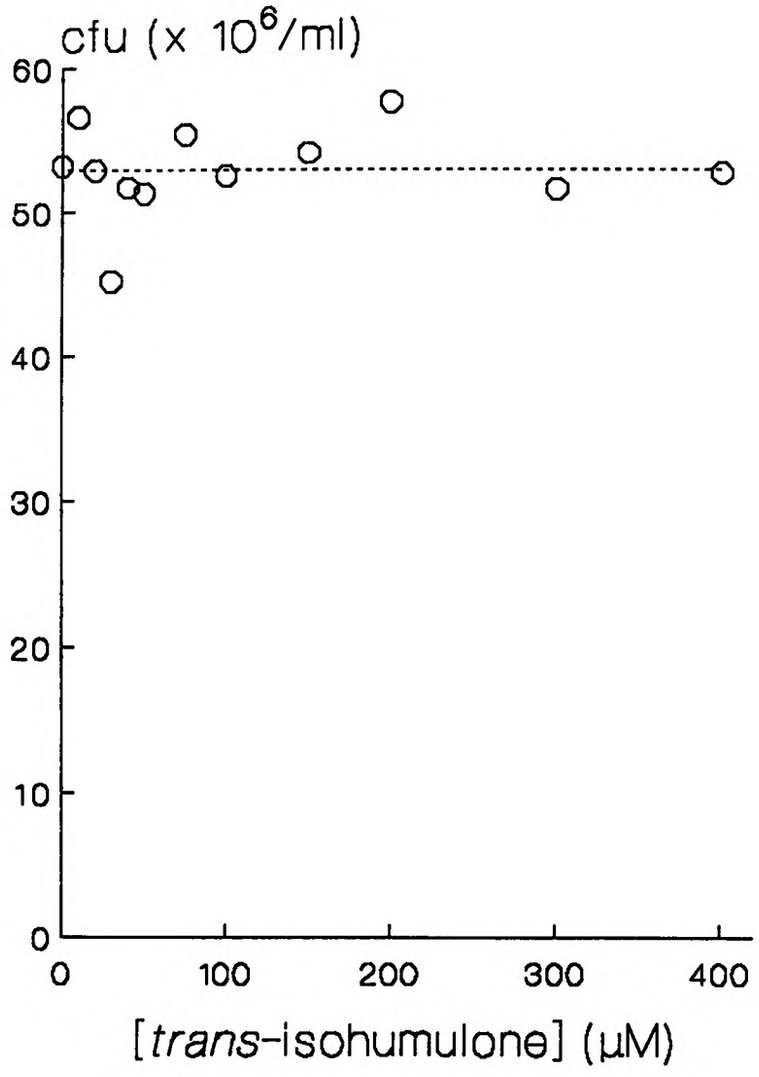


Figure 4.7 Effect of *trans*-isohumulone on viability of non-growing cells of *Lactobacillus brevis* IFO 3960. A standardised suspension of organisms was prepared in 0.1M NaDMG (pH 5.2) containing glucose (10g/l), divided into portions and exposed to different concentrations of *trans*-isohumulone for 60min at 25°C. The viable count was then determined by plating on MRS agar. Each point represents the mean of three determinations.

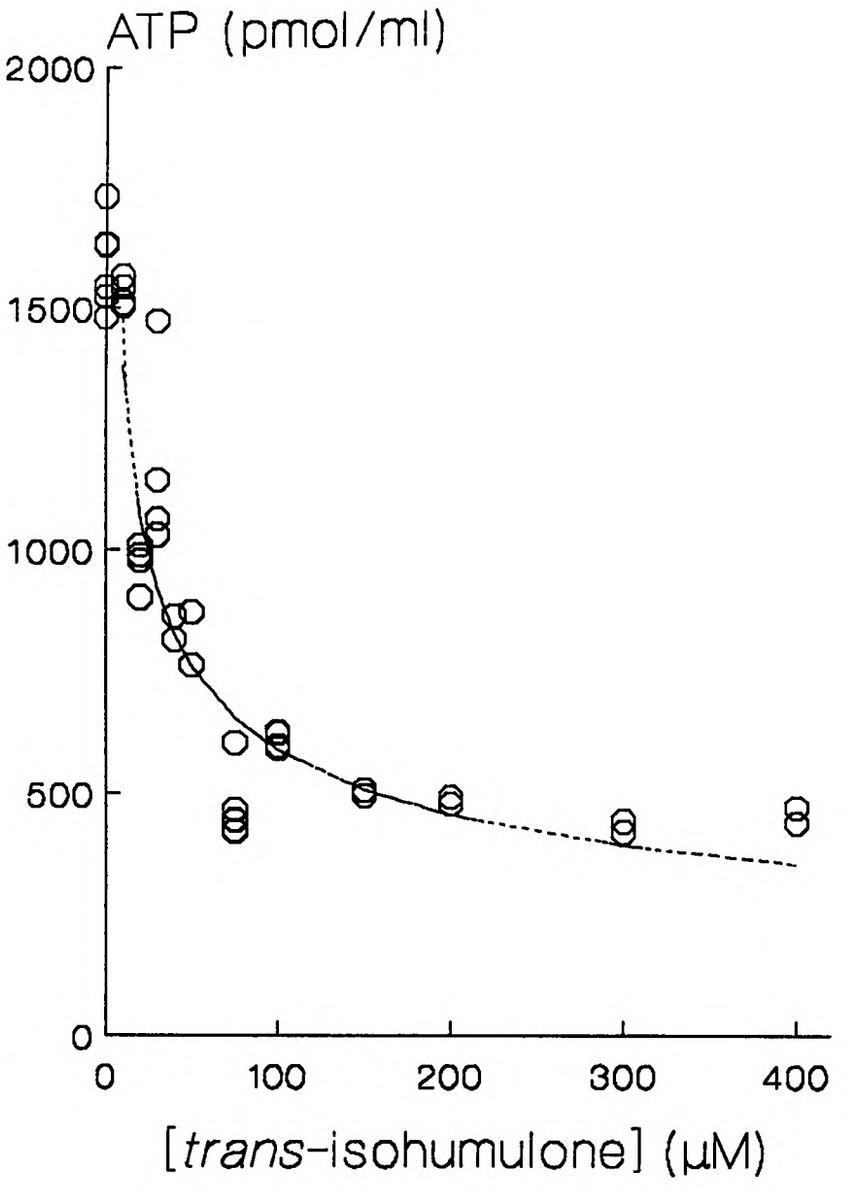


Figure 4.8 Effect of *trans*-isohumulone on the intracellular ATP content of non-growing cells of *Lactobacillus brevis* IFO 3960. A standardised suspension of organisms was prepared in 0.1M NaDMG (pH 5.2) containing glucose (10g/l), divided into portions and exposed to different concentrations of *trans*-isohumulone for 60min at 25°C. The intracellular ATP content was then assayed using the firefly luciferase reaction.

organisms, exposed only to buffer for the duration of the challenge period, had an ATP pool level of 1589pmol ATP/ml (mean of five replicates). Exposure to *trans*-isohumulone at concentrations of 0-100μM resulted in a concentration-dependent reduction in the cellular ATP pool to 610pmol ATP/ml culture (mean of four replicates) at 100μM *trans*-isohumulone while further increases in concentration from 100μM-400μM had little further effect. At a *trans*-isohumulone concentration of 40μM (the MIC in mod. MRS medium), the ATP pool was approximately halved.

Damage to plasma membranes often manifests itself in release of cytoplasmic constituents, the first indication of membrane damage usually being given by detection of K^+ in the extracellular medium (Lambert & Hammond 1973). Inorganic phosphates, pool amino acids and material which absorbs light at λ=260nm are often released (in that order) as the degree of membrane damage increases (Russell & Chopra 1990). In order to assess whether the integrity of the cell membrane was affected by *trans*-isohumulone, the suspending medium was assayed for materials which had leaked from the cells. Analyses were performed on samples from which the organisms had been removed by membrane filtration. Control experiments were performed to quantify the amount of material available for leakage. Both chemical (detergent or acid) and physical (sonication) extraction methods were employed to prepare extracts for these control experiments.

The effect of *trans*-isohumulone on the integrity of the cell membrane to K^+ could not be assessed under the experimental conditions employed. Filtrates from control cell suspensions contained 14mg K^+/l but the number of organisms in the test suspension was too low to permit increases in extracellular K^+ resulting from leakage to be discriminated.

A small amount of material absorbing light at 260nm was released from the cells on exposure to *trans*-isohumulone, amounting to a maximum of 25% of the 'pool' for cells exposed to 400μM isohumulone. Some 260nm material was released on exposure to NaDMG buffer alone, amounting to *ca* 6% of the available pool.

The firefly luciferase assay provides a more sensitive means of monitoring loss of the physical integrity of the plasma membrane than does measurement of material which absorbs light at λ=260nm since ATP concentrations as low as 80fM can be detected (Simpson *et al.* 1990). Typically, nucleotides and nucleosides (major components of the 260nm 'pool') have molar extinction coefficients of *ca* 10,000, making the limit of detection of such assays (assuming no dilution of the sample and the ability to reliably discriminate an absorbance of 0.05 in a cell of 1cm path length) almost 6 x 10^7 times poorer than that of the ATP assay. After allowing for dilution of the sample, as little as 8pM ATP could be detected using the reagents, equipment and protocols employed in the present work. Leakage of the ATP pool, as induced by specific extractants designed to efficiently release ATP from microbial cells, resulted in a typical concentration of ATP in the assay of 2.5μM. No ATP leaked from control or isohumulone-treated cells. Typically, the extracellular ATP concentration was 1-8nM.

These results, like those obtained with exponentially-growing organisms, indicate that the membrane structure of *Lact.brevis* IFO 3960 was not physically disrupted by *trans*-isohumulone.

4.3.3. Uptake of *trans*-isohumulone from solution by *Lact.brevis* IFO 3960

Studies of the uptake of tetrachlorosalicylanilide

(Hamilton 1967), fentichlor (Hugo & Bloomfield 1971), long-chain fatty acids (Galbraith & Miller 1973) and N-dodecyldiethanolamine (Lambert & Smith 1977) by bacteria have been reported and have provided information relating both to the amount of drug required for inhibition and to the way in which drug molecules are arranged at the bacterial cell surface.

The uptake of *trans*-isohumulone by standardised suspensions of *Lact.brevis* IFO 3960 from buffered solutions (0.1M NaDMG; pH 6.5) was essentially instantaneous and no further uptake occurred over the following 30min. Studies of the pattern of adsorption under the same conditions over a range of concentrations were severely limited by the small amount of substance taken up by the organisms. In all cases the amount of *trans*-isohumulone taken up was less than 10% (and in many cases much less than 10%) of that available. Since the amount adsorbed was calculated from the difference in its concentration in the buffer solution, before and after contact with the organisms, and since considerable dilution of the sample was necessary prior to assay, the potential for error was considerable. Consequently, large differences were observed between duplicate data points. At pH 4.5, no uptake of *trans*-isohumulone could be demonstrated. The low solubility of the test compound at this pH limited the concentration of *trans*-isohumulone which could be employed. Similar difficulties have been noted by those studying the adsorption of fatty acids by bacteria. In the case of fatty acids such problems were overcome by the use of radiolabelled (^{14}C) fatty acids which allowed the very small quantities of material bound to microbial cells to be quantified (Galbraith & Miller 1973). No radiolabelled hop compounds or hop-derived compounds (Wright & Howard 1961) were available at the time of this study, however, so further information

relating to the extent to which *trans*-isohumulone was taken up by *Lact.brevis* IFO 3960 was obtained using indirect methods.

For example, the finding that (-)-humulone does not antagonise antibacterial activity of *trans*-isohumulone (section 3.3.1.) lends support to the idea that ionic binding of isohumulone molecules to the bacterial cell surface does not occur. Since the inhibitory effect of *trans*-isohumulone against *Lact.brevis* IFO 3960 was not influenced by the number of organisms present over the range 8 x 10^3/ml - 2 x 10^7/ml (section 3.3.1.) it seems that the number of *trans*-isohumulone molecules which interact with sensitive cells is small, representing a small proportion of those available. In some experiments, in which the number of organisms in the assay was significantly higher (*ca* 8 x 10^8/ml) the availability of *trans*-isohumulone did become limiting (see section 4.3.5).

4.3.4. The effect of *trans*-isohumulone on the permeability of resting cell suspensions of *Lact.brevis* IFO 3960 to protons

The use of a titrimetric technique to study the movement of ions across the cell membranes of *Micrococcus lysodeikticus* and rat liver mitochondria was first described by Mitchell (1961b) and the technique has subsequently been employed to investigate drug-induced movement of protons across the cell membranes of a variety of organisms and organelles. Of direct relevance to the present investigation is the work of Harold & Baarda (1968b) in which protonophore-induced movements of protons across cell membranes of whole cells of *Streptococcus faecalis* (now named *Enterococcus faecalis*) was studied. Upon addition of HCl to a lightly-buffered

suspension of *E.faecalis*, the pH of the cell suspension fell abruptly, then slowly rose as the protons entered the organisms. Approach of the pH value to equilibrium was found to be accelerated by protonophores. Since, in the absence of glucose, the cells were essentially impermeable to both K^+ and Cl^- (ion exchange in this organism, as in most microorganisms, is energy dependent), entry of protons was limited by the development of a membrane potential ($\Delta\psi$). Harold & Baarda (1968b) showed that this restriction on proton movements could be relieved by addition of valinomycin to the cell suspension. Valinomycin, permeablises the plasma membrane of *E.faecalis* to potassium ions. By itself, this ionophore has only a slight effect on the permeability of resting cell suspensions to protons. However, the coincident application of valinomycin and CCCP caused a rapid entry of protons into the organisms as assessed by a rise in pH of the suspension.

Similar experiments to those of Harold & Baarda (1968b) were performed in the present investigation using *Lact.brevis* IFO 3960 as test organism and *trans*-isohumulone or colupulone as test compound. CCCP and valinomycin, both alone, and in combination, were also used to study the response of the organism to a representative protonophore and ionophore respectively. Addition of CCCP (15μM) to a suspension of *Lact.brevis* IFO 3960 in $KCl/MgCl_2$ solution increased the rate of proton influx into the bacteria (as shown by a rise in the pH of the extracellular fluid). Addition of valinomycin (0.45μM) to the suspension had little effect. As expected, the simultaneous presence of CCCP and valinomycin caused a rapid influx of protons into the cells since the electrical restriction on proton influx, imposed by $\Delta\psi$ in this experiment, was removed by

valinomycin (Table 4.3). The effect of *trans*-isohumulone on the pattern of proton influx into *Lact.brevis* IFO 3960 is shown in Figure 4.9. Unlike CCCP, *trans*-isohumulone had the ability to promote a rapid influx of protons into the cells in the absence of valinomycin. Addition of valinomycin to the cell suspension, either before or after addition of *trans*-isohumulone, had no effect on the proton influx rate. This suggests that transfer of protons across the plasma membrane of this organism was an *electroneutral* process.

Unlike simple protonophores, such as CCCP, in which the translocation of protons across the bacterial cell membrane, in response to a concentration gradient, results in a build-up of $\Delta\psi$, *trans*-isohumulone appears to discharge ΔpH while conserving $\Delta\psi$. This could take place in one of two ways. The transfer of a proton across the plasma membrane could be accompanied by the transfer of an ion of equal, but opposite, charge. For example, the simultaneous transfer of a proton and a chloride ion would result in dissipation of ΔpH without affecting $\Delta\psi$. Similarly, dissociation within the cell, of *trans*-isohumulone molecules into protons and *trans*-isohumulone ions could allow $\Delta\psi$ to be conserved. Many weak acid preservatives inhibit bacterial growth in this way. As described in section 4.2.13. this mechanism is taken for granted when weak acids, such as salicylic acid, are used to measure intracellular pH. Generally, however, for compounds such as benzoic, sorbic or propionic acid, an extracellular concentration of >10mM is required to inhibit growth of Gram-positive bacteria (Eklund 1980). Acids of high pKa value are generally less active, since they do not ionise inside the bacterial cell.

In the case of *trans*-isohumulone, which has pKa_{equil}=3.1, this mechanism of action is potentially

Table 4.3: Effect of *trans*-isohumulone on passive proton permeability of *Lactobacillus brevis* IFO 3960

Addition	Description of conditions	$T_{1/2}$ (min)	
		Suspending medium $KCl/MgCl_2$	Suspending medium H_2O
Methanol	Passive permeability of de-energised organisms to H^+	2.8	2.8
CCCP (15μM)	Protonophore-assisted permeability of organisms to H^+ (restricted by build-up of membrane potential)	2.5	-
VALIN (0.45μM)	Ionophore-assisted permeability of organisms to K^+	2.3	-
CCCP/VALIN (15μM/0.45μM)	Protonophore-assisted permeability of organisms to H^+ (membrane potential relieved by ionophore-assisted efflux of K^+)	0.3	-
trans-isohumulone (100μM)	Exposure of organisms to test antibiotic	0.2	2.8

$T_{1/2}$ = time required (min) required to dissipate one-half the difference between the starting and equilibrium pH values. High values of $T_{1/2}$ indicate that the cell membrane has low permeability to protons, low values that permeability has been increased.

CCCP = carbonyl cyanide *m*-chlorophenylhydrazone; VALIN = valinomycin

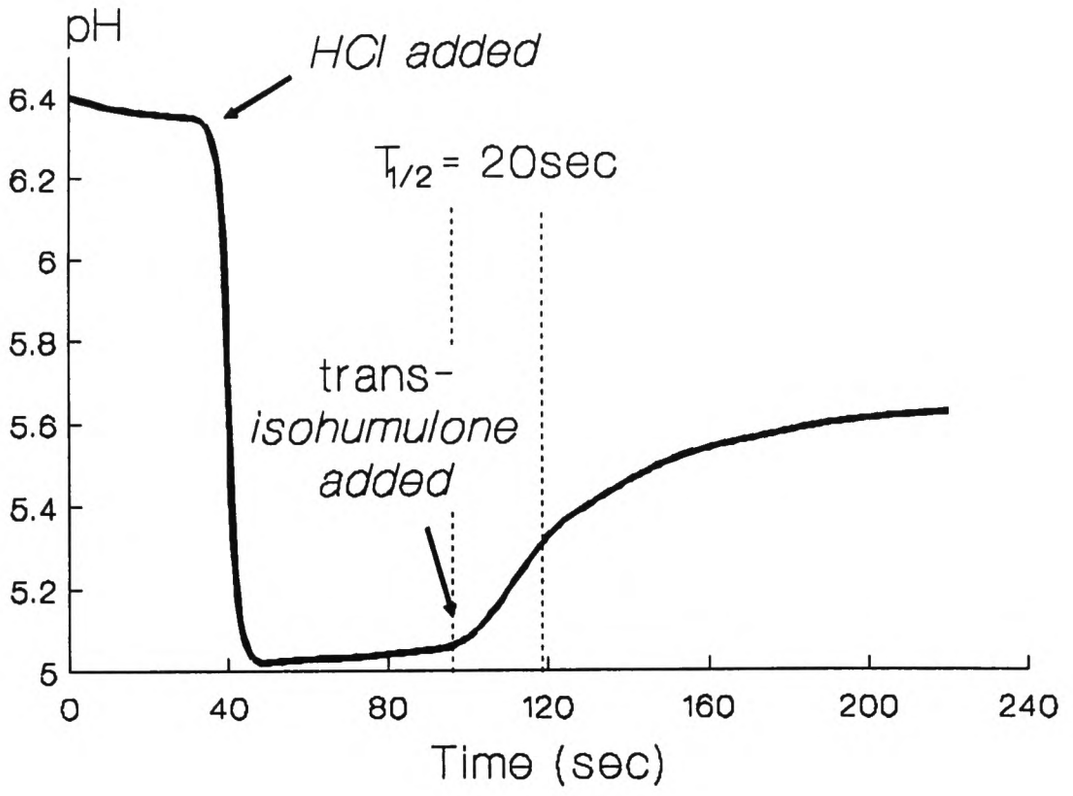


Figure 4.9 *Trans*-isohumulone induces electroneutral influx of H^+ into *Lactobacillus brevis* IFO 3960. Organisms (*ca* 1 x 10^9/ml) were suspended in KCl (150mM), $MgCl_2$ (2mM). Net H^+ movements were monitored by changes in extracellular pH. Addition of HCl to the suspension created an H^+ gradient across the cell membrane (inside alkaline). Addition of *trans*-isohumulone resulted in a time-dependent dissipation of the gradient. $T_{1/2}$ = time required (min) to dissipate one-half the difference between the starting and equilibrium pH. The fact the H^+ movements proceeded in the absence of valinomycin and were unchanged on addition of valinomycin indicated that the process was electroneutral.

feasible. Assuming an intracellular pH under the experimental conditions employed of *ca* 5.0, approximately 99% of the *trans*-isohumulone molecules crossing the cell membrane could dissociate into protons and *trans*-isohumulone ions. The argument is less valid for colupulone, which has pKa_{equil}=6.1 since only 10% of the molecules would dissociate. If $\Delta\psi$ was conserved in this manner then CCCP, which has a pKa value of 6.09 (McLaughlin & Dilger 1980) should have behaved similarly to colupulone. This was not the case. Also, hop compounds and hop-derived compounds display antibacterial activity at concentrations as low as 2μM in contrast to weak acid preservatives which are active at mM concentration.

An alternative mechanism by which electroneutral dissipation of ΔpH could be achieved is through the operation of an antiport mechanism. Thus, transfer of one or more protons across the plasma membrane could be coupled to transfer of one or more positively-charged counter-ions across the plasma membrane in the *opposite* direction. Several experiments were performed to test the validity of this theory. Since such ion-carriers (ionophores) function by equalising the gradients of protons and counterions across the plasma membrane it was reasoned that application of *trans*-isohumulone to the cell suspension in deionised water should lead to efflux of the counterion. The counterion could then have been identified using an appropriate analytical method. However, in deionised water *trans*-isohumulone was devoid of protonophoric activity. Activity was restored if monovalent cations such as K^+ or Na^+ was added but not if Mg^{2+} was added. Thus, for expression of protonophoric activity, the presence of a second monovalent cation, in addition to H^+ was essential for activity.

Of a range of cations tested (K^+, Na^+, NH_4^+, Mg^{2+},

Ca^{2+}, Mn^{2+}) only Mn^{2+} had the ability to reverse proton movements indicating that it could function as a counterion in the ionophoric action of *trans*-isohumulone (Figure 4.10).

Having established that *trans*-isohumulone acted as an ionophore which effected electroneutral exchange of H^+ for Mn^{2+}, it was necessary to differentiate between two possible types of activity. As described in section 1.2.2., it is possible to distinguish between true ionophores, which function as mobile carriers of ions, and *pseudo*-ionophores which form aqueous pores in the plasma membranes of sensitive organisms. *Pseudo*-ionophores are characterised by an ability to catalyse transport of ions when the temperature of the plasma membrane is below the phase-transition temperature of the membrane lipids. In contrast, ionophores of the mobile-carrier type are unable to catalyse ion movements when the membrane lipids are frozen in this way. *Trans*-isohumulone is a true ionophore, since protonophoric activity was arrested if the cell suspension was cooled to 0°C, regardless of whether the compound was added before or after cooling. Increasing the temperature of the suspension restored protonophoric activity (Table 4.4).

4.3.5. Effect of *trans*-isohumulone on the intracellular pH of *Lact.brevis* IFO 3960

The experiments described in the previous section demonstrated that hop compounds and hop-derived compounds act as ionophores and showed that, under physiological conditions, *trans*-isohumulone had the ability to dissipate ΔpH. Confirmation of this effect was obtained by measurement of the pH_{int} (a measure of the proton

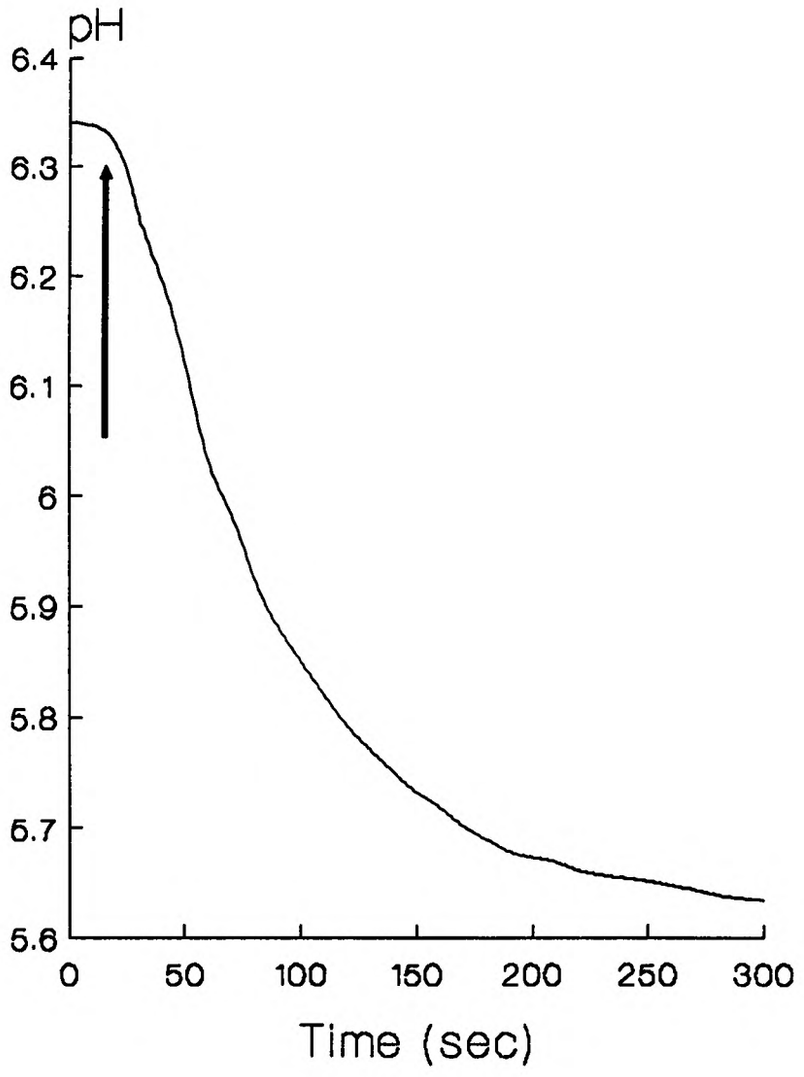


Figure 4.10 Manganese ions reverse the direction of *trans*-isohumulone-mediated H^+ flux in *Lactobacillus brevis* IFO 3960. Organisms (*ca* 1 x 10^9/ml) were suspended in $MnCl_2$ (200mM) and the pH of the suspension adjusted to 6.35 with NaOH. *Trans*-isohumulone (100μM) was then added (arrow). Net H^+ movements, induced by *trans*-isohumulone, were monitored by changes in extracellular pH. A time-dependent efflux of H^+ from the cells, as indicated by a decrease in the suspension pH, was observed. In the absence of *trans*-isohumulone the pH did not change significantly.

Table 4.4: Effect of temperature on ionophoric activity of *trans*-isohumulone

Temperature (°C)	$T_{1/2}$ (min)
0	>30
5	5.6
10	2.0
20	0.3

Lactobacillus brevis IFO 3960 was suspended in KCl/$MgCl_2$ solution and *trans*-isohumulone-induced proton movements examined using the titrimetric method as described in section 4.2.11. $T_{1/2}$ = time required (min) to dissipate one-half the difference between the starting and equilibrium pH values. High values of $T_{1/2}$ indicate that the cell membrane has low permeability to protons, low values that permeability has been increased. Similar results were obtained regardless of whether *trans*-isohumulone was added to the suspension prior to cooling or after cooling.

activity) of control and isohumulone-treated cells. Attempts were made to measure pH_{int} of *Lact.brevis* IFO 3960 in three ways, although two of the methods could not be successfully applied to study of this organism.

Fluorescein diacetate method

Rotman & Papermaster (1966) showed that fluorescein esters of fatty acids provided a viability stain for certain cell types which was dependent on the presence of a functional cell membrane. The method has been widely employed in analysis of both plant (Widholm 1972) and microbial (Paton & Jones 1975) cells and has found a practical application in the brewing industry in the form of a rapid detection method for contaminant micro-organisms (Molzahn & Portno 1975). The technique also forms the basis of a simple method for measurement of pH_{int}. Indeed, it has been used, in conjunction with quantitative fluorescence photography, to demonstrate the presence of discrete domains of differing pH in single cells of the yeasts *Saccharomyces cerevisiae* and *Endomyces magnusii* (Slavik & Kotyk 1984).

Lact.brevis IFO 3960, and several other lactic acid bacteria tested, failed to fluoresce when stained with this substance. It seems that although such organisms had the ability to hydrolyse fluorescein diacetate (since the supernatants fluoresced), the smaller ΔpH which they generate in comparison with organisms such as *Saccharomyces cerevisiae* or *Bacillus subtilis* was not sufficient to allow substantial accumulation of fluorescein ions. This method was therefore abandoned.

^{31}P NMR method

Moon and Richards (1973) showed that the intracellular pH of living cells could be measured from the position of ^{31}P NMR lines arising from intracellular materials and the

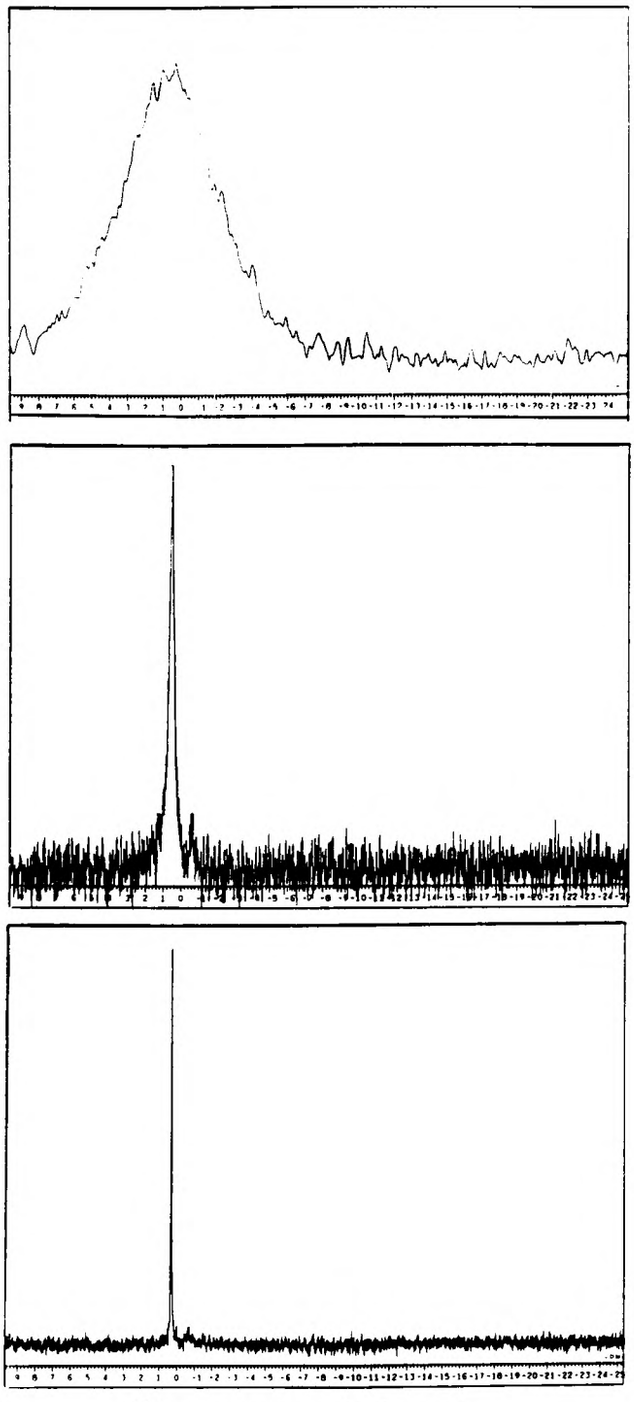
technique has since been widely employed to determine pH_{int} of a variety of cell types (Barton *et al.* 1980; Gillies *et al.* 1981; Slonczewski *et al.* 1981; Belton & Ratcliffe 1985; Axe & Bailey 1987). The basis of the method is that chemical shift values of ionisable phosphate groups of intracellular materials (*e.g.* inorganic phosphate, adenosine 5'-triphosphate, glucose-6-phosphate) are dependent on the pH of the medium in which they are dissolved provided that the pH is within one unit of the pKa of the titratable group. At pH 7, inorganic phosphate exists mainly in monovalent ($H_2PO_4^-$) and divalent (HPO_4^{2-}) forms. The chemical shift values for each of these ^{31}P resonances are 0.58ppm and 3.14ppm respectively. However, in practice, only one resonance line is observed; a weighted average of the chemical shifts of the monovalent and divalent forms. The observed chemical shift value of inorganic phosphate is determined by the relative amount of the two species, and is, therefore, pH dependent. Calibration curves can be constructed by plotting chemical shift against pH. The titration curve must be generated in a medium that resembles the intracellular environment, since the chemical shift of ionisable groups is affected by the ionic strength of the solvent (Campbell-Burk & Shulman 1987).

Intracellular phosphorylated metabolites can be observed by ^{31}P NMR provided they are mobile (*i.e.* not bound to other molecules) and sufficiently concentrated (usually mM concentration) to give narrow spectral lines (Campbell-Burk & Shulman 1987). To be of use, the phosphate moieties must possess pKa values which lie within the range of intracellular pH values under investigation.

Biochemical analysis of cell extracts and literature evidence revealed that several compounds were present in

Lact.brevis IFO 3960 which were capable of giving well-defined spectral lines within a short (<1min) accumulation time. For example, the intracellular concentration of ATP (*ca* 14mM under the conditions employed) should have been sufficiently large to allow well resolved signals to be obtained. Dense suspensions of *Lact.brevis* IFO 3960 (*ca* 10^{10}-10^{11} organisms/ml) were examined but no signals were obtained even after accumulation of several thousand scans. In an attempt to explain this inability to obtain well-resolved signals, several experiments were carried out. Examination of ^{31}P NMR spectra of mod. MRS medium (pH 5.2) revealed that an inordinately large number of scans (2000-4000) had to be accumulated before the major ^{31}P NMR signal from this medium (that of inorganic phosphate) could be resolved (Figure 4.11). Furthermore, the line width (the spectral width, measured in Hz, at half peak height) of the observed peak (530Hz) greatly exceeded that expected from ^{31}P NMR spectra recorded under such conditions. The line width of the phosphoric acid solution used as the secondary chemical shift reference in the experiment was only 8Hz. Addition of further quantities of inorganic phosphate (as H_3PO_4) to mod. MRS failed to give an improved signal response. It was suspected that poor spectral line widths, and hence the very low signal intensities obtained, were due to the presence of paramagnetic ions in the growth medium and not due to lack of phosphate. This was confirmed by recording ^{31}P NMR spectra of batches of mod. MRS which had been supplemented with the chelating agent ethylenediamine tetra-acetic acid (EDTA). The line width of the inorganic phosphate peak in mod. MRS was substantially improved in the presence of EDTA (reduced from 530Hz to 40Hz and 25Hz in the presence of 15mM and 60mM EDTA respectively).

Figure 4.11 ^{31}P NMR spectra of inorganic phosphate in mod. MRS (top), mod. MRS containing EDTA (middle) and mod. MRS without added manganese chloride (bottom). Spectra were recorded at 109.3MHz using an acquisition time of 0.535sec, a pulse delay of 0.3sec and a broadening factor of 1Hz at 25°C. The number of data points was 3192. The phosphate signal from mod. MRS was very broad (line width *ca* 530Hz). Addition of EDTA to the medium improved the line width (*ca* 40Hz) as a result of its metal-chelating properties. If manganese was omitted from the medium, a sharp peak (line width *ca* 9Hz) was observed. The number of scans in each case was 3965, 158 and 214 respectively.



At this stage, it was suspected that manganese ions (which are strongly paramagnetic) were responsible for the effect. To test this hypothesis, ^{31}P NMR signals from a batch of mod. MRS, from which this metal had been omitted, were recorded. A sharp signal (9Hz line width) was obtained thus indicating that manganese ions were responsible for the line broadening observed in other spectra. The line shapes and spectral widths obtained in these experiments are summarised in Figure 4.11.

In an attempt to overcome the problems associated with manganese ions, *Lact.brevis* IFO 3960 was subcultured three times in mod. MRS from which the manganese had been omitted. An unacceptable broadening of the ^{31}P NMR resonance lines was still observed (line width *ca* 400Hz) when such organisms were examined (Figure 4.12). It is likely that *Lact.brevis* IFO 3960 efficiently scavenged manganese from the growth medium thus concentrating this ion within the cellular matrix.

Similar problems concerning the effect of manganese ions on ^{31}P NMR studies of *Rhodopseudomonas sphaeroides* (Nicholay *et al.* 1987), *Staphylococcus aureus* (Ezra *et al.* 1983) and *Lactococcus lactis* (Thompson & Torchia 1984) have previously been reported. Indeed the catastrophic effect of manganese ions on the signal intensity of ^{31}P NMR lines has been exploited to discriminate signals from different cellular compartments of eukaryotic cells and in the study of internal and external surfaces of vesicles. When employed in this way, manganese is used as a 'broadening probe'; its function being to reduce the ^{31}P NMR signal from the manganese-containing compartment to zero while having no effect on the signals from the manganese-deficient compartment (Belton & Ratcliffe 1985).

It is possible that, by persistent cultivation of *Lact.brevis* IFO 3960 in manganese deficient growth media,

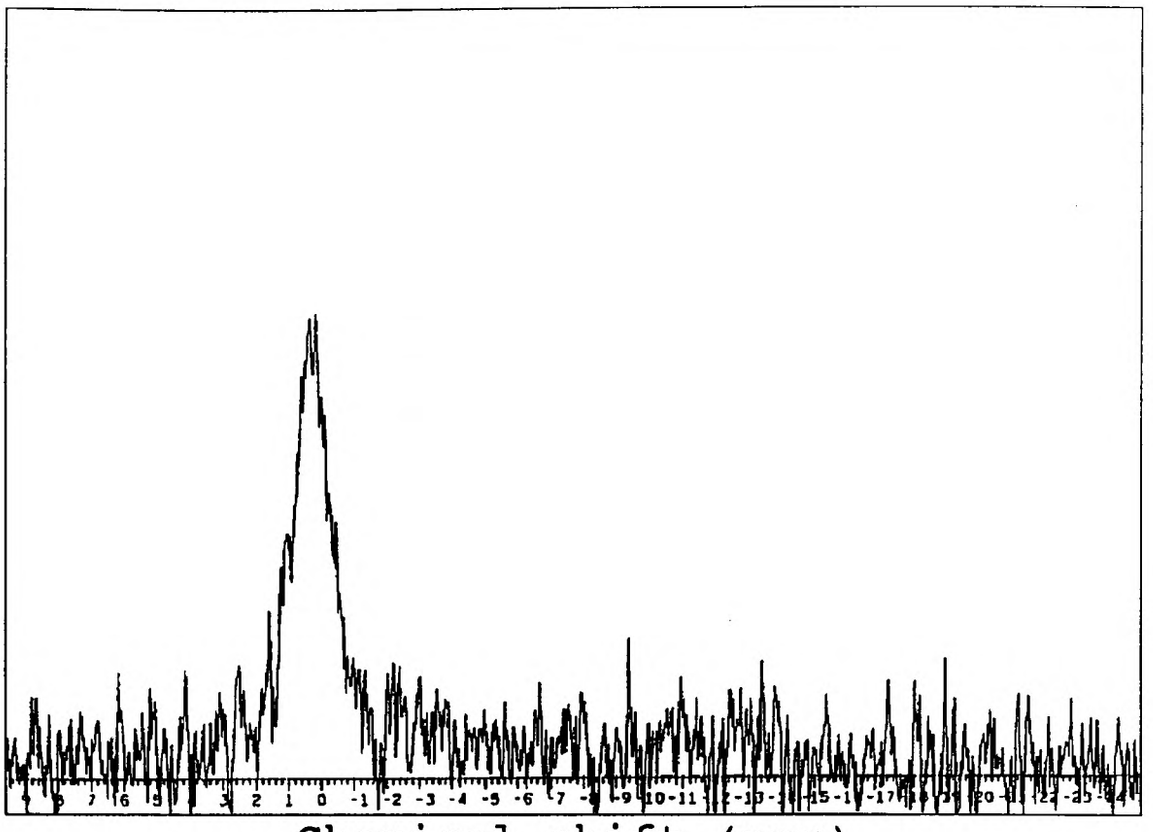


Figure 4.12 ^{31}P NMR spectrum of live *Lactobacillus brevis* IFO 3960 suspended in 0.1M NaDMG buffer (pH 5.2). Organisms were subcultured three times in manganese-deficient mod. MRS, harvested by centrifugation then suspendend in buffer at a concentration of 10^{11} organisms/ml. The spectrum was recorded at 109.3MHz using an aquisition time of 0.535sec, a pulse delay of 0.3sec and a broadening factor of 1Hz at 25°C. The number of data points was 8192. Proton decoupling was not applied. The spectrum shown represents the Fourier transform of 4454 scans. The broad signal obtained (line width *ca* 130Hz) was due to the presence of intracellular manganese ions.

the intracellular manganese content could have been reduced and a corresponding improvement in the line width of the ^{31}P NMR signals achieved. However, this radical step was not warranted, since it would have been difficult to relate any results obtained, regarding the action of *trans*-isohumulone, to the bulk of the experimental work carried out. In addition, the fact that Mn^{2+} had been shown to play a role in the antibacterial action of *trans*-isohumulone made this option particularly unattractive.

In an attempt to use the technique to identify intracellular phosphorylated metabolites, extracts of *Lact.brevis* IFO 3960 were prepared using trichloroacetic acid. Once again, however, paramagnetic ions prevented adequate resolution of the resonance lines being achieved (results not shown). To ensure that the NMR experimental conditions were suitable for detection of such resonances, a solution of ATP (0.1M) was mixed with an equal volume of TCA solution (5% w/v) then examined by ^{31}P NMR spectroscopy using conditions identical to those employed in previous experiments. Figure 4.13 shows the spectrum obtained. The three phosphate peaks, split by their attached protons are clearly visible. Furthermore, similar spectra could be obtained using an accumulation time of <1.5min (representing <99 accumulations). Clearly, the equipment and experimental conditions employed were capable of allowing observation of intracellular metabolites and measurement of intracellular pH, but the test organism, grown under the conditions described, was not suitable.

Radiolabel method (measurement of ΔpH, $\Delta\psi$ and Δp)

As described in section 4.2.13., the average intracellular pH (and hence ΔpH) of a suspension of microorganisms can be calculated from the equilibrium

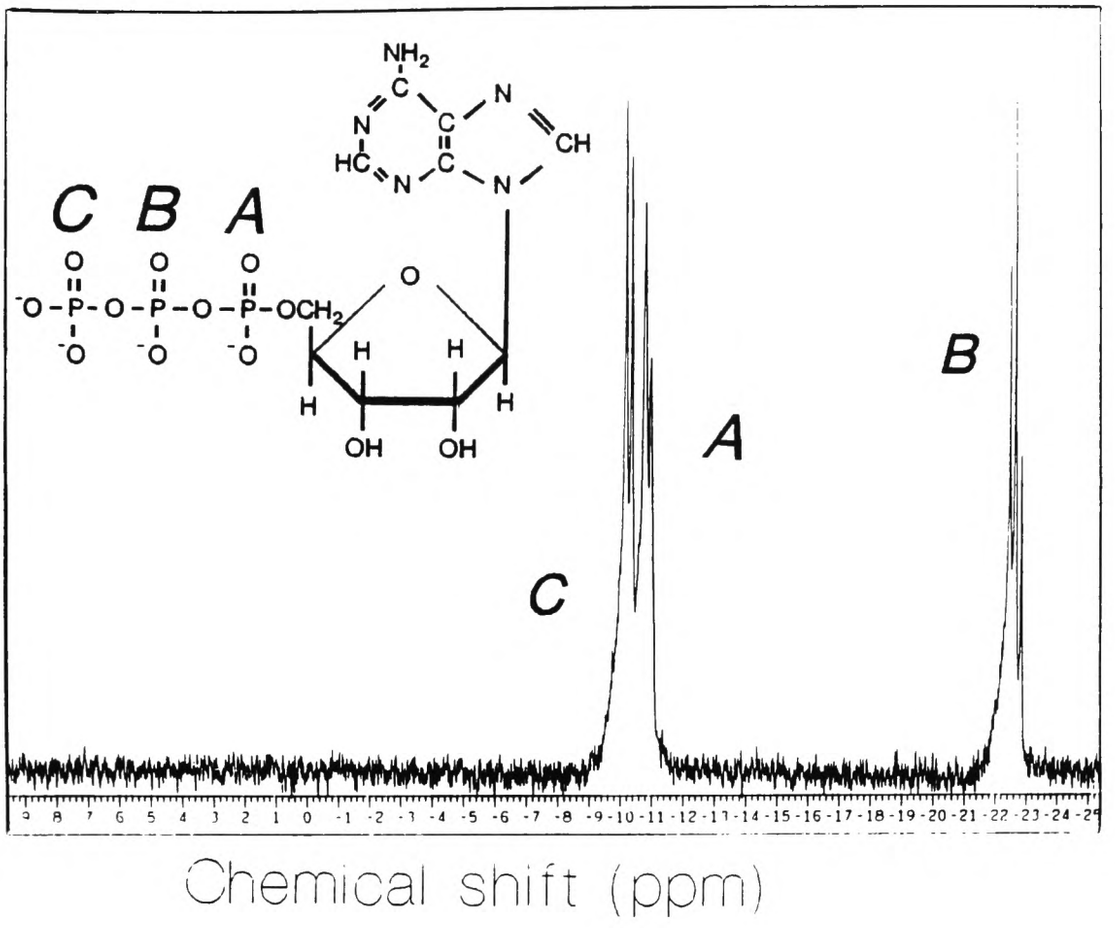


Figure 4.13 ^{31}P NMR spectrum of adenosine 5'-triphosphate (ATP) recorded at 109.3MHz using an acquisition time of 0.535sec, pulse delay of 0.3sec and broadening factor of 1Hz. The number of data points was 8192. Proton decoupling was not applied. The spectrum represents the Fourier transformation of 885 scans. Chemical shift values are given relative to 88% H_3PO_4 (=0ppm). The sample contained ATP (50mM) and trichloroacetic acid (25g/l). Seven peaks originate from the three phosphate groups of ATP (as indicated by *A,B,C*). The signal from the *A* and *C* phosphate groups are each split by their attached hydrogens into a doublet while that from the *B* phosphate group is split into a triplet.

transmembrane distribution of a weak acid and the extracellular pH. The membrane potential ($\Delta\psi$) can be calculated from the transmembrane distribution of a lipophilic cation. The proton-motive force (Δp) can be derived from these two values. The effect of *trans*-isohumulone on pH_{int} and $\Delta\psi$ was studied in NaDMG buffer containing glucose at an external pH of 5.2. The experimental conditions were identical to those employed for study of the effect of this compound on metabolic processes and membrane integrity (section 4.2.9.) with the exception that the number of organisms in the assay was 10x greater. At this cell concentration the MIC was *ca* 10x greater (determined from inhibition of [^{14}C]-L-leucine uptake and reduction in intracellular ATP content at both cell concentrations) although the cellular concentration (Hamilton 1968) of *trans*-isohumulone attained under both conditions was similar.

Cells were incubated in the presence or absence of *trans*-isohumulone (400μM, MIC under these conditions). Control organisms incubated 60min in 0.1M NaDMG buffer, pH 5.2 containing glucose (10g/l) had $\Delta pH=0.52\pm0.002$, those treated with *trans*-isohumulone at MIC had $\Delta pH=0.09\pm0.09$ (mean$\pm$s.e.m.,n=3). Measurements of $\Delta\psi$ confirmed that the ionophoric action of *trans*-isohumulone was electroneutral since, under conditions in which ΔpH was dissipated, approximately 40% of the membrane potential persisted ($\Delta\psi=-71.5\pm3.1$ and -42.7 ± 9.9mV [n=3] for control organisms and organisms treated with MIC respectively). If the process had been electrogenic, $\Delta\psi$ would have been increased. Since $\Delta\psi$ was largely conserved, Δp was not completely dissipated. Values for Δp of -102.3 ± 3.2 and -48.2 ± 7.2mV [n=3] were obtained for control cells and cells treated with 400μM *trans*-isohumulone respectively (cells treated with a

combination of CCCP [30μM] and valinomycin [0.45μM] had $\Delta p = 0.89 \pm 9.3$mV [n=3]).

4.3.6. Effect of *trans*-isohumulone on the proton-translocating membrane-ATPase of *Lact.brevis* IFO 3960

The activity of the membrane-bound proton-translocating ATPase of *Lact.brevis* IFO 3960 was measured (at pH 4.5) in the absence of *trans*-isohumulone and in the presence of 60μM *trans*-isohumulone (approximately 10x MIC at this pH). Activity, assessed by measuring the rate of release of inorganic phosphate from ATP at 30°C, was not affected by *trans*-isohumulone, a value of 266nmol Pi released/mg protein/min being obtained both in the presence and absence of the compound. This value is comparable to those presented by other workers (Bender *et al.* 1986; Sutton & Marguis 1987).

It is interesting to note that Teuber and Schmalreck (1973) reported that the activity of the magnesium-dependent ATPase of *Bacillus subtilis* was not affected by the presence of significant quantities of humulone or isohumulone. While the enzymes of *B.subtilis* and *Lact.brevis* have a similar activity (with respect to their mechanism and structure) they differ markedly with respect to their primary cellular function. In *B.subtilis*, which generates ATP primarily by oxidative phosphorylation, the purpose of the membrane-bound ATPase is to *synthesise* ATP in response to an electrochemical gradient of protons. In *Lact.brevis*, however, the function of this enzyme is to *generate* an electrochemical gradient of protons at the expense of cellular ATP. The enzyme thus plays a role in both energy transduction and maintenance of pH_{int} in this organism.

4.3.7. Effect of *trans*-isohumulone on the ability of *Lact.brevis* IFO 3960 to accumulate L-leucine

Cells of *Lact.brevis* IFO 3960 suspended in NaDMG buffer (0.1M, pH 5.2) at 25°C accumulated [^{14}C]-L-leucine when supplied with a source of energy (glucose 10g/l). After approximately 20min the compound was accumulated 10-fold with respect to the extracellular concentration. Uptake of [^{14}C]-L-leucine was partially inhibited by CCCP and completely inhibited by the combined application of CCCP and valinomycin (Table 4.5). As is the case for uptake of L-leucine by lactic streptococci (Poolman *et al.* 1987) transport of L-leucine was driven by the proton electrochemical gradient (Δp), with a contribution being made by both ΔpH and $\Delta\psi$. *Trans*-isohumulone inhibited uptake of [^{14}C]-L-leucine (Figure 4.14). Low concentrations caused slight inhibition, while higher concentrations caused more pronounced inhibition. At the MIC, uptake was retarded by about 60%. This behaviour is consistent with the contention that *trans*-isohumulone abolishes ΔpH but has less effect on $\Delta\psi$. The uptake of [^{14}C]-L-leucine which proceeded in the presence of *trans*-isohumulone at the MIC can be attributed to $\Delta\psi$-driven transport.

When coupling between energy generation (ATP synthesis) and its utilisation for transport (via the electrochemical gradient of protons generated by the membrane proton-translocating ATPase) is broken, the ability to maintain a concentration gradient of L-leucine is lost. Efflux of L-leucine on treatment with protonophore and protonophore/ionophore combinations was, therefore, to be expected. Figure 4.15 shows that addition of CCCP or a combination of CCCP and valinomycin to cells which had been preloaded with [^{14}C]-L-leucine induced a time-dependent efflux of the radiolabelled

Table 4.5: Effect of ionophores on uptake of [^{14}C]-L-leucine by *Lactobacillus brevis* IFO 3960.

Addition	Concentration (μM)	Accumulation ratio ([^{14}C]-L-leucine$_{inside}$/ [^{14}C]-L-leucine$_{outside}$)
none	-	11.0, 11.0
CCCP	30	5.5, 5.5
CCCP	60	4.9, 4.9
CCCP	150	4.3, 2.6
CCCP	300	3.9, 3.8
CCCP/valinomycin	30/0.45	1.1

Organisms were suspended in 0.1M NaDMG (pH 5.2) containing glucose (10g/l) to a final concentration of 8 x 10^8/ml at 25°C then ionophores added (in MeOH) as indicated. After 5min [^{14}C]-L-leucine (1.85 x 10^4Bq; 1.62μM) was added and the distribution of radioactivity determined after 20min incubation at 25°C by liquid scintillation counting after separation of cells and buffer by silicone oil centrifugation. Chloramphenicol (50mg/l) prevented incorporation of [^{14}C]-L-leucine into protein.

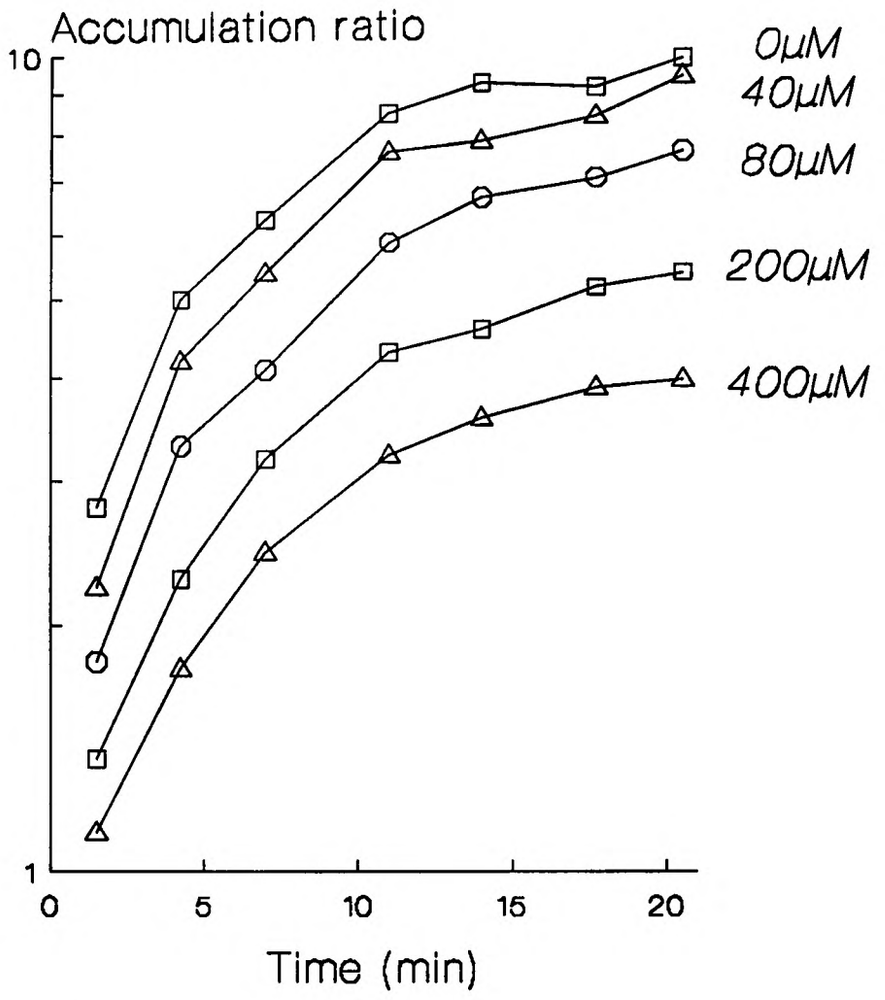


Figure 4.14 *Trans*-isohumulone inhibits uptake of [^{14}C]-L-leucine by *Lactobacillus brevis* IFO 3960. Organisms were suspended in 0.1M NaDMG (pH 5.2) containing glucose (10g/l) to a final concentration of 8 x 10^8/ml at 25°C then *trans*-isohumulone added (equivalent to 0, 0.1, 0.2, 0.5, 1 x MIC at this cell density). After 5min [^{14}C]-L-leucine (1.85 x 10^4 Bq, 1.62μM) was added and the distribution of radioactivity determined at various times by liquid scintillation counting after separation of organisms and buffer by silicone oil centrifugation. Chloramphenicol (50mg/l) prevented incorporation of [^{14}C]-L-leucine into protein. Each point represents the mean of four determinations.

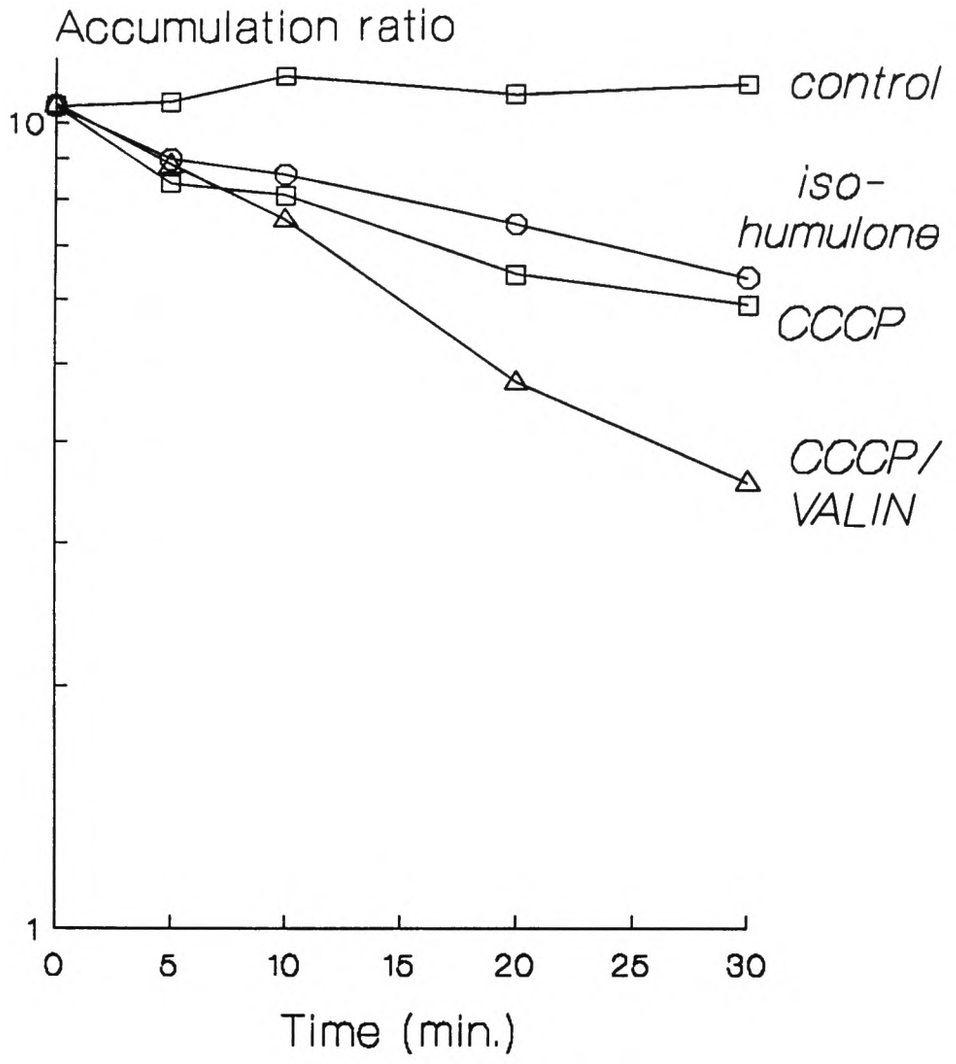


Figure 4.15 *Trans*-isohumulone promotes efflux of [^{14}C]-L-leucine from preloaded cells of *Lactobacillus brevis* IFO 3960. Organisms were loaded with [^{14}C]-L-leucine as described in Figure 4.17 for 20 min. *Trans*-isohumulone (400μM), carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP, 300μM) or CCCP (30μM) and valinomycin (0.45μM) was then added to the suspension (T=0). Control organisms received solvent only. Distribution of radioactivity was determined at various times by liquid scintillation counting after separation of cells and buffer by silicone oil centrifugation.

material. In the absence of CCCP or valinomycin, the intracellular content of [^{14}C]-L-leucine remained constant. *Trans*-isohumulone induced a similar efflux of [^{14}C]-L-leucine lending further support to the view that this compound dissipates ΔpH.

4.4. DISCUSSION

As described in section 1.2.1. antibacterial agents exert their effects on sensitive organisms by one, or more, of a number of mechanisms. The purpose of the experiments described in this Chapter was to identify the site(s) of action of hop compounds and hop-derived compounds in sensitive lactic acid bacteria and to elucidate the mechanism by which the sensitive site is affected by such compounds.

4.4.1. An undissociated form of each of the hop compounds and hop-derived compounds is the antibacterial species

All of the compounds studied (colupulone, (-)-humulone, *trans*-isohumulone, *trans*-humulinic acid) are weak acids, possessing pKa values ranging from 2.7-6.1. In section 3.3.1. it was shown that the MIC of each of the compounds for *Lact.brevis* IFO 3960 varied with the pH of the medium in which they were applied. Calculation of the proportion of each of the materials present in the ionised and undissociated forms at each pH showed that undissociated molecules were responsible for inhibition of growth. Having established a common functional relationship between all of the different compounds tested, the bulk of the remaining experiments were carried out with *trans*-isohumulone. This compound was chosen for further extensive study because, (i) it is the

one of the major bittering components of beer, (ii) it can be obtained in a pure form (*i.e.* a specific stereoisomer free of contaminant stereoisomers and related congeners) via a specific photochemical reaction, (iii) it is the most effective of the antibacterial hop compounds and hop-derived compounds yet studied (when the effectiveness of the undissociated forms is considered).

When applied at a lower concentration than the MIC, *trans*-isohumulone reduced the growth rate of the test organism. At, or above, the MIC it inhibited growth but did not kill the organisms during the first 24h contact in mod. MRS (pH 5.2) at 25°C.

4.4.2. Cell or cell membrane structure is not grossly impaired: energy generation is not disrupted

Growth of *Lact.brevis* IFO 3960 was immediately arrested on contact with isohumulone. Initially the action was bacteriostatic but later a bactericidal effect was noted, probably due to a secondary effect of the antibacterial action. Inhibition of growth did not result from inhibition of energy generation since the cellular concentration of ATP was not markedly reduced in *trans*-isohumulone-treated cells. Indeed, in the early stages of application of the compound at the MIC, a stimulation (*ca* 3-fold) in the cellular ATP pool was observed. In addition, studies employing a genetically manipulated light-emitting strain of *Lactococcus lactis* subsp. *diacetylactis* F712 which had been transformed with the *lux*A/B gene, indicated that the availability of $FMNH_2$ (which, in turn, is dependent on the availability of NADH and/or NADPH) was not seriously affected by the presence of any of the hop compounds or hop-derived compounds (Simpson & Hammond 1991).

The physical integrity of the cell membrane of growing and non-growing organisms was not grossly impaired by *trans*-isohumulone since, (i) no cellular ATP, and little material absorbing light at λ=260nm, leaked from the the cells at any of the *trans*-isohumulone concentrations tested; and (ii) intracellular pools of [^{14}C]-L-leucine were released only slowly from cells which had been preloaded with the compound prior to challenge with *trans*-isohumulone.

Teuber & Schmalreck (1973) suggested that the membrane integrity of *B.subtilis* was impaired by hop compounds and hop-derived compounds. The present results are consistent with those obtained by these workers but can be interpreted in a different way. Consider the case of ATP which has a relative molecular mass (M_r) of *ca* 500. Many membrane-active antibacterial agents (*e.g.* benzethonium chloride, chlorohexidine gluconate) induce leakage of ATP from cells. Indeed, the ability of such compounds to cause rapid and complete leakage of the intracellular ATP pool forms the basis of commercially-available extraction reagents for assay of the ATP content of microorganisms developed by the present author (NRM/LUMIT QM reagent system, Lumac bv, the Netherlands). Unlike mitochondria, which possess a transport system to exchange cytoplasmic ADP for mitochondrial ATP (Smagula & Douglas 1988), intact bacterial cell membranes are impermeable to ATP. Thus, observation of the effect of drugs on the integrity of the bacterial membrane to both intracellular ATP and to certain other intracellular compounds (which, in contrast, are maintained as concentrated cellular pools as a result of the existence of a proton-motive force or, as a result of the operation of other energy-dependent transport systems) can give an indication as to whether the antimicrobial agent has disrupted the physical integrity of the cell membrane or

merely disturbed coupling between energy generation and its utilisation for nutrient transport (*e.g.* by disturbing the distribution of ions across the cell plasma membrane). In the former case the action of the drug is likely to be irreversible, while in the latter case, restoration of coupling between energy generation and its utilisation for transport (*e.g.* restoration of ion gradient(s) as a result of cellular metabolic activity) should lead to recovery.

The results obtained indicate that impairment of membrane integrity induced in sensitive cells by *trans*-isohumulone is of the type in which coupling between energy generation and transport is broken rather than that in which the physical integrity of the plasma membrane is disrupted. The results of Teuber & Schmalreck (1973) can be interpreted in a similar way. This hypothesis is supported by the results described in section 4.3.7. which showed that the ability of the cells to retain an intracellular pool of [^{14}C]-L-leucine was not seriously impaired by *trans*-isohumulone (although a ΔpH-linked efflux was observed).

In general, contact between *Lact.brevis* IFO 3960 and *trans*-isohumulone produced little morphological change. Examination of non-growing organisms treated with *trans*-isohumulone revealed no differences due to treatment. On prolonged exposure, growing organisms responded differently in that, although initially unaffected, most organisms exposed to isohumulone for longer periods (<40h) became elongated and some lysed. Schmalreck *et al.* (1975), working with a strain of *B.subtilis*, observed almost complete lysis as a result of contact between growing cells and hop compounds and hop-derived compounds. They reported that, in a minimal medium, concentrations of humulone, isohumulone and humulinic acid which exceeded the MIC by *ca* one third

induced complete (>96%) lysis of cell suspensions within 270min at 37°C. It is possible that lysis of actively metabolising bacteria in such experiments was a consequence of the redistribution of ions across the plasma membrane. For example, osmotic swelling (Maloney *et al.* 1975) or activation of autolytic enzymes (Ghuysen 1968), mediated by the change in intracellular pH, may have caued lysis. Induction of 'giant' cell morphology in *Pediococcus cerevisiae* by humulone (Nakagawa & Kitahara 1962) (a phenomenon in which, in the presence of humulone, bacterial cells swell from their normal size of *ca* 0.8μm diameter to as much as 20μm diameter) may similarly be attributable to ionophoric action and consequential ion distribution.

4.4.3. The transmembrane proton gradient (ΔpH) of *Lact.brevis* IFO 3960 is dissipated by *trans*-isohumulone

Lactic acid bacteria maintain a pH gradient (ΔpH) across their cell membranes (Kashket 1987; McDonald *et al.* 1990). They do not, however, maintain a constant pH_{int}, unlike bacteria such as *E.coli* or *B.subtilis* which maintain pH_{int} over a wide range of external pH values (Padan *et al.* 1981; Booth 1985). Instead, they maintain a ΔpH of 0.5-1.0 over an external pH of 4.0-6.5 (Kashket *et al.* 1980; Kashket 1987; McDonald *et al.* 1990).

The transmembrane proton gradient, a component of the proton-motive force, is the driving force for accumulation of many nutrients, including sugars and amino acids (Pavlasova & Harold 1969; Kashket & Wilson 1973, Simoni & Postma 1975; Ramos & Kaback 1977; Poolman *et al.* 1987) and also for accumulation of inorganic ions such as K^+ (Bakker & Harold 1980; De la Peña *et al.* 1982). The intracellular pH also plays an important role

in regulation of nutrient transport processes in such bacteria (Poolman *et al.* 1987; van Boven & Konings 1987).

In lactic acid bacteria, ΔpH is established by the action of a membrane-bound proton-translocating ATPase and by excretion of metabolic end-products such as lactic acid. The membrane-bound ATPase converts energy derived from fermentation of sugars (which is in the form of ATP) to an electrochemical gradient of protons which can be put to work for transport of nutrients. The energy recycling model (Michels *et al.* 1979) proposes that carrier-mediated excretion of metabolic end products (such as lactic acid) can occur in symport with protons resulting in Δp generation. It is generally believed that both processes operate in lactic acid bacteria, but that the role of the membrane ATPase is dominant (ten Brink *et al.* 1985). An essential prerequisite to establishment of ΔpH by either means is that the plasma membrane should be essentially impermeable to protons.

The proton motive force is not essential for bacterial growth. Harold & van Brunt (1977) demonstrated that growth of *Enterococcus faecalis* can proceed under conditions in which there is no ΔpH or $\Delta\psi$ provided that the growth medium is rich in nutrients, has a slightly alkaline pH and a high concentration of K^+.

Many compounds have the ability to dissipate ΔpH or Δp and, as a result, possess antimicrobial activity. Examples include solvents such as ethanol (Cartwright *et al.* 1986); weak acid food preservatives such as benzoic acid and sorbic acid (Freese *et al.* 1973; Sheu *et al.* 1975; Eklund 1980; Salmond *et al.* 1984; Cole & Keenan 1986, 1987); protonophores such as dinitrophenol (Mitchell 1961b); detergents such as quaternary ammonium compounds (Hugo 1978) and ionophores such as nigericin (Harold & Baarda 1968a). The ability to interfere with Δp or ΔpH generation can result from one, or more, of

several mechanisms of action, (i) the compound may inhibit the activity of the proton-translocating ATPase, (ii) the compound may decrease the natural impermeability of the cell plasma membrane to H^+ or other cations (*i.e.* increase membrane permeability to H^+). This may result from significant physical disruption of the membrane or from effects on the permeability to specific ions, (iii) the compound may inhibit energy generation causing a reduction in ATPase activity due to a lack of ATP.

Several facts pointed to the possibility that hop compounds and hop-derived compounds exert their antibacterial action by dissipating one, or more, components of the proton-motive force, *viz.*, (i) an undissociated form of each compound was essential for activity; (ii) in the early stages of contact, the action of the compounds was bacteriostatic and reversible; (iii) the physical integrity of the cell membrane was not affected by the compounds; and (iv) they did not inhibit energy generation.

Experiments were therefore performed to demonstrate the effect of such compounds on components of the proton-motive force of *Lact.brevis* IFO 3960 and to discriminate between the possibility that (i) Δp was dissipated as a result of inhibition of the membrane ATPase and (ii) inhibition of Δp was the result of specific changes in the natural permeability of the bacterial plasma membrane to specific ions. The titrimetric method revealed several features associated with the antibacterial action of *trans*-isohumulone. When the experiments were conducted in a medium containing both KCl and $MgCl_2$, *trans*-isohumulone displayed pronounced protonophoric activity. But, when applied to a cell suspension in deionised water, the compound was devoid of protonophoric activity. Movements of protons across the plasma membrane of *Lact.brevis* IFO 3960 were catalysed by

trans-isohumulone only in the presence of monovalent cations such as K^+, Na^+, NH_4^+, Li^+, or Rb^+, in addition to H^+. In contrast, addition of Mn^{2+} reversed the direction of net proton flux. Secondly, proton movements catalysed by isohumulone were electroneutral since, unlike those induced by the protonophore CCCP, they were not dependent on the simultaneous presence of the potassium-selective ionophore valinomycin. Thirdly, the fact that *trans*-isohumulone acted as a mobile-carrier of ions was demonstrated by the finding that ionophoric activity was prevented if the plasma membrane of the test organism was solidified, or frozen, by cooling the cell suspension to 4°C. Immobilisation of the membrane lipids in this way presumably prevented isohumulone from shuttling back and forth across the plasma membrane thus inhibiting ionophoric activity.

Trans-isohumulone has thus been found to act as an ionophore of the mobile-carrier type which catalyses transfer of protons across the plasma membranes of sensitive cells in an electroneutral process in which H^+ is exchanged for Mn^{2+} and a second monovalent cation (K^+, Na^+ *etc.*) is required. The demonstration of movements of H^+ across the plasma membrane of *Lact.brevis* IFO 3960 did not necessarily indicate that pH_{int} would be affected by *trans*-isohumulone. This is because pH values indicate the *activity* of H^+ rather than its *concentration*. Thus, a cell whose cytoplasm possesses a high buffering capacity will initially show little change in intracellular pH on challenge with ionophores. In contrast, a cell whose cytoplasm possesses a low buffering capacity will show more marked changes in pH_{int}.

When applied at the MIC, *trans*-isohumulone completely dissipated ΔpH within 60min, indicating that coupling between energy generation and its utilisation for transport was impaired. Measurements of $\Delta\psi$ under the

same conditions confirmed that the ionophoric action of *trans*-isohumulone was electroneutral since, under conditions in which ΔpH was completely dissipated, approximately 40% of Δψ persisted. The reduction in Δψ in glucose-energised cells may arise from increased demands on membrane-bound ion pumps resulting from loss of ΔpH. That the effects observed were solely due to ionophoric action and not to inhibition of the membrane bound proton-translocating ATPase was confirmed by the fact that no inhibitory effect of *trans*-isohumulone on this enzyme could be demonstrated.

4.4.4. Proton gradient-linked solute transport in *Lact.brevis* IFO 3960 is inhibited by *trans*-isohumulone

Solute transport systems in lactic acid bacteria can be classified into three groups according to the mechanism by which they are coupled to energy generation (Poolman *et al.* 1987), namely, (i) ATP-dependent transport systems which utilise chemical (phosphate-bond) energy to drive solute translocation; (ii) secondary transport systems which utilise electrochemical energy to energise solute translocation; (iii) group translocation systems which couple translocation to the chemical modification of the solute. For some systems in lactic streptococci, a role for both ATP *and* the electrochemical gradient of protons has been implicated in energisation or regulation of transport.

As shown above, hop compounds and hop-derived compounds dissipate the proton-motive force of sensitive bacteria (primarily the ΔpH component of Δp) and consequently would be expected to inhibit those solute transport processes which depend on an electrochemical gradient of protons. This was demonstrated for the case

of uptake of L-leucine by *Lact.brevis* IFO 3960. Uptake of [^{14}C]-L-leucine was shown to be sensitive to the combined action of CCCP and valinomycin. Together these compounds dissipate the proton-motive force. Organisms exposed to both these compounds could not accumulate [^{14}C]-L-leucine; instead the concentration of [^{14}C]-L-leucine at equilibrium was equal on both sides of the cell plasma membrane. On its own, CCCP reduced but did not abolish the uptake of [^{14}C]-L-leucine by *Lact.brevis* IFO 3960. These observations indicate that uptake of L-leucine (at low external concentration) is mediated by a Δp-dependent carrier which is energised by both ΔpH and $\Delta\psi$ components.

Uptake of [^{14}C]-L-leucine by *Lact.brevis* IFO 3960 was, indeed, found to be sensitive to the action of *trans*-isohumulone. Like CCCP, however, *trans*-isohumulone was incapable of completely inhibiting L-leucine uptake since it only dissipates ΔpH. Presumably, the driving force for L-leucine transport which remained in CCCP-treated and *trans*-isohumulone-treated cells was provided by $\Delta\psi$. As expected, addition of *trans*-isohumulone to cells which had been preloaded with [^{14}C]-L-leucine resulted in slow leakage of radiolabelled material from the cells, since coupling between energy (ATP) generation and transport had been broken by the protonophoric action of *trans*-isohumulone.

CHAPTER 5

Conclusions

(a brief reminder)

Und was in schwankender

Ercheinung schwebt,

Befestiget mit daurnden Gedanken.

(And what in fluctuating

appearance hovers,

Fix by lasting thoughts.)

(Goethe)

Although the molecular structure of the major hop resin materials and their derivatives was known prior to the present study, knowledge of the NMR behaviour of the compounds was incomplete. In the case of one of the compounds examined (dehydrated humulinic acid) no NMR data had previously been reported. The NMR assignments of colupulone, (-)-humulone, (-)-cohumulone, *trans*-isohumulone, *trans*-isocohumulone, *trans*-humulinic acid and dehydrated humulinic acid have, in the present work, been significantly improved and clarified.

From the literature, the reader can be left with the impression that ionisation of hop compounds and their derivatives can be described by single pKa values. However, the present investigation has revealed that at least two different values are obtained for each compound depending on the measurement method employed. The anomalous behaviour of hop compounds and hop-derived compounds with respect to their ionisation, spectroscopic and solubility behaviour can be explained on the basis that, in aqueous solution, such compounds exist in a state of equilibrium between hydrated and dehydrated forms. As a consequence, *equilibrium* pKa values (pKa_{equil}) rather than true pKa values are measured using potentiometry or conductimetry. Analysis by spectrophotometry or by measurement of solubility gives the equilibrium dissociation constant for the dehydration reaction. The possibility that such anomalous behaviour was due to keto-enol tautomerism was ruled out using NMR spectroscopy.

In methanolic solution, *trans*-isohumulone forms a 1:1 complex with Mn^{2+}. The ability to form such complexes is an integral part of its antibacterial action.

The minimum inhibitory concentration (MIC) of hop compounds and hop-derived compounds for *Lactobacillus brevis* IFO 3960 was dependent on the pH of the growth

medium. Calculation of the concentration of undissociated molecules in each of the tests (using the pKa_{equil} values determined experimentally) showed that undissociated molecules were the active antibacterial species and that ionised molecules had little antibacterial activity. As little as 0.1-0.4μM of the undissociated form of *trans*-isohumulone was sufficient to inhibit growth of *Lact.brevis* IFO 3960 at pH 4.0-7.0.

Metal cations also influenced the antibacterial activity of the compounds. In the case of *trans*-isohumulone, activity was enhanced in the presence of monovalent cations such as K^+, Na^+ and Rb^+, but reduced in the presence of the divalent cation Mn^{2+}.

These observations could be reconciled by the finding that hop-derived compounds, such as *trans*-isohumulone, act as ionophores of the mobile-carrier type. Inhibition of growth of *Lact.brevis* IFO 3960 was caused by the ability of *trans*-isohumulone to lower intracellular pH. The ionophoric action of *trans*-isohumulone was electroneutral; charge translocated in the form of H^+ was compensated for by the movement of cations of equivalent charge in the opposite direction. In a model experimental system it was shown that *trans*-isohumulone could exchange H^+ for Mn^{2+} in this way. The presence of a monovalent cation other than H^+ in the growth medium was essential for ionophoric activity of *trans*-isohumulone. As a consequence of ionophoric activity, nutrient transport and protein synthesis in *Lact.brevis* IFO 3960 were inhibited, but metabolism of fermentable carbohydrate and generation of ATP continued. The role of covalent hydration in the ionophoric action of hop compounds and hop-derived compounds such as *trans*-isohumulone, is not yet clear.

A number of practical applications suggest themselves as a result of the work reported in this

thesis. These include an improved means of assuring the bacteriological stability of beer by controlling beer pH and a new method of selecting beer-spoilage bacteria on the basis of their resistance to hop-derived materials. The shortcomings of traditional plate count techniques for determining the numbers of viable lactic acid bacteria in media such as beer have also been highlighted. These applications have been discussed in more detail elsewhere (Simpson & Fernandéz 1991; Simpson & Hammond 1991).

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