# STUDIES ON ASCORBIC ACID AND ITS DERIVATIVES

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BY

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# P R E F A C E

The studies reported in this thesis were made in the context of the earlier work done in this laboratory on ascorbic acid and its derivatives. Although the chemistry of this vitamin has been under study for a very long time, there are still some aspects of it that are not quite well understood and need clarification. It was considered important to investigate these matters in view of the possibility that some of the results may have serious biochemical and physiological implications.

The earlier work was restricted to spectroscopic studies. This work has now been extended, and, in addition, the relative reactivities of the vitamin and some of its derivatives and analogues in a few reactions have been studied. The general background of entire work is set forth and the status of the background information available relating to every one of the studies is briefly surveyed. The reactions studied include, autoxidation, benzylation and Udenfriend hydroxylations. Some allied stereochemical problems have been solved.

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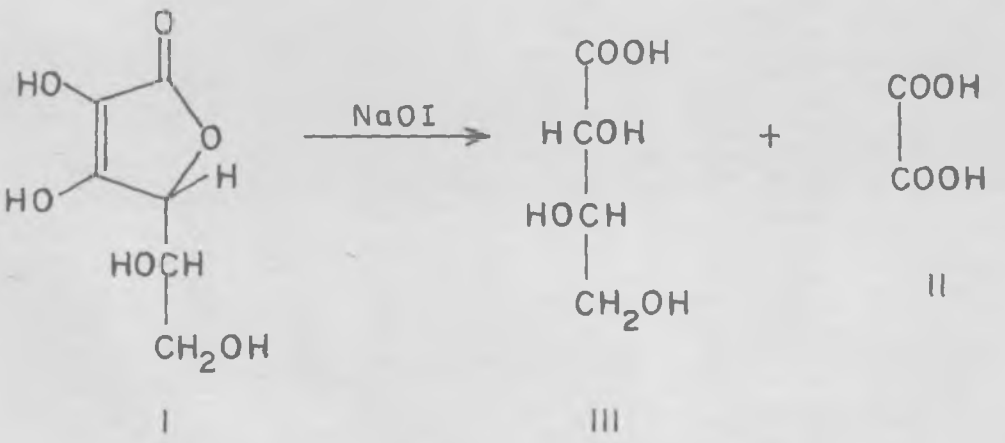
# Chapter I

## GENERAL INTRODUCTION

Ascorbic acid (Vitamin C, AA) is one of the most extensively studied of vitamins and a vast literature has been accumulated on it[1,2]. However, even today, the mechanisms of its action in its various biological roles are not clearly understood at the molecular level. At least, to a limited extent, the situation holds true for the chemistry of the substance. Even though the compound and its derivatives have been studied for more than four decades, there are still a number of instances in its chemistry where the observed behaviour has not been accounted for satisfactorily. Each of the six carbon atoms of the molecule is chemically different in character and each carries a functional group of different reactivity. This situation can give rise to relatively complex behaviour in certain chemical reactions and it is presumably for this reason that some aspects of the chemistry of the vitamin still remain to be clarified. The studies reported in this thesis have been made in this context. Such an effort may be justified for its own sake, but, more importantly it may have some bearing on the mechanism of biological action of the vitamin.

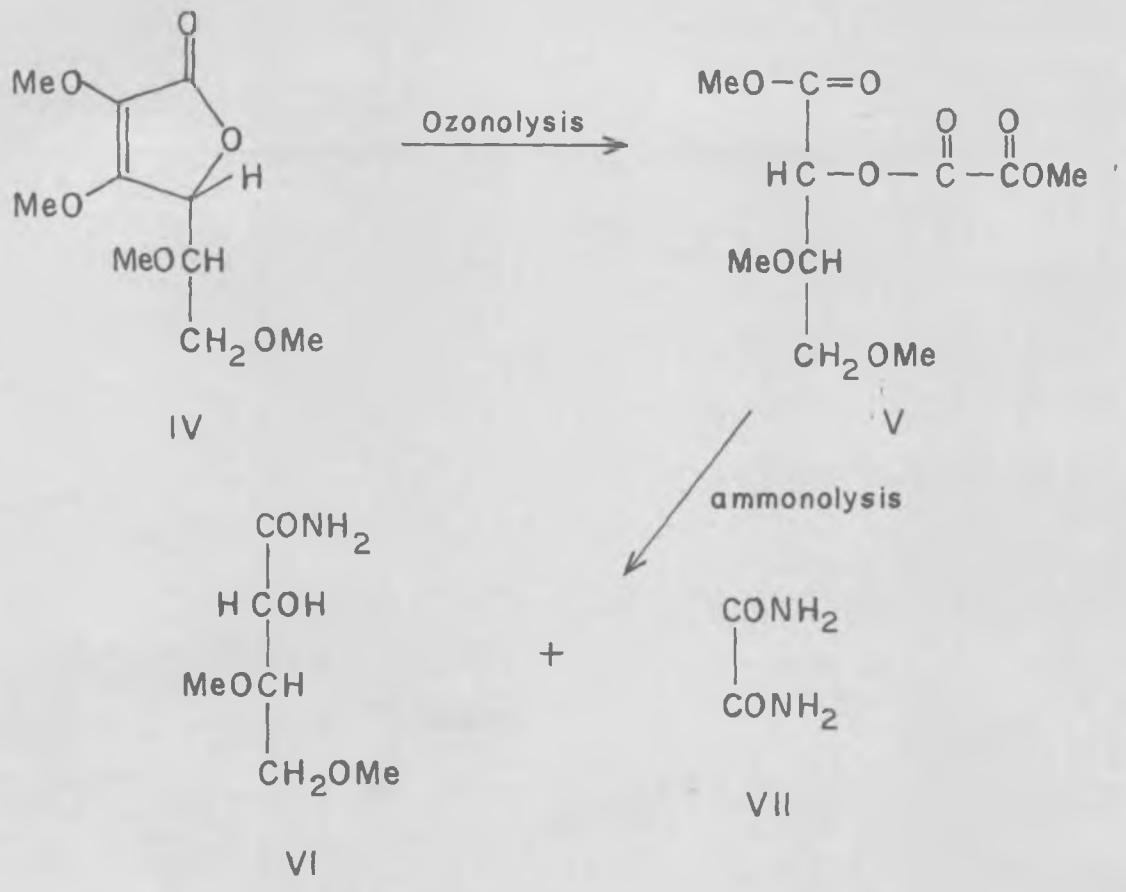
AA was first isolated by Zilva[3] from lemon juice and characterised as a nitrogen free unstable substance with powerful reducing properties. Soon afterwards, Szent-Gyorgi[4a,b] isolated from the adrenal cortex of oxen a crystalline substance (m.p. 192°C) with formula $C_6H_8O_6$. With the demonstration of its antiscorbutic activity[4c], this substance was recognised to be the same as that isolated by Zilva.

The structure of AA was first established as L-threo-2,3,4,5,6-pentahydroxy-2-hexenoic acid-4-lactone (I) by Herbert, Hirst and coworkers[5]. Formation of furfural and carbondioxide on treatment of the substance with strong hydrochloric acid indicated the unbranched nature of the molecule[6]. The presence of four hydroxyls was shown by the formation of a tetraacetate[7] and a tetramethyl ether[5]. Two of the hydroxyls were acidic in nature, with pKa's of 4.25[8] and 11.79[9], and, further, these were the ones that got methylated in the reaction with diazomethane[5,10-12]. The presence of a 1,2-glycol system out of which one was a primary alcohol was indicated by the reaction with lead tetra-acetate giving formaldehyde[13,14]. The UV absorption of the compound and its change with addition of alkali suggested the presence of an α:β unsaturated system with a hydroxyl at the β-position[5,15]. The formation of an isopropylidene derivative with the reducing properties of the vitamin confirmed the nature of glycol system[16]. With the oxidation of the vitamin itself to oxalic acid (II) and L-threonic acid (III) by hypoiodite[5,17]




I III II

and the ozonolysis of the tetramethyl ether (IV) to give a product (V) which could be decomposed to the dimethyl ether of L-threonamide (VI) and oxamide (VII)[5,18], the structure of the vitamin could be put down as (I), and it was consistent with




the formation of a neutral oxidation product[5], dehydro ascorbic acid (DHA, VIII and many other aspects of the chemistry of the substance that were investigated[5]. This structure has been confirmed by X-ray and neutron diffraction studies[19].

The importance of the study of vitamin C may be realised by even a cursory consideration of what happens in scurvy in

I

AA

VIII

DHA

man[20-22,29]. The degeneration that sets in is so extensive and all encompassing that no part of the human body is left unaffected. Vitamin C is present in all human tissues and is necessary for their proper functioning. AA is a labile substance which passes rapidly from the plasma into the white cells where it is involved in immunological defence and is also stored. It passes from the plasma into body tissue where it plays important roles in liver metabolism, haemopoesis, collagen formation, endocrine control and metabolism including ovulation and other metabolic processes. General tissue metabolism is maintained by AA, and tissue anabolism and growth is stimulated by a supply of the vitamin in adequate amounts to maintain tissue saturation. Many enzymes and hormones require AA as a cofactor for their functioning[20,22,23].

It is unlikely that in all these diverse functions of AA, there is one chemical feature of AA that serves as the common denominator for them all. It looks more likely that we would

require a set of them for a satisfactory understanding of the totality of all actions of AA. It is not clear how many elements are involved in this set. It is of interest here to note that this situation makes it difficult to assess the significance and reliability of the estimates of the anti-scorbutic activities of AA analogues and other materials. The index of antiscorbutic activity[22,24] has necessarily to be confined to a limited number of relevant observations for practical purposes[24], and, for this reason, "antiscorbutic" activity is not a well defined term even today. The ability of a substance to serve as an ascorbic acid substitute in a particular biochemical role, for instance, as a mediating agent in hydroxylation[51], need not necessarily imply that it will be able to play all the roles of AA with equal efficiency[25].

Because of the variety of roles played by ascorbic acid, there can be considerable doubt as to man's requirement for the vitamin which are to be met entirely from exogeneous sources[26]. The mega-vitamin controversy that has arisen in recent years may be mentioned in this connection[27-29]. It is apparently a well established fact that animals that are able to synthesise their own requirements of vitamin C increase their production of the vitamin about ten fold when subjected to biochemical stress, such as drugs[30]. The increased production is apparently a response to a need. This implies that animals like man, who cannot meet their own needs of the vitamin from endogenous sources, would also require increased amounts of it under

biochemical stress, and, unless the required amount is ingested, their physiologies would be adversely affected[27a,31-35]. There is considerable merit in this argument. However, the actual needs for meeting specific stresses will have to be estimated only on the basis of experience. In actual practice, possible harmful effects of AA metabolites like oxalic acid and their variations in individuals will also have to be taken into account in determining therapeutic dosage.

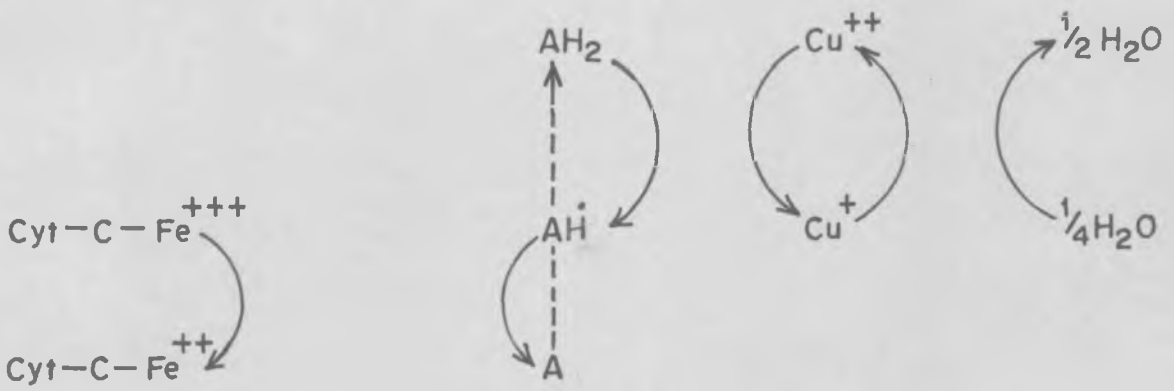
Of greater interest to us here are the suggestions as to the likely paths by which AA is able to potentiate the different activities it has been recognised to have. The notion that intake of AA increases resistance to bacterial infection has been widespread and it has considerable experimental basis[29]. This is so even for viral infections[29], and, apparently, there is evidence of the toxicity of AA to some types of cancer cells[27b,29,36]. In the case of resistance to bacterial infection, it has been suggested that the role of AA consists of the help it gives for the synthesis of the required -globulins. AA presumably helps to degrade cystine disulphide linkages to SH groups i.e. to form cysteine which goes into the making of -gluobulins. The disulphide linkages of the gloublins themselves could be formed by the action of DHA that is formed. The formation of -globulins is apparently genetically restricted to the initial formation of chains with SH groups only since no trinucleotide is known to code for cystine[29]. Similarly, it has been suggested that, in the resistance to

viral infections, AA action might manifest through a physical effect, namely surface tension, and make the virus cell walls vulnerable to attack by body nucleases[29]. It has also been suggested that some of the beneficial effects of AA may manifest through the increased formation of the cyclic nucleotides cyclic-AMP[37-39] and cyclic-GMP[40], which, in turn, potentiate hormonal action[29]. Several of these suggestions are apparently important; but, they will have to be placed on firm experimental footing before we can consider more detailed mechanistic implications.

The effect of vitamin C on every function of the human body, seems to have been investigated[20]. Most of this has been empirical in nature and little is known about their inter-relationships. "Contributions that may be of great importance have been lost under a deluge of clinical reports, many of which have little significance"[41]. Nevertheless, some progress towards an understanding of AA functions at the "molecular level" has emerged from the work done so far. Some of the typical bio-chemical functions of AA that have been known are briefly considered below.

The most outstanding property of AA is its capacity for reversible oxidation and reduction, and this property has dominated the search for its role in metabolism[42]. From the fact that both the reduced and oxidised forms are usually found in plant and animal tissues possessing high metabolic activity,

it appears probable that it may play an important role in the reactions involved in electron transfer in the cells. It has been suggested that oxidation by numerous agents such as enzymes or metal catalysts involves as electron transfer occuring in two stages. The first step is the formation of an unstable semiquinone like radical formed as an intermediate between the fully reduced and fully oxidised form, DHA. The evidence for the existence of this unstable and very reactive species comes from studies of Kern and Racker[43] and Nason et al.[44] with plant enzymes on animal tissues[49] and from ESR studies[45]. The second step is the formation of DHA by dismutation of monodehydro AA. Thus, the reduction of cytochrome C by AA which involves a cuprous-cupric cycle may be visualised as follows:[46]




(Scheme for cytochrome C reduction catalysed by AA oxidase. Dotted lines show dismutation reaction of free radicals).

The role of AA in tyrosine metabolism has been extensively studied[50]. From the reports, it appears that AA catalyses the reaction of p-hydroxyphenylpyruvic acid to homogentisic acid in the presence of the enzyme, p-hydroxyphenylpyruvic acid oxidase. The reaction, which involves hydroxylation of the benzene ring, oxidative decarboxylation and migration of the side chain, proceeds more rapidly in the presence of AA. The role of AA in this reaction has, however, been shown to be nonspecific, and substances other than AA have been found to be equally effective[51].

Staudinger[47,52] and Kersten et al.[48] have indicated that AA is involved in the biosynthesis of corticosteroids in adrenal homogenates and that it is required for 11-β-hydroxylation. Kahnt and Wettstein[53] and Kernsten et al.[54] have also shown that the rate of hydroxylation of deoxycorticosterone is increased by AA. Staudinger et al[49] have also demonstrated that an AA dependent NADH oxidation in rat liver and kidney microsomes requires only catalytic amounts of AA.

The participation of AA in the formation of inter cellular substances of cartilage, dentine, bone and collagen of fibrous tissues has been established beyond doubt[42]. The essential function of AA in collagen formation is its ability to catalyse the hydroxylation of proline which forms 14% of collagen[55]. Cooper[56] has suggested that AA is required for

the conversion of tryptophan to 5-hydroxytryptophan. It has been suggested that a hydride ion is first removed from the 5-position of the tryptophan ring and this is replaced by OH from water. The two protons and electrons then reduce the true acceptor DHA, and the cycle is complete by the oxidation of AA to DHA.

AA has been found to be associated with the functioning of other vitamins and some types of enzymes[42]. In some cases it activates, e.g. arginase[57], liver esterase[58], etc. and, in others, it inhibits, e.g. urease[59].

It has been observed that AA is concentrated in the more actively growing regions of the plant, and hence it has been suggested that it promotes the growth of certain plants[60]. Aberg and Johnson[61] have indicated that the effects of AA on growth are due to the formation of $H_2O_2$ that occurs in the oxidation of the vitamin by metal catalysis.

It has been shown that the transfer of plasma iron, present as a protein iron complex, and its incorporation into tissue ferritin requires the presence of AA[62].

Because of its multifunctional ability, AA has been widely used by food processors in following ways[63]; as a vitamin[64], an acid, a stabiliser in wine making, and soft drinks, a flour improver in bread making, an antioxidant and nutrient in soft drinks and beverages, a curing agent in meat

preservations, an agent of quality in food processing, an oxygen scavanger, etc.[65]. Its 6-esters with fatty acids find wide applications in antioxidant formulations for edible oils and fats and also in various cosmetic formulations[63b].

We may now turn our attention to some aspects of the reported chemistry of AA and its derivatives where the observed behaviour is not accounted for satisfactorily or requires further elaboration. The systems involved are much less complex than in biochemistry, but their study may conceivably give information of appreciable relevance for the biochemistry of AA. Herbert, Hirst and coworkers[5], in their study on the structure elucidiation of AA, have indicated that AA is capable of reacting in different forms and the structure assigned by them takes this fact into full consideration. An example is the formation of 1-O-methyl-hetero-AA in the reaction with diazomethane[10]. This indicated the existence of an isomeric form of AA in methanolic solution. However, the significance of several other observations was not discussed[5].

2,3-Di-O-methyl AA, on treatment with alkali was reported to give an isomeric compound which was suggested to be a bicyclic 3,6-anhydro derivative[66]. The formation of only one epimer of the product, its unusual stability and the possible stereochemistry at $C_{(2)}$ were not explained. The 2,3,5-trimethyl and 2,3-dimethyl-6-trityl ethers of AA could also be isomerised by alkaline treatment. The natures of the products obtained in these cases have not been studied. A

later study reported the formation of two epimeric products in the isomerisation of 2-O-benzyl-3-O-methyl-AA[67].

Another interesting instance which requires further clarification is the reported formation of only one isomer of the $C_{(2)}$-benzyl derivative in the benzylation of AA. Attack by the benzylating agent would normally be expected from both sides of the ring[68].

AA has also been reported to mediate not only enzymatic hydroxylations but also nonenzymatic ones, the products obtained being the same as the ones obtained under enzymatic conditions[69]. Various substrates have been reported to be hydroxylated by the Udenfriend system, which consists of AA, a metal ion, a chelating agent and molecular oxygen or $H_2O_2$. The most interesting from our present point of view are those at $sp^3$ sites where the products of hydroxylation have been claimed to be highly stereo and regiospecific. No satisfactory mechanism has been suggested for the high specificities obtained which are surprising, particularly in the context of the notion that the active part of the molecule is the ene-diol - -lactone system. Another property of AA which is quite opposite to this is its efficiency as an antioxidant[63b]. How the same compound is acting as an oxidation mediator and an oxidation inhibitor is not clearly understood.

The rapid fall in the UV absorption of AA in aqueous

solution was noted even in the earliest studies on AA[5]. This was attributed to its rapid oxidation under the usual conditions of measurement where no special measures are adopted for exclusion of air. However, there is strong indication that the issue here is more complicated. Pronounced fall in the intensity of absorption of very dilute sodium ascorbate solutions has been observed in conductivity water under an inert atmosphere[29]. Also air oxidation of AA in aqueous solution is extremely slow in the absence of a catalyst. The results of the mechanistic studies in this regard are not consistent[70]. DHA, the first oxidation product of AA, has been shown to exist in different forms depending on the manner of its generation and subsequent chemical treatment. However, some of the reports in this regard are contradictory and there is need for further study of the matter[71].

Among the aspects of the behaviour of AA which we have listed as requiring further clarification, several involve the formation of isomeric bicyclic forms explicitly. We have also seen that there is evidence to show that AA is capable of existing in the hetero form which has a carbonyl group at the 3-position. This would suggest the possibility that bicyclic forms of AA itself may be involved in some of the reactions of ascorbic acid, although they may exist merely as transient intermediates. The studies reported in this thesis have been made in the context of the clarifications

needed and the difficulties mentioned above, and an attempt has been made to see if they could possibly be connected with the existence of AA in bicyclic forms.

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69. Introductory review of Chapter VI of this thesis, p.

70. Introductory review of Chapter III of this thesis, p.

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# Chapter II

## SPECTROSCOPIC STUDIES ON ASCORBIC ACID, DEHYDRO ASCORBIC ACID AND MONOMETHYL ETHERS

## 1. Introduction

The review on the structure, chemistry and physiological action of AA shows that the tremendous number of investigations in this regard have enabled us to understand the mechanistic aspects of its diverse functions only in a very limited way, and only the broad outlines have been obtained[1]. In order to understand the mechanisms involved in detail at the molecular level, a thorough understanding of the chemistry of the molecule is necessary. The possibility of AA or some of its derivatives existing and reacting in different isomeric forms is an important aspect of its chemistry which needs to be studied in detail in this context.

One method of tackling a problem of this type is by means of spectroscopic studies which can be very helpful. The requirement for the direct observation or indirect recognition of isomeric forms by this method, however, requires their presence in appreciable amounts. Several spectroscopic studies of various types have already been reported, mainly on AA itself. Although it is well-known that the proportion of such isomeric forms are generally functions of the solvent used, not much attention has been devoted to this matter mainly because of the lack of a suitable method of study. It was therefore considered worthwhile making some attempts in this regard.

But, it is useful to take stock of what has already been done by way of spectroscopic work before the present study is described. The required background information is briefly summarised below.

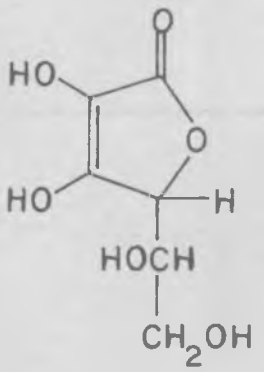
In their work on the structure of Vitamin C, Herbert[2] et al. reported that an aqueous solution of AA has a very intense absorption band in the UV with its head at 245 nm, which moved to 263 nm with addition of alkali. This band confirmed the presence of an α,β-unsaturated carbonyl system, with an .OH in the β-position. This band showed up at 245 nm even in ethanol and methanol[3]. Both the 3-O-methyl and 2,3-di-O-methyl derivatives showed strong absorption bands at 230-235 nm, which shifted to 270 nm in the case of 3-O-methyl-AA[4,5]. However, there was no absorption in the case of 2,3-di-O-methyl-AA on addition of alkali[5]. The 1-O-methyl-hetero-AA had an absorption at 280 nm[4], which shifted to 320 nm on addition of alkali. The 1,2-dimethyl ether had an absorption band at 270 nm in water which on standing at 50°C slowly shifted to 245 nm, as it readily hydrolysed to give 2-O-methyl-AA, in which the double bond had shifted back to its original place between $C_{(2)}$ and $C_{(3)}$ as in AA[4,5].

An interesting feature of the absorption of AA in the UV was the rapid fall in the intensity of the band[2]. It was more persistent in acid solution (pH $\leq$ 3).

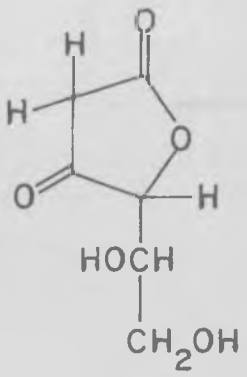
The fall in intensity was attributed to air oxidation by Herbert et al[2]. However, recent studies have shown that this phenomenon was observed even when oxygen free conductivity water was used for recording the spectrum. The optical absorbance of 50 of sodium ascorbate at pH 6.1 and 25°C was found to decrease with time even when an inert atmosphere was maintained over the solution by bubbling nitrogen, free from oxygen and carbondioxide.[6] The absorption at neutral pH could not be measured in the usual manner (i.e. where no precaution was taken to exclude oxygen) without a stabiliser.[7] A 0.001% KCN solution has been used as a stabiliser. The role of KCN is regarded as the complexation of trace amounts of metal ions present which catalyse the oxidation of AA. The system was found to obey Beer-Lambert law in the concentration range 2-10 /ml.[7]

The position of absorption maximum and the value of extinction coefficient have been reported to be pH, concentration and solvent dependent.[3,8,9] The $_{max}$ has been found to vary from 245 nm to 263.5 nm. Decreasing concentration and increasing pH gave a bathochromic shift. The UV absorption of AA has been assigned to a $\pi \rightarrow \pi^*$ [3,8] excitation. The shift in $_{max}$ with change in concentration of AA in aqueous solution has been attributed to association[9,10] and/or the presence of a keto form of AA.[11,12]

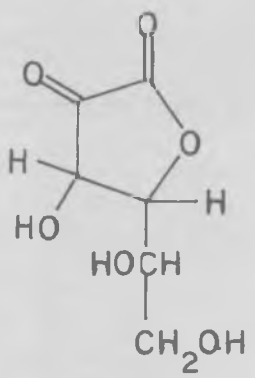
However, Ogata and Kosugi[3] have ruled out the possibility of association and/or presence of a keto form. The two possible keto forms have been considered by them out of which II may be expected to be more stable than I.




I




II




III

They attributed the bathochromic shift to the dissociation of AA with increasing dilution or increasing pH. The high value of $_{max}$ (9600) can be attributed only to a $\pi \rightarrow \pi^*$ transition. The keto forms I or II will have $_{max}$ for an $n \rightarrow \pi^*$ transition at a longer wavelength than for the enol form and a weaker intensity (smaller $_{max}$). Also, the keto forms, which have strained rings, are not stabilised by the conjugation of C=O to C=C. There is no evidence of any absorption of the type expected for I or II.

UV absorption of AA in acid solution, namely $H_2SO_4$, has also been reported to be dependent on the $H_2SO_4$ concentration.[3,13] The $\lambda_{max}$ which is at 262 nm in $1x10^{-5}$N aqueous $H_2SO_4$ has been reported to shift to 245.5 nm in $1x10^{-1}$N $H_2SO_4$.[3] Increasing concentration from 45 to 95% of $H_2SO_4$ decreased the intensity of absorption.[13] It was also reported that AA in highly acidic solution does not reduce iodine and 2,6-dichlorophenolindophenol because it mainly exists as a non-reducing keto form.[12]

A semiempirical LACO-MO-SCF method was used to calculate the charge distribution, bond parameters and UV spectra of AA, its monoanion, hydroxy tetronic acid and its monoanion. The calculated $\lambda_{max}$ values agreed with the experimental; but, the bond length agreement was not so good.[14]

It was noted that 2,3-di-O-methyl ether of AA lost its absorption on addition of alkali. It is also known that alkali treatment of the compound leads to isomerization to isodimethyl AA, which does not have any UV absorption indicating the absence of double bond.[5] This is apparently something different from the loss of absorption of sodium ascorbate in very dilute solution.

The vibrational spectra of crystalline AA and sodium ascorbate have been discussed in terms of the known structural parameters, previously determined by

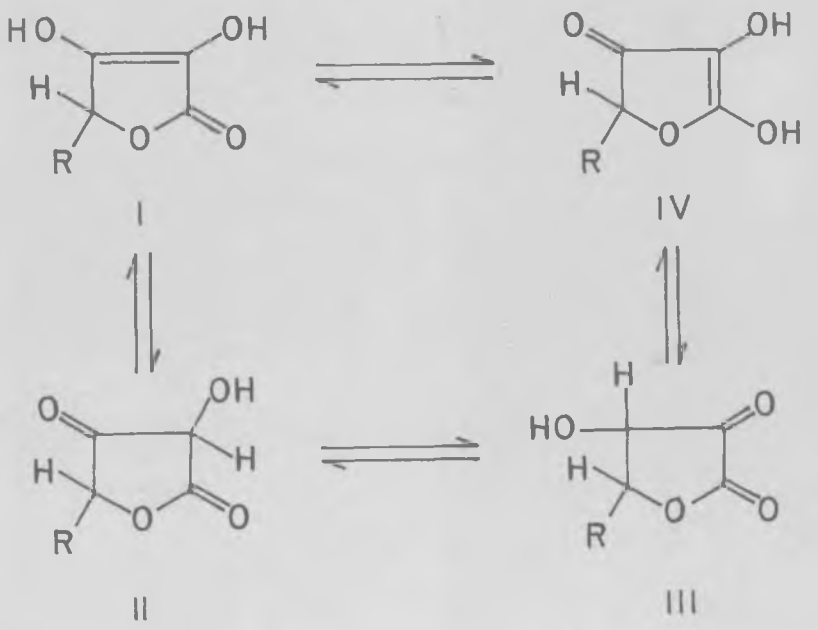
X-ray diffraction analyses. Particularly, the observed O-H stretching frequencies of AA and the corresponding O-D frequencies of the deuterated material have been correlated with the O ...O distances and the varying degrees of the H-bonding that would be involved. Various changes in Raman spectra were observed on dissolution of AA and its sodium salt in water; but there was no evidence for any drastic rearrangements.[15] IR and Raman bands for the intermolecular and intramolecular hydrogen bonded OH groups and for the free and intramolecular H-bonded C-O group of AA have been studied.[16]

IR spectrum of AA aqueous solution in the pH range of 1.8 to 13.5 were used to determine the frequency of fluctuations in functional groups of AA in the unionised, monoionic and dianionic forms. Both AA and ascorbate formed proton bridges between the carbonyl oxygen and the neighbouring H-atoms of the ene-diol group.[17]

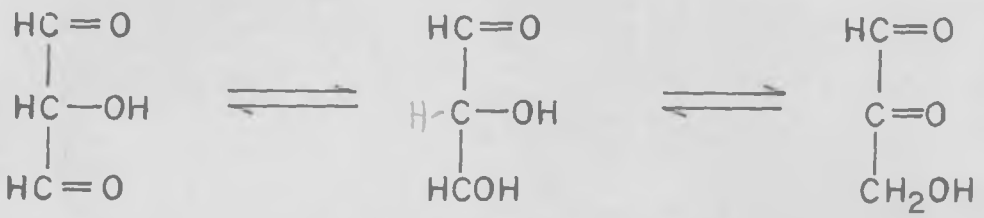
It was reported by Weigl[18] that deuterated AA prepared by crystallisation of AA from $D_2O$ shows a C-D stretching frequency in its high resolution IR spectrum. He has attributed the presence of C-D band to the exchange of the $C_{(4)}H$ for deuterium. The sample, examined in a KBr disc, was prepared in the usual manner which involves application of very high pressures.

Various proton and $^{13}C$ NMR studies have also been reported. An attempt was made to study the keto-enol tautomerism of AA and sodium ascorbate.[19] Keto-enol tautomerism in a number of 1,3-diketo systems was studied successfully with the help of $^{13}C$-FT NMR spectroscopy. The isomer ratios found by this technique were in good accord with those found by others. However, for AA and Na-ascorbate no keto-tautomer could be detected by this technique.

Berger[20] has made a systematic $^{13}C$ NMR study of the bio-redox pair AA-DHA. From the $^{13}C$-chemical shifts obtained for AA at various pH values, he has shown that form I is predominant out of the four forms (I to IV).




$$R = -CH(OH) - CH_2(OH)$$



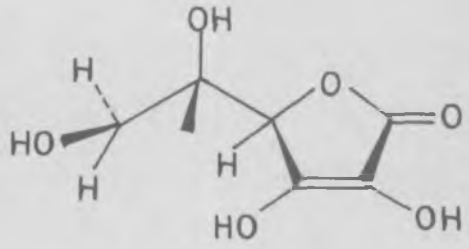
V

A proton decoupled carbon spectrum showed only six signals and an off resonance spectrum showed the presence of one primary, two secondary and three tertiary carbons. Structures (II) and (III) could be eliminated on this ground as well as the observation that only one carbon showed the characteristic shift for a carbonyl group. Further a proton coupled carbon spectrum of the substance showed a 5.7 Hz geminal proton-carbon coupling constant for the less shielded of the two non-carbonyl tertiary carbons. This was consistent only with structure (I). The parent reductone, the threose reductone (V), showed only two carbon signals, indicating rapid interconversion of the tautomeric forms.

In AA, the equilibrium is apparently heavily shifted to the ene-diol and the rate of interconversions is apparently too rapid for observation of any of the minor component that could conceivably be present. It appears as if keto forms (II and III) are to be neglected at least from the

point of view of proportions as far as aqueous solutions are concerned.

A recent study of $^{13}C$ NMR of AA and 5-C-deutero AA was reported by Ogawa et al. who have established the correct conformation of the AA side chain with the help of the carbon-proton coupling constants. The long range $^{13}C-^{1}H$ spin-spin coupling constants were obtained from proton coupled $^{13}C$ NMR spectra. The most favoured rotamer of AA in aqueous solution appeared to be VI.




VI

Iacobucci and Goldsmith[22] have studied the $^{13}C$-NMR of many 2-and 3-substituted derivatives of AA. The $^{13}C$ shifts of the 3-O-methyl ether and 2- and 3-phosphate esters of AA have been measured. A modification of the proposed structure of AA monophosphate has been suggested. Confirmatory evidence for the structure of 3-O-methyl AA

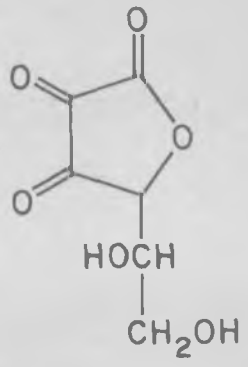
was obtained by chemical modification and study of mass spectra of its hydrogenated product and its comparison with that of 1,2,4,5,6-penta-O-acetyl-3-O-methyl-D-glucitol.

There has been a lot of controversy about the form in which DHA, the first oxidation product of AA, exists in solution. This issue is very important in connection with our own work where we are concerned with the possibility of AA existing in different isomeric forms. It is very important to know the properties of DHA, since it can have serious implications for the behaviour of AA.

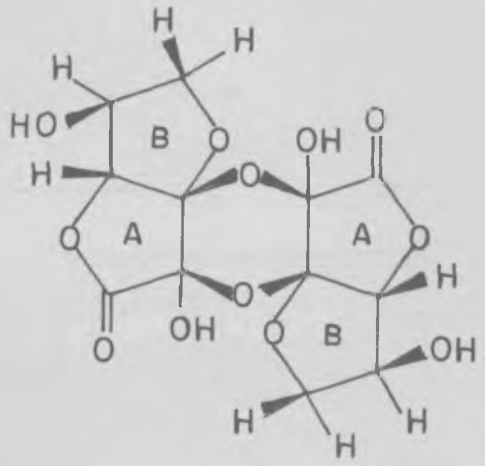
Earlier workers could not isolate DHA due to its unstable nature.[23] They studied the properties of this compound only in solution. Attempts to isolate this compound from aqueous solution did not succeed. These gave dark syrups which were complex mixtures. Hirst and Woodward[24] isolated it for the first time by oxidation of an ethanolic solution of AA in presence of lead carbonate. The purity was only about 65-70%. Later, Kenyon and Munro[25] showed that the product prepared by Hirst and Woodward after drying and dissolving in ethanol at room temperature and allowing it to stand deposited a crystalline colourless compound, which had the characteristics of anhydrous DHA. They suggested the possibility of existence of DHA in polymeric form.

In 1951, Pecherer[23] showed that it is extremely unlikely that DHA could be successfully isolated from aqueous solutions. He oxidised AA in methanol with chlorine gas to get a methanol adduct of DHA of very high purity which was in accordance with the result of Kenyon and Munro.

The triketo structure (VII) assigned to DHA in earlier days has been criticised by several authors because the three carbonyl groups in the ring are hardly compatible with the colourlessness of the crystalline solid.[24,26] Albers, Muller and Dietz[27] and Tiechmann and Ziebarth[28] based their criticism on the nature of the IR spectrum of solid and proposed a polymer form. Molecular weight determinations to ascertain the extent of polymerisation were complicated by poor solubility in most solvents.[28] But cryoscopic measurements in DMSO lead to the conclusion that DHA is a monomer in solution.[28,29] Albers, Muller and Dietz in 1964 prepared several derivatives of DHA and showed that DHA exists in dimeric form (VIII). Muller-Mulot[30] proposed an equilibrium in solution between a monomeric and a dimeric form and based his conclusion upon the dependence on time of the optical rotatory power.




VII

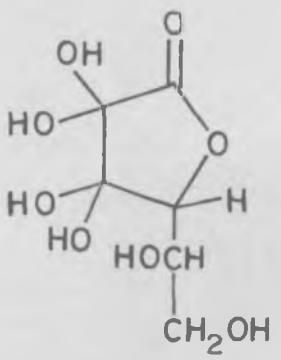



VIII

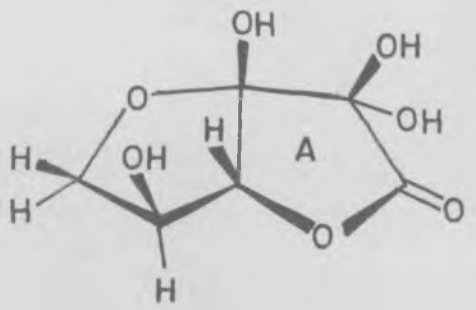
Dietz[29] (1970) emphasized the role of solvent in determining the nature of DHA in solutions. Apparently, the crystalline dimer could be precipitated when certain acids were added to the solution of the monomeric DHA. In the same year, Muller-Mulot[31] gave a standard procedure for preparing a stable dimer of DHA. AA dissolved in anhydrous DMF, DMSO or dimethyl acetamide when oxidised by p-benzoquinone, chloranil or mercury acetate gave DHA. On addition of pure formic acid and another organic acid the crystalline DHA precipitated immediately. He also gave a reliable procedure for recrystallisation.

Hvoslef[32] in 1972, showed that the dimeric structure (VIII) suggested by Albers, Muller and Dietz[27] for crystalline DHA was correct by X-ray crystallography. The material used for the study was obtained by dissolution of crystalline DHA in DMSO and reprecipitation with acetonitrile. The dimeric structure for crystalline dimer appeared to be constant with the $^{13}C$ NMR spectrum obtained by Berger[20] in aqueous solution. However this dimer in aqueous solution was shown to go over to the open chain dihydrated monomer in about two days. In the same study it was shown that the spectrum of the material freshly prepared by iodine oxidation of AA in aqueous solution seemed to exist in an open chain dihydrate (XVI) of the triketo form (VII).

In a similar study Matusch[34] suggested that the first product of iodine oxidation was the dimer which soon broke down to the triketodihydrate form. When the reaction was done in DMSO, it appeared that the triketo form was formed first which then changed over to the 3,6-anhydro form (X) and finally to the dimer (VIII). An appreciably different result was obtained by Pfeilsticker, Marx and Bockish[33] who employed benzoquinone and molecular oxygen for oxidation and PMR, UV and IR spectroscopy for the observation of structural changes.



XVI

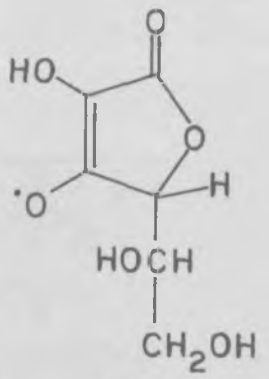


IX

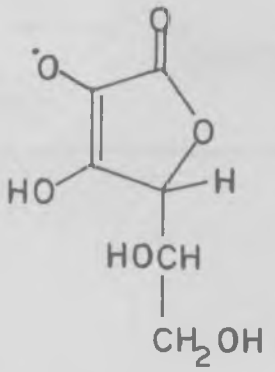
It appears that the product in aqueous solution was 2,2,3,5-tetrahydroxy-3,6-anhydro-threo-hexuronic acid-lactone (IX). The stable dimer of DHA could also be broken down to the monomer by warming its aqueous solution. All 8 structural possibilities were considered. In a method worked out for the gas chromatographic analysis of AA and its oxidation products, the aqueous system were fixed by a cold shock, lyophillised and the compound permethyl silylated in anhydrous medium. The GLC of the solution of stable dimer in warm water showed a single peak. The permethyl silylation indicated the presence of four silylable hydroxyls.

There are a number of ESR spectroscopic studies of the free radicals generated during AA oxidation in aqueous as well as non-aqueous media. Two neutral radicals (X and XI) and two ion radicals (XII and XIII) have been reported to be formed[35-39]. These radicals have been generated

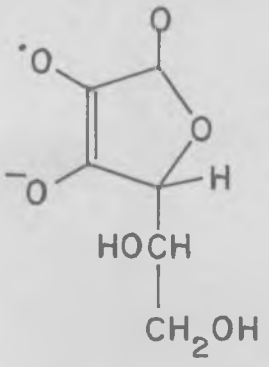
using different oxidising agents under different conditions.[35,36] There appears to be a little confusion regarding the usage of the term "monodehydro AA". What we refer to as monodehydro AA is the neutral species (X or XI).



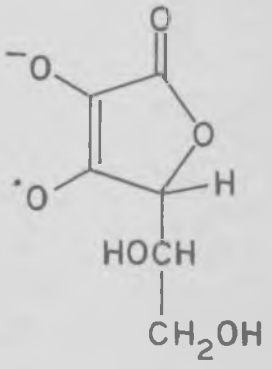

X



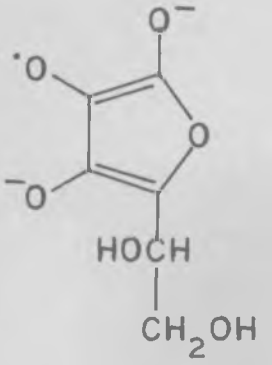

XI




XII




XIII




XIV

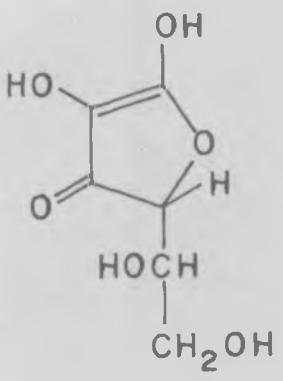
Russel et al. detected a paramagnetic intermediate during oxidation of AA.[39] On the basis of hyperfine splitting pattern, they suggested that the trione radical (XII, XIII) anion exists as a dianion (XIV) or enol. Similar dianion structure has been considered by others for the radicals generated in the reaction of AA with hydrazine[38] which was later shown to be wrong[37]. Kirino and Kwan[36] have identified

two types of species labelled as l and m. The l-type species is stable and is produced by a number of oxidising agents, while that of the m-type is unstable and is formed only when $Ti^{3+}$-$H_2O_2$ is the oxidant. They compared the radicals of AA with that of hydroxytetronic acid. They suggested that the species formed from AA are (X) or (XI) and a monoanion radical (XII).

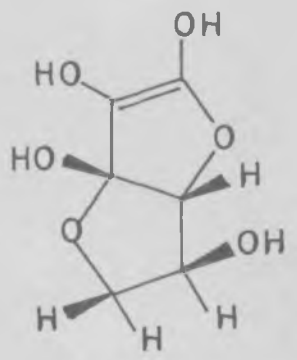
This report was further supported by Kirino and Kwan[40] who studied the oxidation of AA, its analogues and derivatives, where 3-OH is free, by ESR. With $Ti^{3+}$-$H_2O_2$, they reported two species, one a neutral radical (X or XI) and the other an anion radical (XII). Only anion radicals are generated when oxidising agents other than $Ti^{3+}$-$H_2O_2$ were used. Radicals generated in water MeOH, EtOH, PrOH and DMSO have also been reported.[41] Radicals have also been generated by photolysing AA solutions. An effect of solvent was observed and interpreted in terms of intramolecular H-bonding. AA is probably able to scavenge all radicals by H-atom transfer.

Differences in g-factor and shifts in proton and $^{13}C$-hyperfine coupling constants were observed for radicals from AA and its analogues in aprotic solvents as compared with those in aqueous solution.[42] It is interesting to note that the $C_6$-protons became magnetically nonequivalent in the radical from AA, while they were equivalent in the radical from D-iso-AA.

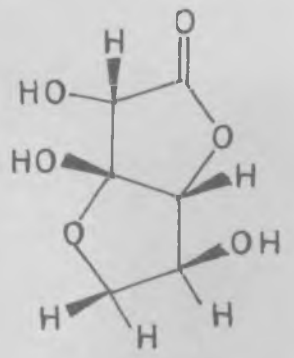
The foregoing account of spectroscopic work on AA and DHA leaves some questions unanswered. Studies on DHA show that the uncertainty in this case is limited to the question of the structure of the monomeric form in aqueous solution. Two studies report divergent results. There is need to sort out the confusion here. However, all the structures considered have tetrahedral carbons at the 2- and 3-positions. Even the 2-carbonyl is presumably converted to the 2-bis-hydroxy or 2-hydroxy-2-alkoxy derivatives in aqueous or other hydroxylic solvents. Since there is proof for the existence of AA in the hetero form (IV), the question of the possible existence of AA also in bicyclic forms (XVII & XVIII) has to be examined,




IV




XVII




XVIII

particularly in the background of the information available on DHA and the need for furnishing an explanation for the disappearance of UV absorption of AA in dilute solution. Even if these are present only in extremely small amounts, the occurence of these species will have important

consequences for the reactivity of the vitamin. Most of the studies that have so far been made have been limited to aqueous solutions. It appeared that it would be worthwhile to examine AA and some of its derivatives in some non-aqueous solvents spectroscopically to begin with. The present study on AA, its monomethyl ether and DHA was undertaken in this context.

Results and discussion

The pH and concentration dependences of the UV absorption of AA, which have been studied earlier[3], were re-examined. At a concentration of 20 mg/100 ml, the $\lambda_{max}$ was at 250 nm and the apparent $\lambda_{max}$ shifted with neutralization in the manner shown in Fig. 1. This corresponded to the appearance of a second $_{max}$ at around 268 nm. With excess alkali, a third $\lambda_{max}$ at 300 nm indicating probably a doubly ionized species was also seen. From the pKa of AA, it could be calculated that the starting solution should be dissociated to the extent of over 19 percent. With the addition of increasing amounts of alkali the intensity increases observed for the second maximum did not quite correspond with the anticipated amounts of ascorbate in solution. One is reminded here of the recent report[6] on the behaviour of sodium ascorbate in aqueous solution. Even in conductivity water and a completely inert atmosphere, ascorbate solutions showed pronounced decrease in absorption intensity with time.

The behaviour of pure AA solutions themselves as a function of concentration is illustrated in Fig. 2. The $\lambda_{max}$ shifted progressively to longer wavelengths. In very dilute solutions, the problem of oxidation made intensity measurements of doubtful value. At a concentration of 1 mg/100 ml in water, the absorption maximum disappeared in about 2 hrs (Fig. 3) under aerobic conditions.

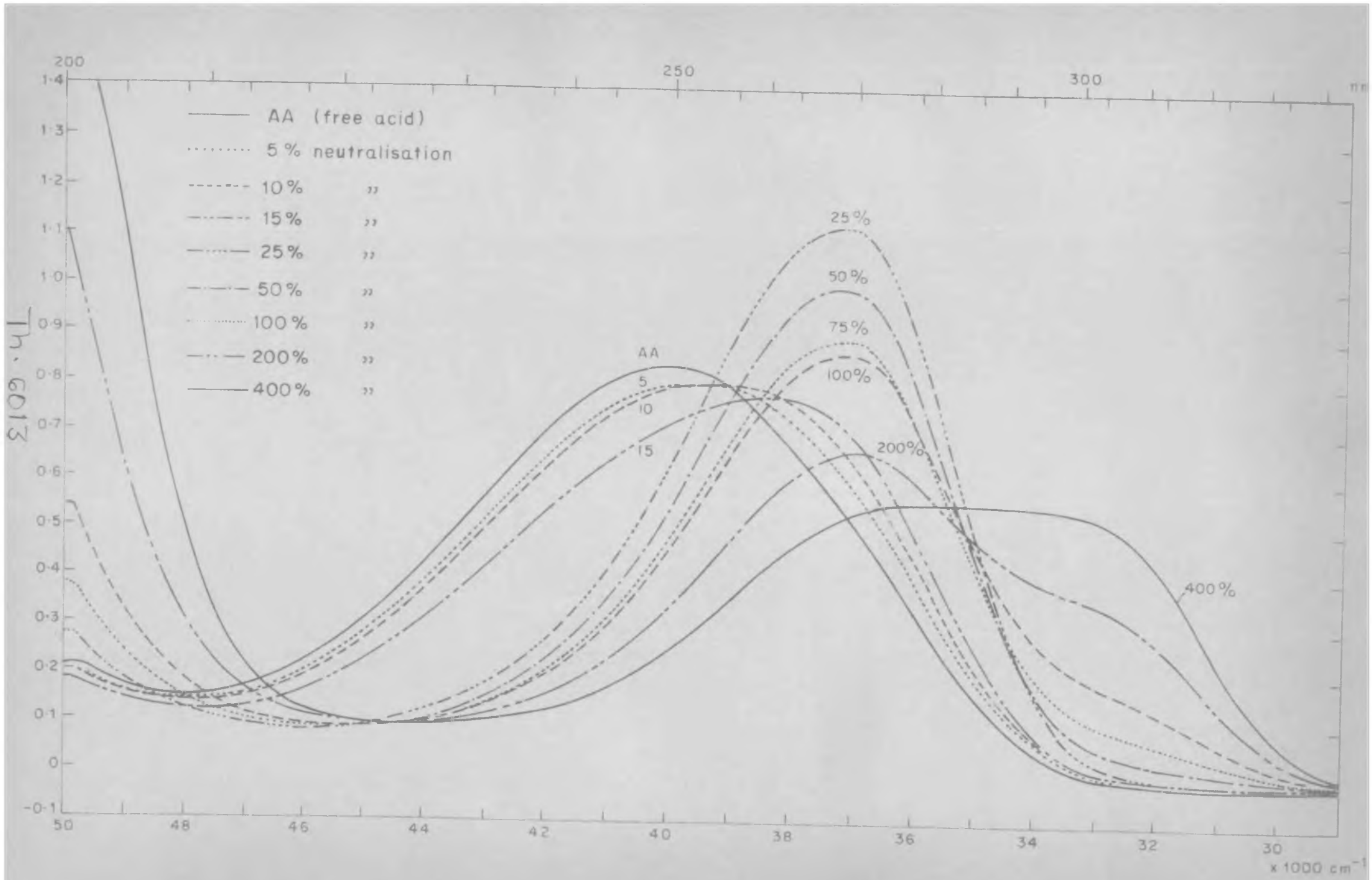



Fig. 1 Variation of UV absorption of AA with neutralisation

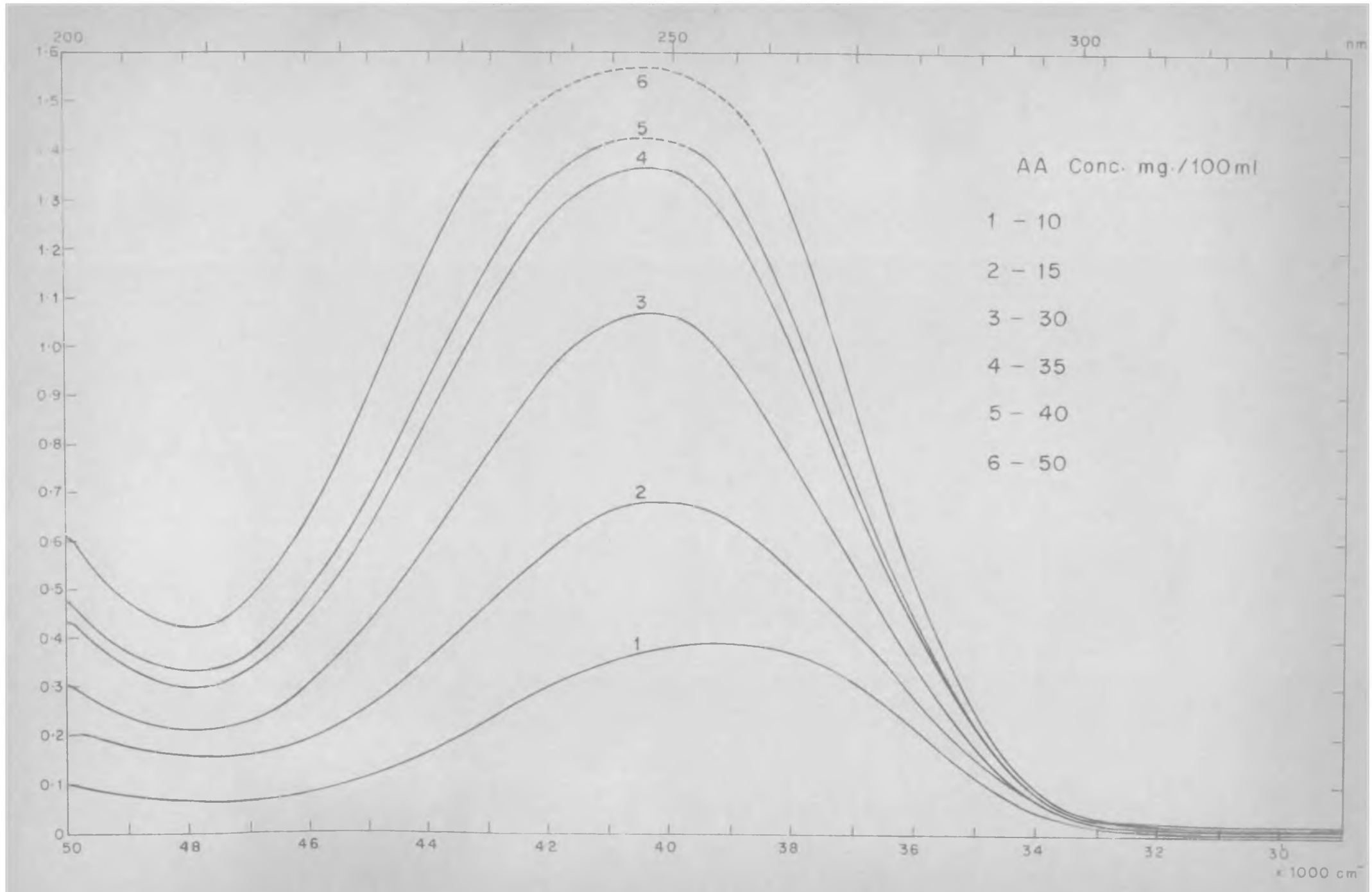



Fig. 2 Concentration dependence of UV Spectrum of AA in $D_2O$

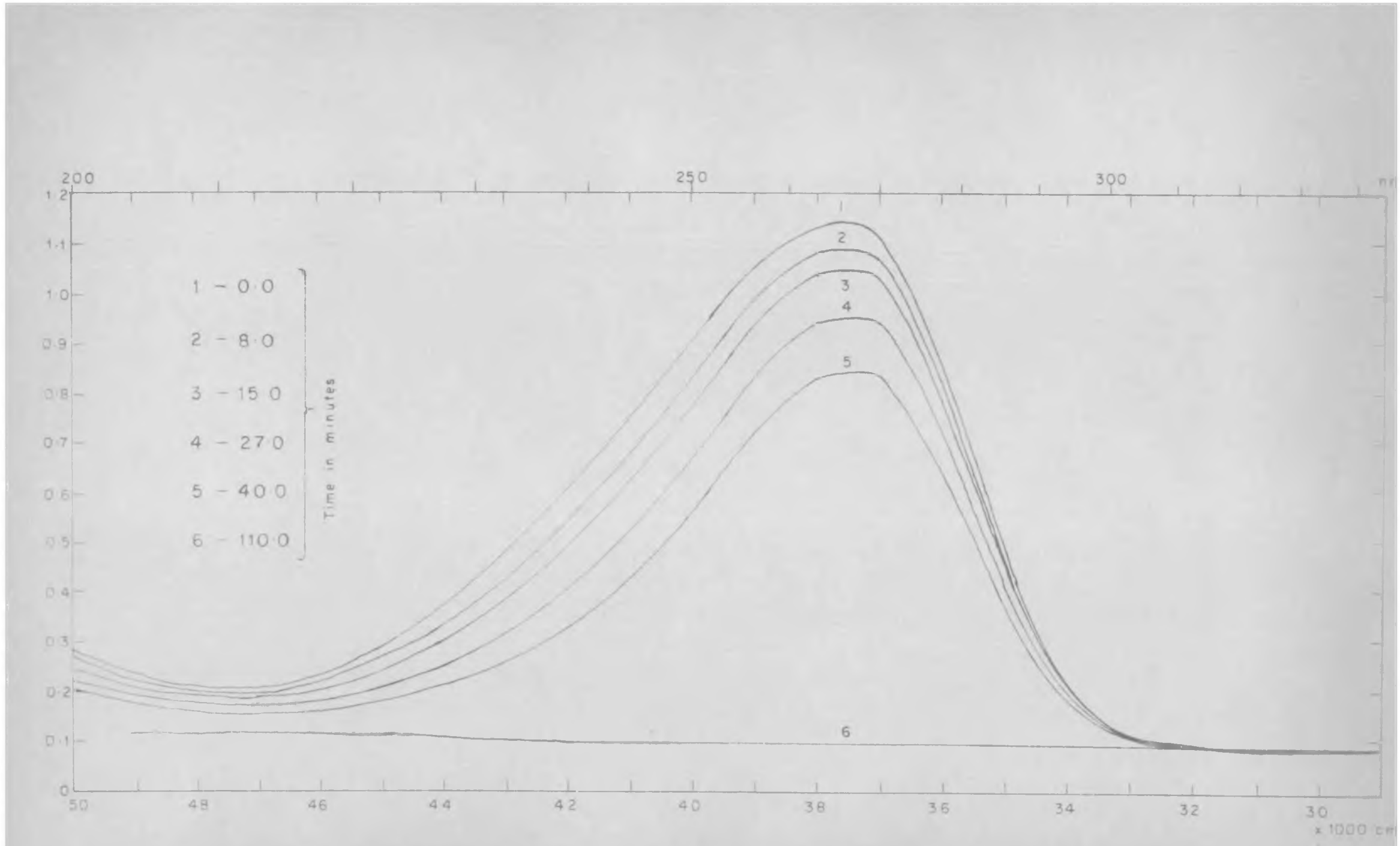
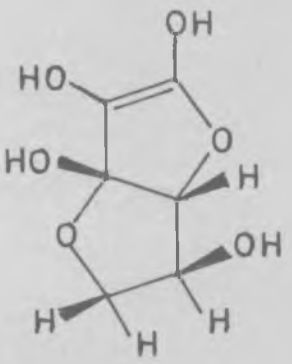


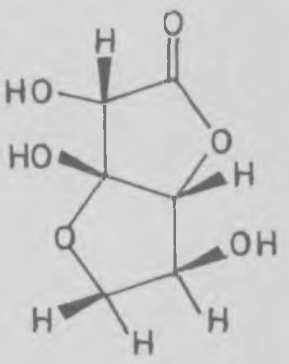

Fig.3 Fall in intensity of the UV absorption of AA with time under aerobic conditions

However, even with total exclusion of air, a very appreciable fall in intensity was still seen, although this was smaller than that under acrobic conditions. This result appeared to be similar to the reported behaviour of sodium ascorbate in dilute solution which has been already referred to.

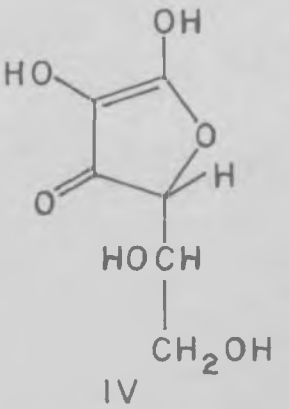
No explanation has so far been suggested for this observation. It is suggested here that the fall in intensity may be understood in terms of the formation of the bicyclic forms (XVII) and (XVIII) of AA.



XVII



XVIII



IV

The molecule has been shown to exist in the hetero form (IV) in methanol by the isolation of the corresponding 1-O-methyl derivative in the reaction with diazomethane in the original work of Herbert, Hirst et al[4]. As is well recognized, the ascorbate ion is ambident in nature and there is no reason why it cannot protonate the lactone carbonyl oxygen which is involved in the resonance of the

anion. One should expect the hetero-acid to be formed in aqueous solution also, and if this form is present, the occurence of the bicyclic forms (XVII) and (XVIII) is also likely because of the availability of a hydroxyl group on $C_{(6)}$- which is to the $C_{(3)}$-carbonyl in (IV). Thus, an unconjugated ene-diol and/or its keto form may be obtained. The ease of interconversion between these bicyclic species may be facile, very much like it is in the case of the various forms of triose reductone. Form (XVII) having only an isolated double bond and form (XVIII) which is a saturated lactone cannot have UV absorption above 220 nm. Forms (XVII) and (XVIII) should be weaker acids than the normal form of AA, and their formation under high dilution can give rise to an anomalous increase in pH which seems to have been actually observed[6].

A PMR spectrum of AA in $D_2O$ is shown in Fig. 4. The $C_{(4)}$-proton absorption is a doublet ($J = 1.86$ Hz) at 4.96 . The multiplet absorptions of $C_{(5)}$- and $C_{(6)}$-protons are around 4.07 and 3.76 respectively. The small value of $J_{4,5}$ shows that there is no free rotation around the $C_{(4)} - C_{(5)}$ bond. The conformational preference is apparently for (XX) or (XXI), since in the rotamer (XIX) the $C_{(4)}$- and $C_{(5)}$-protons are trans to each other[43]. If repulsion between the adjoining oxygens is the determining factor, then (XX) would be the preferred conformation.

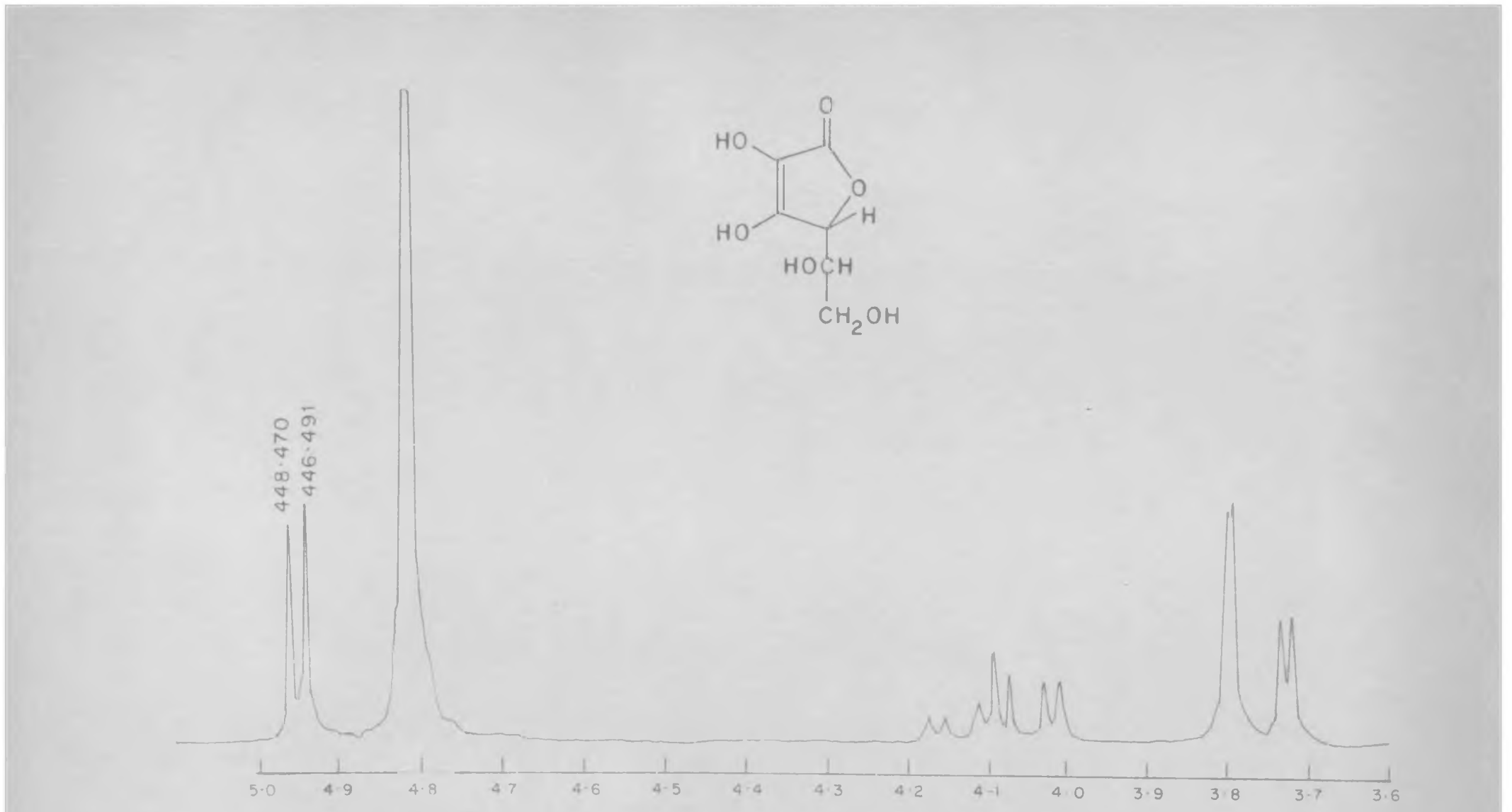
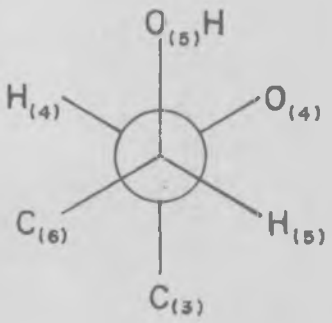
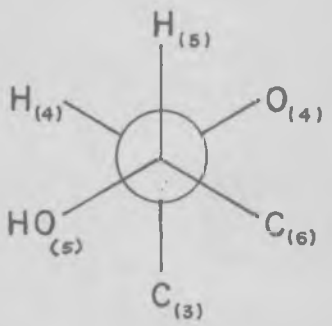


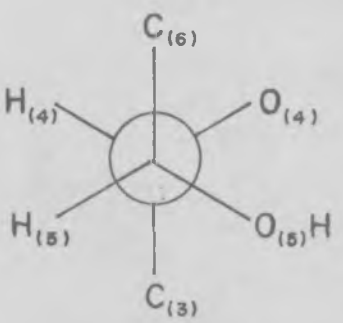

Fig. 4 90 MHz PMR Spectrum of AA in $D_2O$




XIX

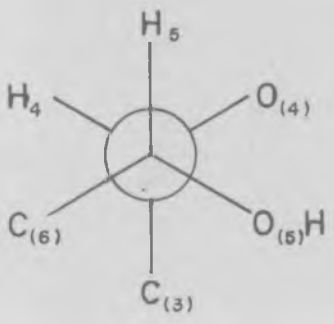



XX

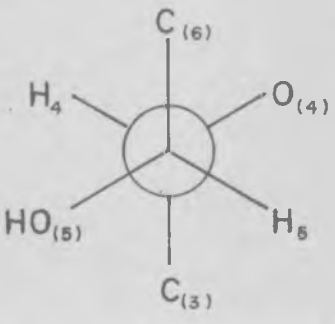



XXI

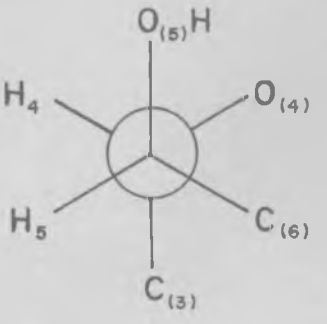
On the other hand, if the $C_{(5)}$-hydroxyl is involved in hydrogen bonding with the $C_{(4)}$-oxygen (lactone ether oxygen), then (XXI) would be the preferred form. A consideration of the PMR spectrum of D-iso-ascorbic acid presented in Fig. 5 shows that the second of the two possibilities mentioned is correct. The rotamers for the D-iso-AA are (XXII), (XXIII) and (XIV).




XXII




XXIII




XXIV

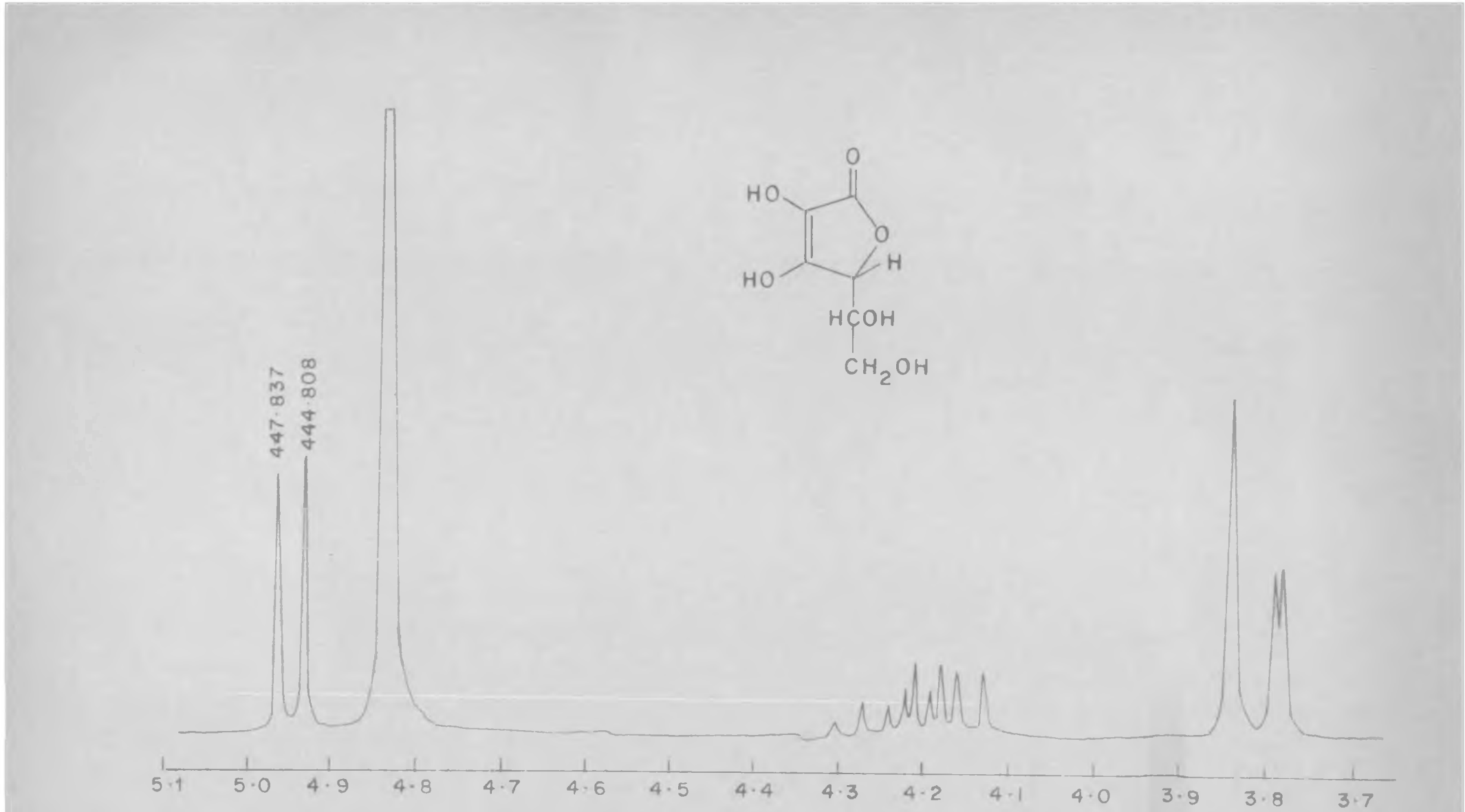
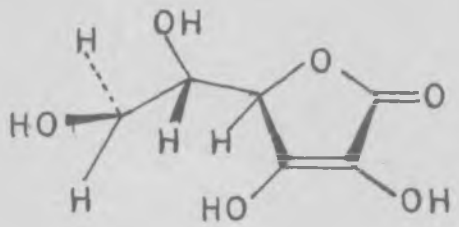



Fig. 5 90 MHz PMR Spectrum of D(−)−iso−AA in $D_2O$

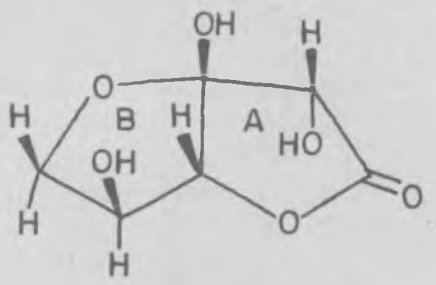
Fig. 5 shows that $J_{4,5}$ is small (3 Hz) in this case as well. If the oxygen-oxygen repulsion is the deciding factor, the favoured rotamer would be (XXIII) in which the hydrogens are also trans. Since the strong coupling expected for this is not seen, it is clear that hydroxyl hydrogen bonding is the deciding factor. The favoured conformation for AA is as shown in (VI), where the oxygen are gauche to each other. For D-iso-AA it may be either (XXII) or (XXIV).



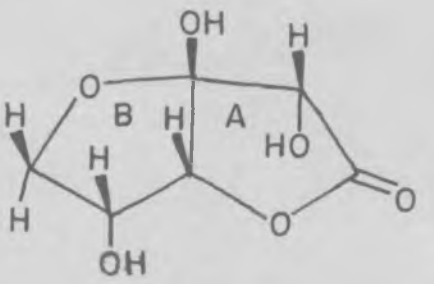

VI

The preferred conformation for AA has actually been established[21] by a more laborious, but sophisticated way by measurements of $^{3}J_{^{13}C\text{-}H}$ coupling constants on AA labelled with deuterium at $C_{(5)}$. In ascorbic acid, the $C_{(6)}$-carbon is thus situated away from the $C_{(3)}$-carbon. From the point of view of the ease of cyclization of (IV) to (XVII), it may therefore appear, at first sight, that the conformational position is not favourable. However, the barriers to rotation involved here are too small to be able to prevent cyclization from taking place and the energy of the cyclized

form itself would be more important for our considerations here. In fact, such differences in configuration do not prevent the formation of furanose forms in carbohydrate chemistry. In the bicyclic structure (XVII) the hydroxyl is <u>trans</u> to the $C_{(4)}$-oxygen with respect to ring B and at a




XVIII



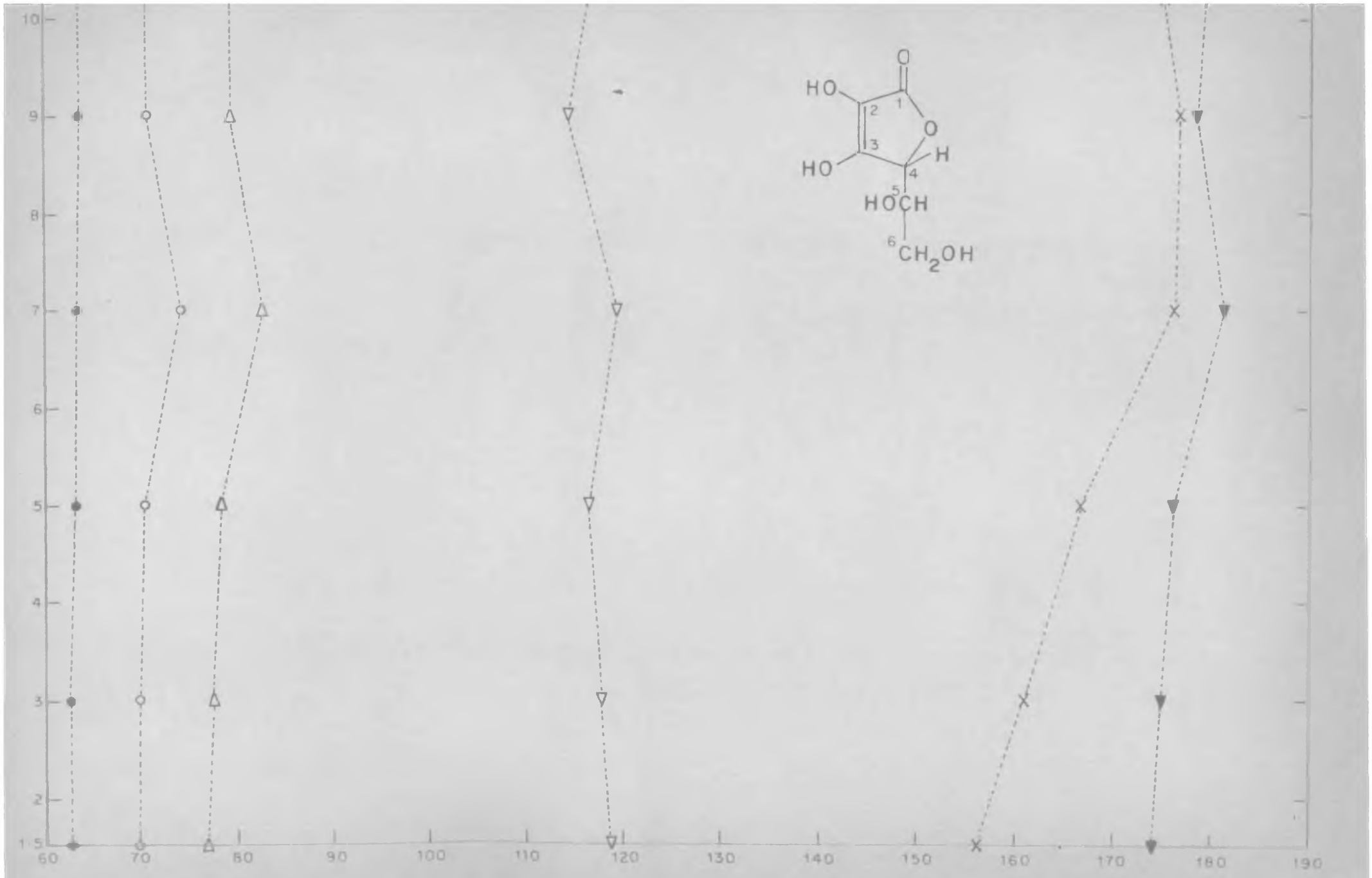

XXV

dihedral angle close to 120°. In a corresponding bicyclic structure for D-iso-ascorbic acid (XXV) the $C_{(4)}$- and $C_{(5)}$-oxygens will eclipse and the repulsion energy involved will be more than the hydrogen bonding energy. This is not to say that a cyclic structure like (XXV) is not possible for D-iso-ascorbic acid. It only means that such a structure will be less stable and will probably be less readily formed.

The $^{13}C$ spectrum of AA was obtained in $D_2O$ solution at various pH values. The spectra of solutions of the pure acid and samples of the same with 25,50,75 and 100 percent neutralizations were recorded. Also, a spectrum with excess

alkali was obtained. The chemical shift variations for all the six carbons are shown as functions of pH in Fig. 6. It is found that it is the $C_{(3)}$ shielding that shows the largest change. Up to the full neutralization stage, the shift is to the low field side. With excess alkali, there is a minor change in the reverse direction. It is not clear what significance should be attributed to this. In any case, no pronounced structural change is indicated.

The formation of 1-O-methyl hetero-ascorbic acid in the methylation of AA in methanol with diazomethane has already been referred to[4]. This result is suggestive of the presence of the hetero form (I.V.) in solution. The methylation experiments are preferably done at low temperatures. An attempt was therefore made to see if any of the hetero form or its modifications can be detected in methanol. The $^{13}C$ chemical shifts of AA in methanol did not show any change with temperature indicating the absence of any important structural change. However, the intensity of $C_{(4)}$, $C_{(5)}$ and $C_{(6)}$ signals decreased with decreasing temperature, while as that of $C_{(1)}$, $C_{(2)}$ and $C_{(3)}$ were unaffected. This is readily understood in terms of the slowing down of internal rotations in the side chain at $C_{(4)}$ at low temperatures. This clearly confirmed the conclusion that no structural change was involved. It was also thought that it might be useful to check the behaviour in DMSO in which AA has good solubility.




Fig·6 pH dependence of chemical shifts of AA in $D_2O$

No evidence for a second form could be obtained either from PMR spectra in DMSO or CMR spectra in methanol. In very dry DMSO solution some changes were seen for the proton signals, particularly for those of the hydroxyl groups. Immediately on dissolution, the enolic hydroxyls could be seen separately for very brief moments, if the ascorbic sample was extremely pure. But, for ordinary A.R. grade material, the enolic hydroxyls appeared as a broad absorption at 650 Hz and the alcoholic hydroxyls as another broad signal at 306 Hz. The $C_{(4)}$-, $C_{(5)}$- and $C_{(6)}$-proton signals were similar to those in water and centered around 282, 224 and 206 Hz respectively. After two days, both the hydroxyl absorptions tended to broaden out and a new absorption started appearing at 334 Hz. The new signal moved to 347 Hz in 4 days and to 372 Hz after 13 days. This did not change the position significantly on further standing. All these changes are shown in Fig. 7. The exchange averaged signal of the hydroxyl groups was to be expected at 478 Hz [(650x2 + 306x2)/4 Hz]. This large difference between the position expected for the exchange averaged signal and the one actually found was indicative of some structural change in solution. However, it was not clear what exactly was happening. Since it was desirable to have very dry DMSO and our deuterated samples contained a fair amount of moisture, we had opted for an experiment in the undeuterated material. The interference from spinning side bands was eliminated by changing their positions, and

an attempt was made to see if there was any change in the nature of the $C_{(5)}$- and $C_{(6)}$-proton absorptions. The provisional reading was that there was no evidence for any change. The significance of the change seen for the hydroxyl protons in the absence of any change for CH absorptions is not clear. A parallel study of time dependence of DMSO spectrum in the infra-red also showed changes. These changes are shown in Fig. 8. The carbonyl absorption and the C=C absorption at 1750 $cm^{-1}$ and 1660 $cm^{-1}$ respectively, first decreased with time with the simultaneous enhancement of a new absorption in the 1610 $cm^{-1}$ region. But on prolonged standing the change was reversed.

When a similar examination was done with 3-O-methyl AA the hydroxyl signal obtained on standing was very close to the exchange average signal. The PMR spectrum of 3-O-methyl in dry DMSO is shown in Fig. 9. The doublet at 284 Hz was assigned to the $C_{(4)}$-H and multiplets around 209 Hz were assigned to the $C_{(5)}$- and $C_{(6)}$-protons. The low field signal at 500 Hz integrating to one proton was due to the chelated $C_{(2)}$-OH. The $C_{(5)}$, $C_{(6)}$-OH protons together gave a signal at 279 Hz. The 3-O-methyl signal appeared at 240 Hz. After standing for about 20 hrs both the OH- signals became more and more sharp. After 30 hrs they again became broad and finally after 6 days completely disappeared. After almost 12 days, a new signal started appearing at 326 Hz. This became much sharper after about 20 days and was at

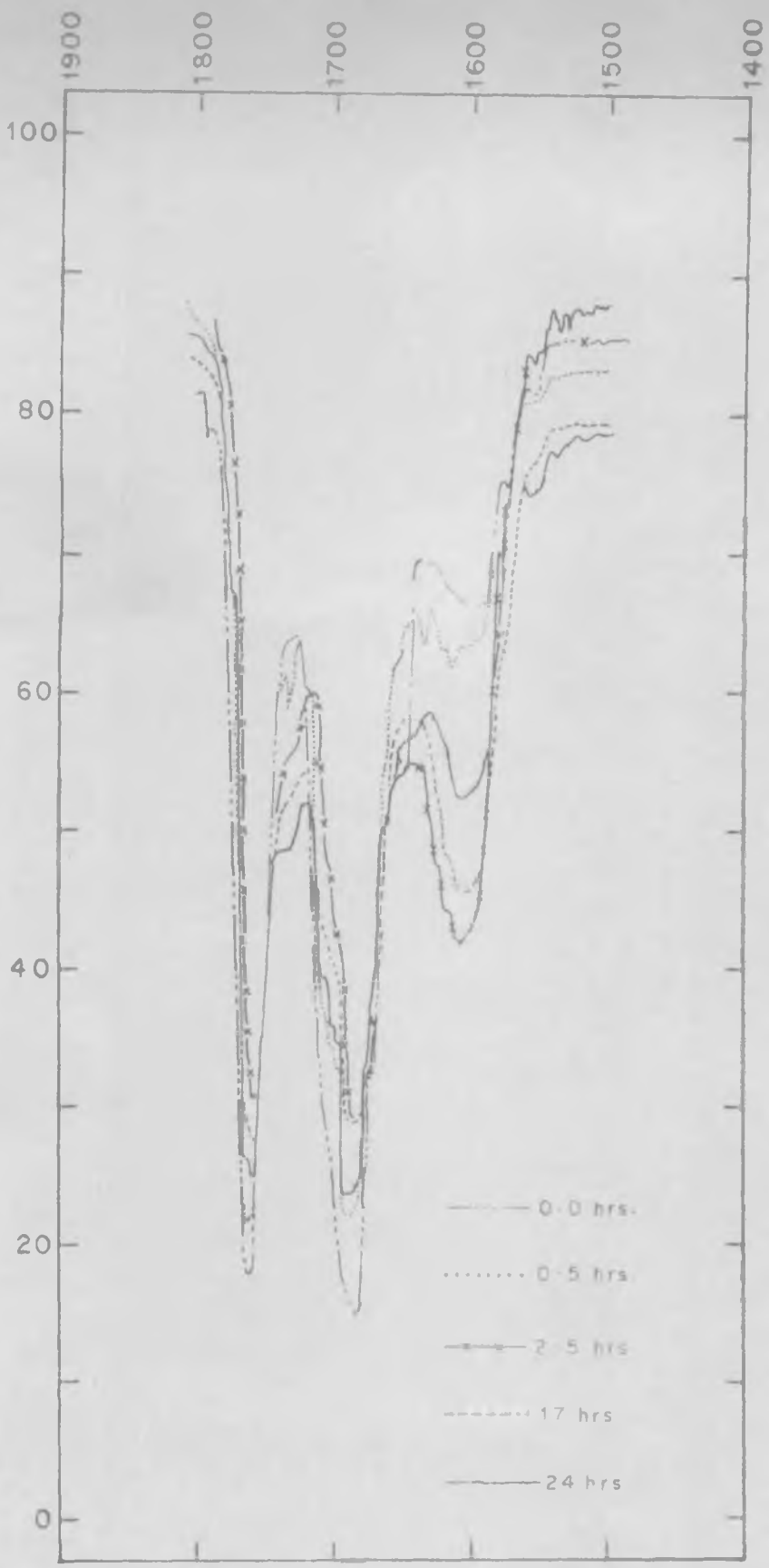



Fig. 8· Time dependence of the IR absorption of AA in dry DMSO

336 Hz integrating for almost 4 protons. Here the exchange averaged signal should be expected at 346 Hz. The observed hydroxyl absorption appeared to be close to the exchange averaged signal. No other important change could be recognized. It was therefore concluded that no major structural change had taken place. In any case, no cyclization was involved. The cyclic 3-O-methyl has been successfully isolated by a different method[44] and its PMR is discussed elsewhere in this thesis.

The 1-O-methyl hetero AA reported by Hirst et al. (m.p. 162°C) showed a very interesting change which could be attributed to a definite structural change. This was prepared in this laboratory by an earlier worker. The spectrum of this sample (m.p. 162°C) in dry DMSO is shown in Fig. 10. The hydroxyls gave two broad signals, one around 450.0 Hz due to chelated hydroxyl and the other centered around 289.0 Hz from the side chain hydroxyls. The $C_{(4)}$-proton signal was at 275.0 Hz and $C_{(5)}$- and $C_{(6)}$-protons absorbed around 210.0 Hz. The methoxyl absorption was at 244.5 Hz. But on standing in DMSO, the spectrum changed considerably. The hydroxyl signals broadened and $C_{(4)}$-proton gave two signals centered around 282.0 and 276.0 Hz respectively. The $C_{(5)}$- and $C_{(6)}$-protons gave broad signals around 221.0 Hz. The methoxyl absorption was split into two, one at 244.5 Hz and the other at about 192.0 Hz. After about 5 days, the spectrum indicated the completion of the

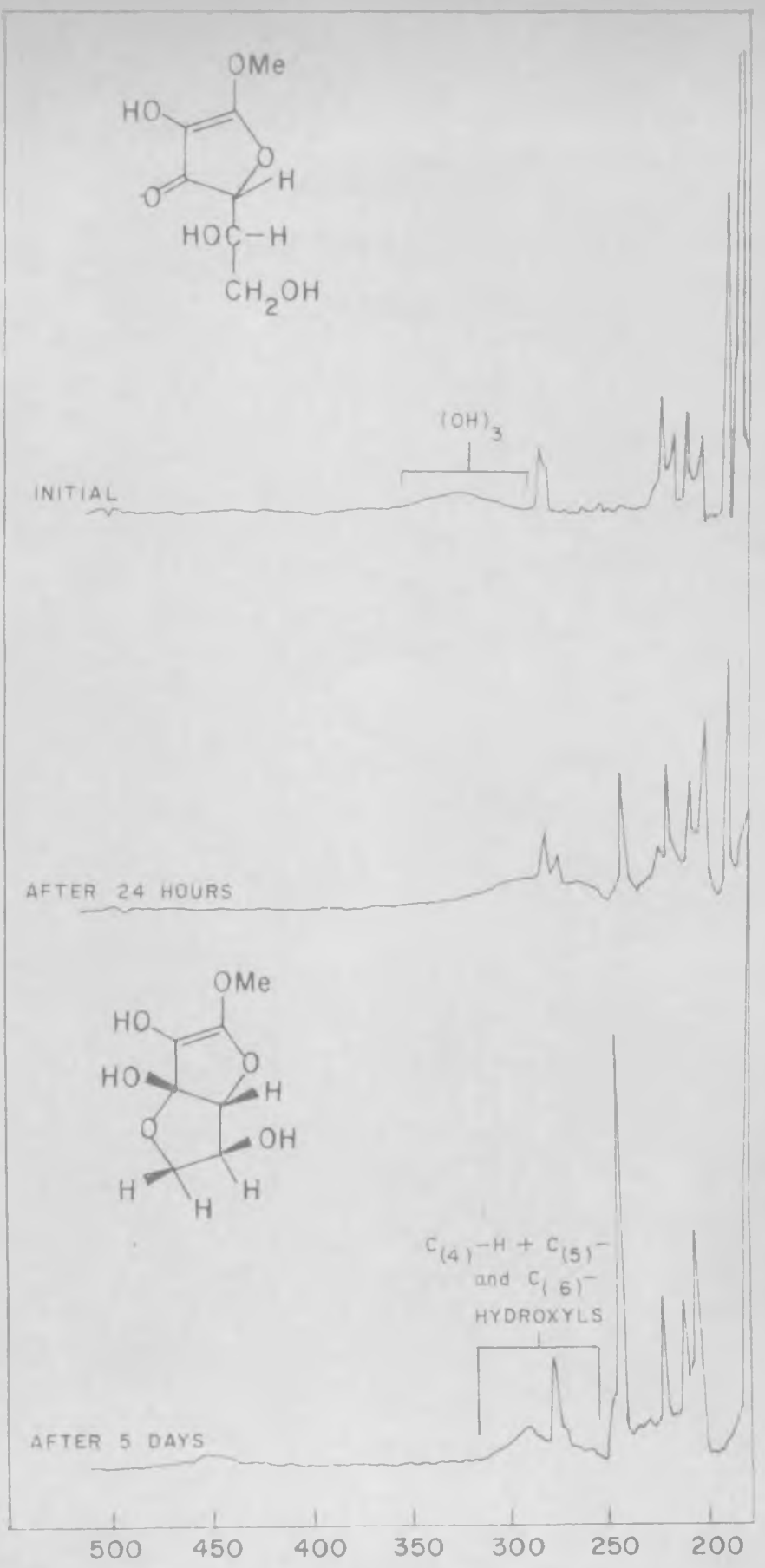
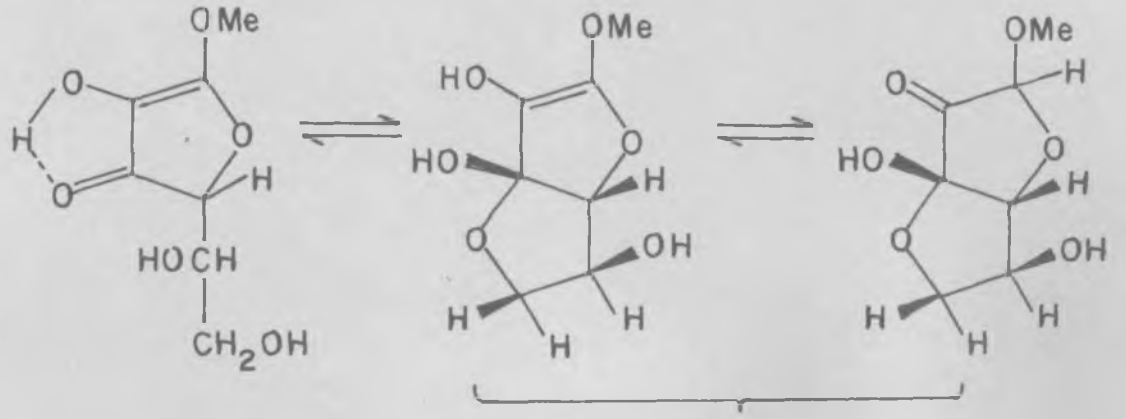



Fig. 10 NMR Spectra of 1-O-Methyl-hetero-Ascorbic acid (m.p.163°C) in DMSO

transformation. The only hydroxyl signal was a broad absorption, centered around 325.0 Hz indicating that all the hydroxyls had become exchangeable. The $C_{(4)}$-proton absorption was shifted down-field to 287.5 Hz. The $C_{(5)}$- and $C_{(6)}$-protons gave separate groups of signals at about 212.0 Hz and 224.0 Hz respectively. The initial methoxyl signal disappeared completely and the one obtained was upfield at 191.5 Hz. This spectrum did not change on further standing. The transformation responsible for the spectral changes was presumably the conversion of (XXVI) into (XXVII). The




XXVI XXVII

addition of the $C_{(6)}$-OH to the carbonyl at the 3-position was expected to be quite facile and susceptible to catalysis by the basic solvent. A simple exchange averaged signal would have appeared only at 342.0 Hz. The actual hydroxyl position eventually obtained was at higher field. The upfield shift of the methoxyl signal was readily attributed to the

destruction of the electron withdrawing carbonyl function.

However, the 1-O-methyl AA that was isolated by us corresponded to the one isolated by Reichstein[45]. Apparently, the formation of this isomeric derivative is not easily reproducible. The PMR spectrum of this in dry DMSO is shown in Fig. 11. The $C_{(4)}$-proton was a doublet at 274 Hz, the $C_{(5)}$ and $C_{(6)}$-protons were at 204 Hz and 210 Hz respectively. The O-Me was at 244.0 Hz. There was only one broad OH absorption which was centered at 290 Hz. This 290 Hz signal however broadened out within an hour. After about 12 hrs a new broad signal appeared at 284 Hz but no change was observed in the rest of the spectrum. On further standing the OH signal at 284 became more sharp and the rest of the spectrum remained unchanged. It appears that the difference between the two samples of 1-O-methyl-hetero-ascorbic acid is most likely to be that the present sample is a hydrated one, i.e. one where water has added to the 3-carbonyl function. The IR spectrum (Fig. 12) of the derivative showed no carbonyl absorption and seemed to support such an assignment. The UV spectra of both samples in water were identical and changed similarly on addition of alkali (i.e. the $\lambda_{max}$ was shifted to 325 nm from 281 nm). If the 3-carbonyl is hydrated, the side chain cannot of course, be involved in a cyclization. The spectrum of the 2,3-dimethyl ether of AA in DMSO seemed to show changes on long standing even in the $C_{(5)}$- and $C_{(6)}$-proton absorption region,

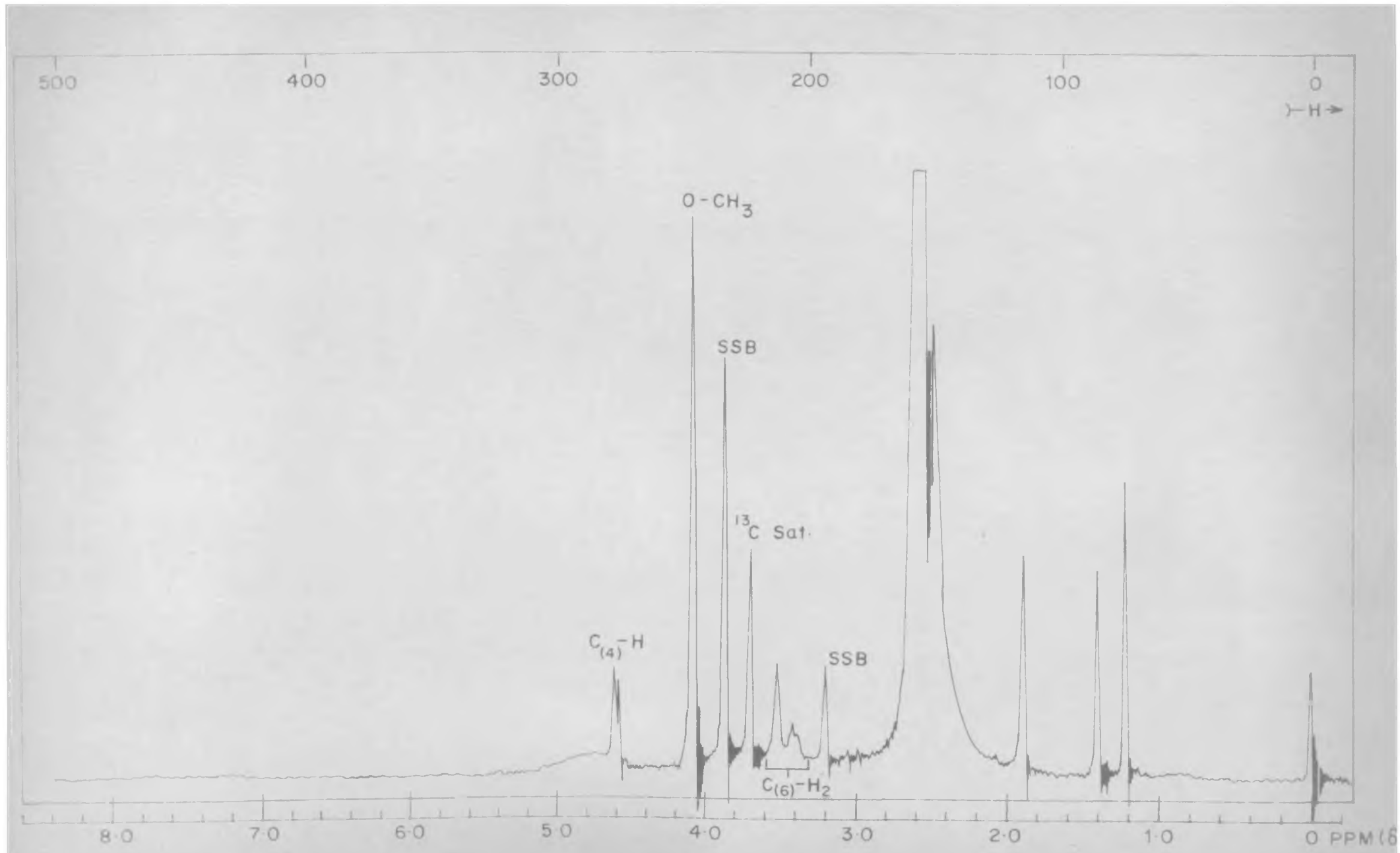



Fig. 11 60 MHz PMR Spectrum of 1-O- Methyl hetero AA (m.p. 152°C) in DMSO after standing for two days

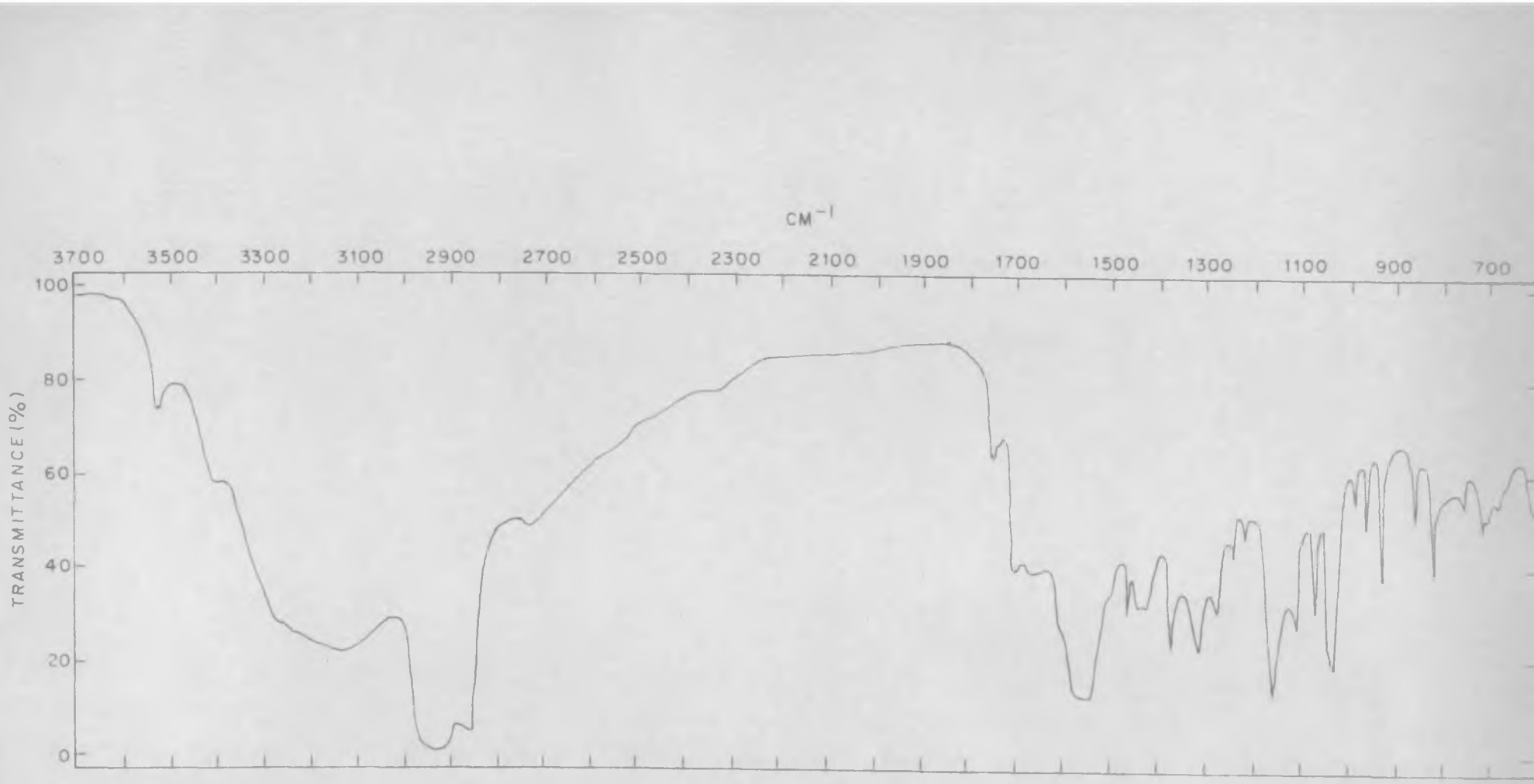
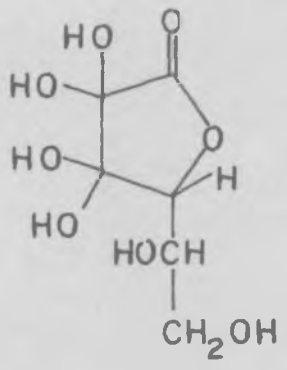



Fig. 12. IR Spectrum of 1-O-methyl hetero AA (m.p. 152 °C)

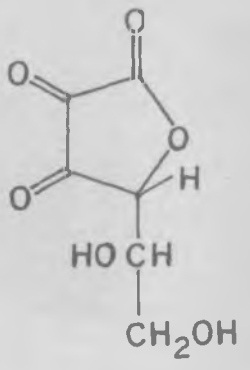
but their significance is not yet clear.

In connection with the question of the ease of cyclization of the ascorbic acid side chain when there is a carbonyl group at the 3-position, NMR studies on DHA are relevant. We have already seen that the stable dimeric form is of the 3,6-anhydro monomer[33]. This appears to have been obtained both by the warming of aqueous solutions of the stable dimeric form as well as by fresh oxidation of AA[33]. However, we have seen that there are very appreciable differences in the nature of the results obtained in three recent studies on freshly formed DHA[20,33,34].

Berger[20] has suggested that the product obtained on iodine oxidation of AA in aqueous solution is the dihydrate (XVI) of the open chain triketo form (VIII). On the basis



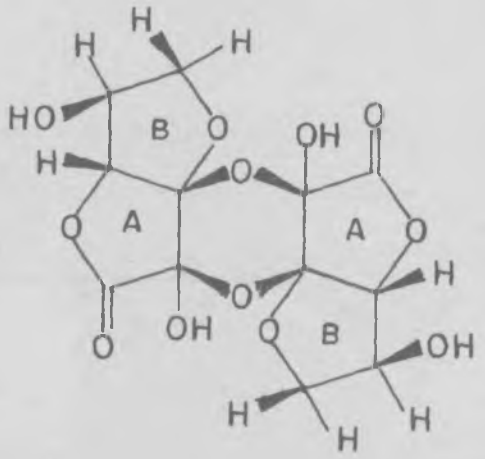

XVI



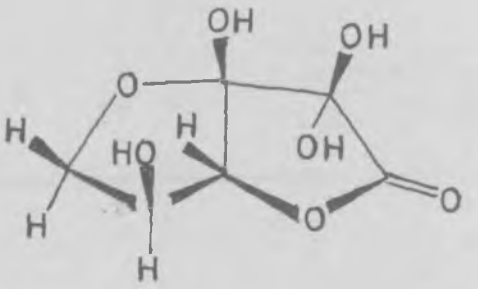

VII

of $^{13}C$ chemical shifts. He has compared these shifts with those obtained for an authentic sample of dimeric DHA (VIII) in $D_2O$. The shifts measured by him are shown in Table I

along with some measured in our study. Matusch[34] has, however, claimed that what is first obtained in $I_2$ oxidation is the dimer (VIII) of DHA and that this changes over to



VIII



IX

dihydrate of the open chain triketo form. What makes matters more complicated is the report of Pfeilsticker[33] and coworkers who prepared DHA samples by oxidation of AA with benzoquinone and $O_2$. In either case, the proton spectrum of the sample suggested a 3,6-anhydro structure with the 2-carbonyl in the hydrated form(IX).

In the present study one of the methods employed for making DHA in aqueous solution was by oxidation of AA with mercuric acetate which was extremely easy to do. Our $^{13}C$ shift data for AA and DHA are shown in Table I. We also prepared aqueous solution of pure DHA by the quantitative oxidation of AA with iodine in the presence of two mole equivalents of sodium bicarbonate. This gave a very good sample without any residual AA. Our data are consistent with a bicyclic

Table - I

| Sample, origin, solvent | $^{13}C$- chemical shifts | | | | | |
|---|---|---|---|---|---|---|
| | $C_1$ | $C_2$ | $C_3$ | $C_4$ | $C_5$ | $C_6$ |
| 1. AA (present study), $D_2O$ | 174.0 | 118.9 | 156.1 | 77.09 | 69.87 | 62.99 |
| 2. *AA (Taylor), $D_2O$ | 174.0 | 118.9 | 156.3 | 76.9 | 69.8 | 63.0 |
| 3. AA (Berger), $D_2O$ | 174.0 | 118.8 | 160.8 | 77,8 | 70.2 | 63.5 |
| 4. DHA (dimer. Berger) $D_2O$ | 174.2 | 92.0 | 106.3 | - | 88.2 | 76.7 |
| 5. DHA (Berger, $I_2$ oxidation), $D_2O$ | 172.0 | 95.6 | 97.4 | 75.2 | 68.7 | 63.4 |
| 6. DHA (present study, $I_2$ oxidation), $D_2O$ | 174.3 | 92.0 | 106.3 | 88.4 | 73.6 | 76.9 |
| 7. DHA (present study, Hg (II) acetate oxidation) $D_2O$ | 174.3 | 92.0 | 106.3 | 88.3 | 73.6 | 76.8 |

Reported shifts converted to $\delta$ ppm from TMS using $\delta_{CS_2}$ 193.7

3,6-anhydro structure for DHA with the 2-carbonyl in the hydrated form. In other words, our conclusions from $^{13}C$ shift data are in agreement with those of Pfeilsticker[33] and coworkers, obtained from PMR studies. The bicyclic nature of the species is indicated by the large shift suffered by $C_6$ in the conversion of AA to DHA. The proton decoupled $^{13}C$ NMR spectrum of a freshly prepared DHA in aqueous solution by iodine oxidation is shown in Fig. 13a. It is clear that there are no carbonyl groups at $C_{(2)}$ or $C_{(3)}$. There are also significant changes in shielding for $C_{(4)}$ and $C_{(5)}$. Our measured shifts are very close to those obtained by Berger for "dimeric DHA" except for $C_{(5)}$.

Dimeric DHA cannot be obtained in an aqueous oxidation of the type we have done. Since our sample is undoubtedly the bicyclic monomer, it is clear that Berger's values are for the same species which was apparently formed readily in aqueous solution from the dimer. We believe Berger's assignment for $C_{(5)}$ is erroneous. He does not cite a shift for $C_{(4)}$. Our assignments for $C_{(4)}$ and $C_{(5)}$ are based on the relative magnitudes of $^{13}C$-H coupling constants for the two cases. The difference could be made cut even from a $^{13}C$-spectrum with off-resonance broad band decoupling of protons, which is shown in Fig. 13b. The direct coupling is larger for $C_{(4)}$ since it is attached to a more electronegative oxygen. Our shifts are consistent with data for other bicyclic

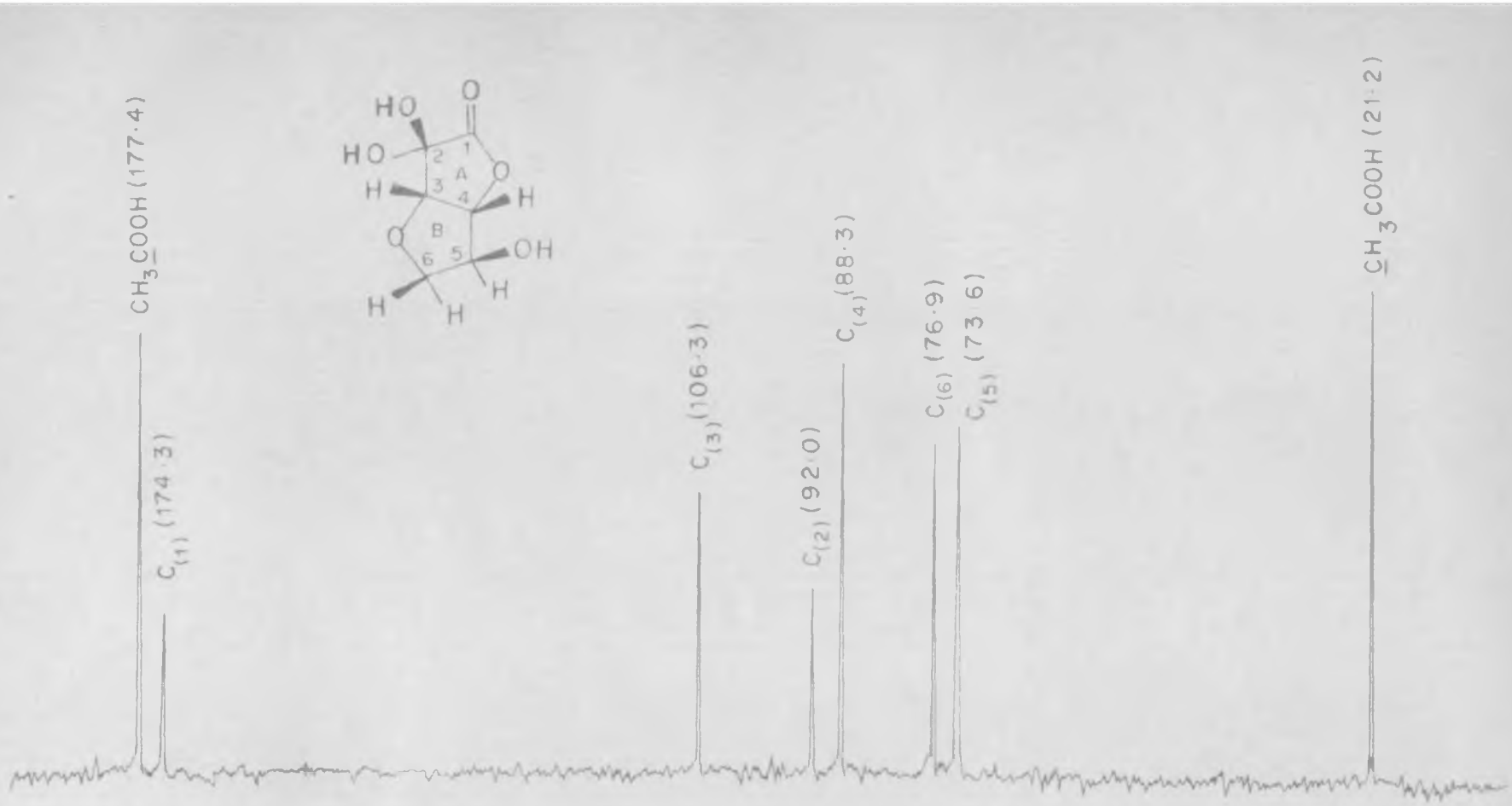



Fig. 13 a Proton decoupled 22·63 MHz $^{13}C$ NMR Spectrum of a freshly prepared DHA in $D_2O$

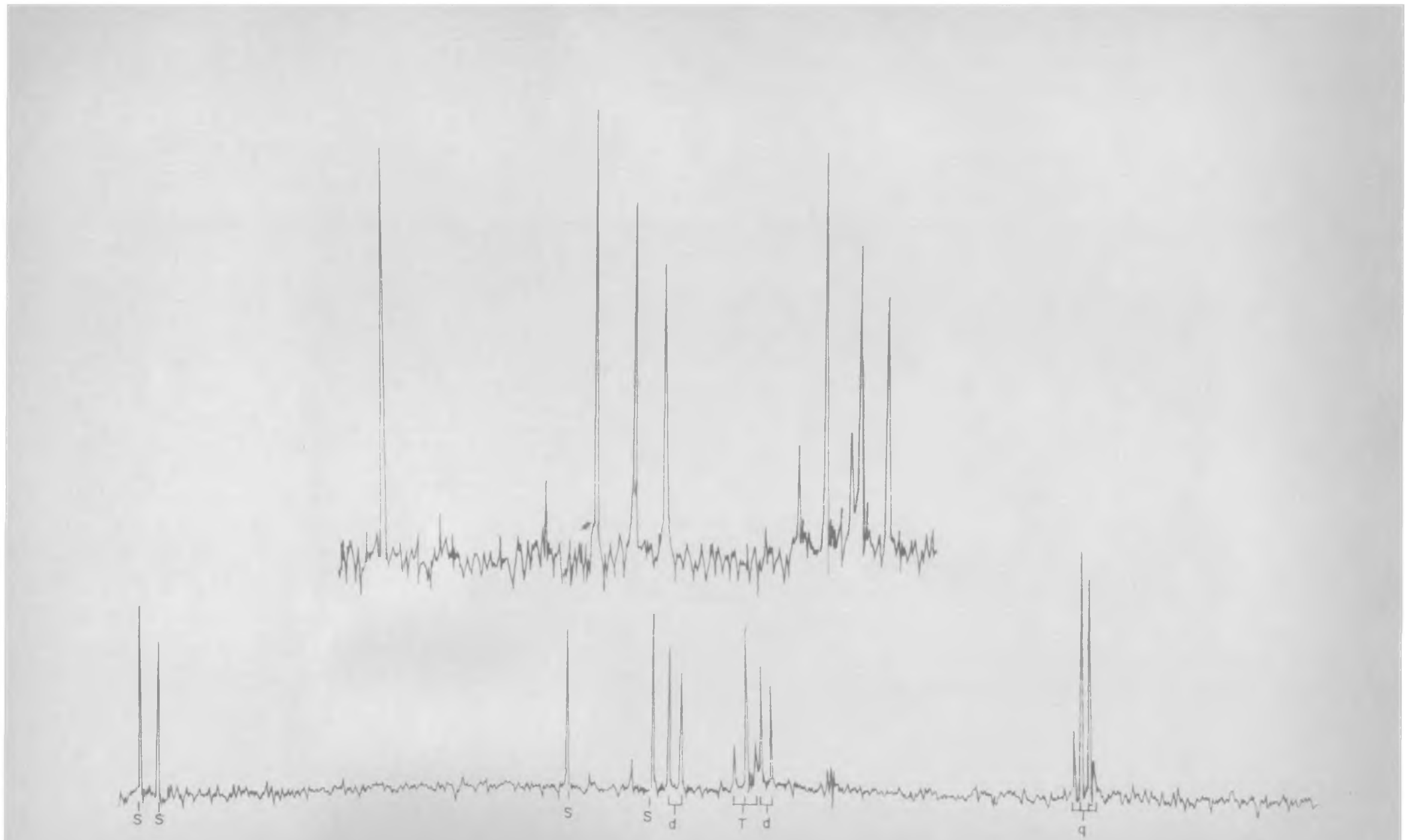



Fig. 13 b Off resonance $^{13}C$ Spectrum of DHA in $D_2O$

derivatives studied in this thesis and discussed elsewhere. The PMR spectrum of DHA prepared in aqueous solution by mercuric acetate oxidation is shown in Fig. 14. The change in the $C_{(6)}$-$H_2$ multiplicity pattern and the down field shift for $C_{(5)}$-H and $C_{(6)}$-$H_2$ observed is in agreement with the 3,6-anhydro structure. The $^{13}$C-NMR spectrum of the DHA prepared by mercuric acetate oxidation of a methanolic solution of AA is also in agreement with the 3,6-anhydro monomeric structure. DHA when generated in methanol apparently forms the methanol complex. The methanol addition at $C_{(2)}$ may be either from the α or from the β side. This is presumably the reason for the doubling of all the DHA carbon signals in the spectrum which is shown in Fig. 15. The two signals each for the acetate carbons are probably due to acetic acid and some methyl acetate that is presumably formed in the processing of the sample.

This conclusion is of relevance to the study of autoxidation of AA discussed elsewhere. It is not clear to us how Berger managed to get an open chain form of DHA by iodine oxidation. Details of his procedure are not available. Since the reaction is reversible, we had employed bicarbonate for neutralisation of the acid produced. The method of oxidation apparently affects the nature of the species obtained.

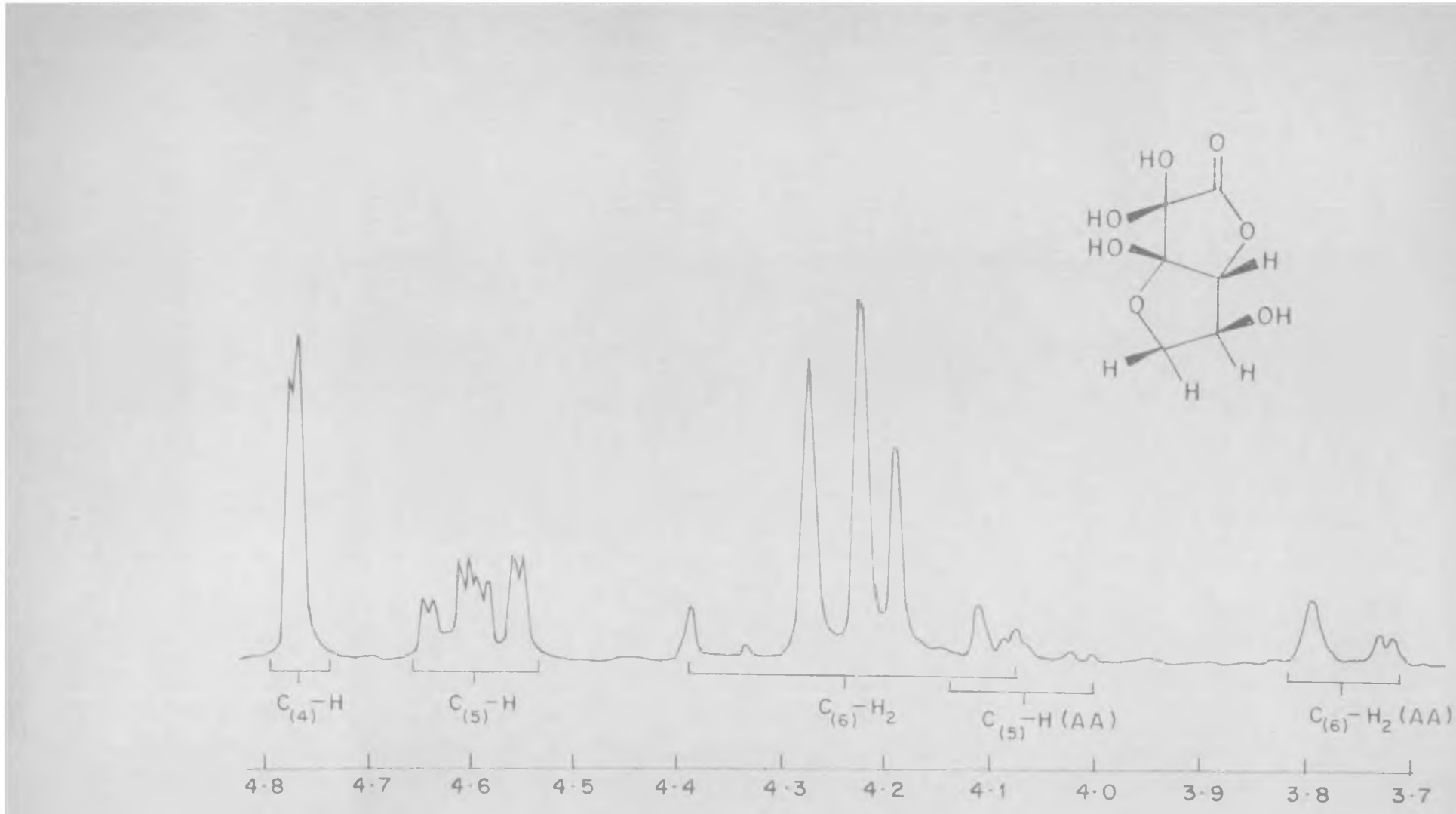



Fig. 14. 90 MHz PMR Spectrum of DHA in $D_2O$

Conclusions

With the help of the PMR spectra of AA and D-iso-AA, particularly the $J_{C_{(4)}-H,\ C_{(5)}-H}$ coupling constants, it has been possible to assign the conformation of the side chain at $C_{(4)}$. This is in agreement with the one made by Ogawa and coworkers[21].

It appears from the spectroscopic evidence that bicyclic form (XVII) is not populated significantly. There does not seem to be any evidence for the hetero form (IV) also in any sizeable amount. Since this species will be involved in rapid exchange with the normal form, the decision in this regard is more difficult. Apparently, as seen from UV spectral studies, evidence for a change in structure seems to appear only in very dilute solutions. These are concentration regions not suited for study by other spectroscopic techniques (NMR and IR). It looks as if the concentration of the type of species we are looking for in the case of AA are so low that their presence can be demonstrated only by reactivity studies.

Our studies on DHA have indicated that, irrespective of the nature of the oxidising agent, the DHA formed in aqueous solution exists in the monomeric 3,6-anhydro form. This structural assignment has been made on the basis of $^{13}C$ and $^{1}H$ spectra.

Experimental

The water used for all the UV experiments was prepared in the following manner. Double distilled deionised water was boiled for 15 minutes under a current of nitrogen to expell all the $O_2$ present in it. It was cooled under a current of $N_2$. Solutions of AA and the partially neutralised solutions were prepared in an atmosphere of nitrogen.

1. pH Dependence of the UV absorption

50.0 Mg of AA (AR grade recrystallised twice from double distilled water) was exactly weighed into a 100 ml volumetric flask. It was dissolved in water prepared as described above and made up to the mark.

4.0 Ml of this stock solution was treated with different amounts (5,10,15,25,50,75,100,200,400 molet%) of 0.1 N NaOH solution prepared in the purified oxygen free water and diluted to 10.0 ml. The UV spectra of these solutions were recorded on a Carl Zeiss "Specord" UV spectrophotometer using a quartz cell of 0.1 cm path length.

2. Concentration dependence of the UV absorption

Different volumes (3.0, 4.0, 6.0, 8.0 ml) of the AA stock solution were diluted to 10.0 ml and their UV spectra were recorded under the above mentioned conditions.

3. Decay of AA under very dilute conditions

The fall in the absorption intensity of the UV

absorption of AA at 1 mg/100 ml concentration was also studied. For this experiment, double distilled water was used with deoxygenation. The oxygen normally present in the water was found to be enough to bring about the oxidation of AA present in it. The UV spectra were recorded in a quartz cell of 1.0 cm path length.

4. pH Dependent $^{13}C$ NMR studies of AA

500 Mg of AA was dissolved in 3.0 ml of $D_2O$ and the $^{13}C$-NMR spectrum was recorded. A 5.0 mm coaxial tube containing dioxane in $D_2O$ was inserted in to the 10 mm tube containing AA solution. The $^{13}C$ chemical shifts of AA were recorded with respect to dioxane signal whose shift from TMS was taken as 67.4 ppm ( $_{TMS}$ - $_{dioxane}$ = 67.4 ppm). Dioxane served only as an intermediate reference and the calculated shifts were with respect to TMS. For measuring $^{13}C$ shifts at different pH's, 500 mg of AA was neutralised to 25, 50, 75 and 100% with 3N solution of NaOD and the total volume made up to 3.0 ml and the spectra were recorded as mentioned above.

5. $^{13}C$ NMR studies of AA in methanol at different temperatures.

200 Mg of AA was dissolved in 3.0 ml of dry methanol and the chemical shifts measured using a variable temperature probe. The changes in chemical shifts with temperature were negligible for all the carbons. Table II shows the

chemical shifts of AA in MeOH.

Table II

| No. | Compound/ solvent | $^{13}C$ chemical shifts | | | | | |
|---|---|---|---|---|---|---|---|
| | | $C_1$ | $C_2$ | $C_3$ | $C_4$ | $C_5$ | $C_6$ |
| 1. | AA/MeOH | 172.4 | 118.1 | 153.5 | 75.1 | 68.7 | 61.8 |
| 2. | AA/DMSO | 171.7 | 119.3 | 153.9 | 75.8 | 69.3 | 63.3 |
| 3. | D-iso-AA/MeOH | 172.4 | 119.3 | 153.9 | 77.7 | 71.9 | 62.7 |

The same observations were made even for D-iso-AA, which are also shown in Table II.

6. Timd dependent $^{13}C$ NMR studies of AA in dry DMSO

400 Mg of AA was dissolved in dry DMSO and the solution was taken into a 10.0 mm tube. All the operations were done in a dry box. The dioxane tube was inserted into this and was tightly sealed to prevent DMSO absorbing any moisture. The sample was scanned at different time intervals. There was absolutely no change in the chemical shifts of all six carbons with time. The $^{13}C$ shifts are shown in Table II.

7. Time dependent PMR studies of AA in dry DMSO

30.0 Mg of AA was dissolved in a 0.5 ml of dry DMSO. A drop of TMS in $CCl_4$ was added to this solution. The tube was sealed well and spectrum was recorded at various time

intervals. The same procedure was adopted even for 3-C-methyl and 1-O-methyl AA.

8. Time dependent IR studies of AA in DMSO

A 5% solution of AA was prepared in dry DMSO in a dry box. This solution was stored in a flask with a rubber septum and kept in $P_2O_5$ desiccator. At definite time intervals, samples of the solution were drawn with the help of a syringe and the IR spectra recorded using a NaCl cell of 0.025 mm thickness.

9. Preparation of DHA

(i) By mercuric acetate oxidation

AA (0.600 g) and mercuric acetate (1.0864 g) were weighed into a conical flask. 3.0 Ml of $D_2O$ was added and the solution stirred on a magnetic stirrer for about 15-20 minutes. It was filtered into the NMR sample tube using Whatman No.42 filter paper.

(ii) By iodine oxidation

AA (0.5 g) was dissolved in $D_2O$ (1.5 ml) to which $NaHCO_3$ (0.477 g) was added. $I_2$ (0.721 g) was dissolved in $D_2O$ (1.5 ml) containing 1.0 g of KI. This solution was slowly added to AA solution with stirring. This solution was then transferred to a NMR sample tube and the shifts recorded using dioxane as an intermediate standard.

(iii) By mercuric acetate in methanol

[illegible] (0.6 g) was dissolved in dry MeOH (20.0 ml). To this mercuric acetate (1.0864 g) was added with stirring. After the completion of the addition of mercuric acetate the solution was stirred for 1/2 hr more. The solution was then filtered through Whatman No.42 filter paper. The clear solution was concentrated under vacuum at 30°C to get a light yellow syrup. This syrup was dissolved in dry methanol and its spectrum recorded.

All the 60 MHz spectra discussed here were recorded on a T-60 Varian spectrometer. Internal TMS was used as a reference wherever feasible. For $D_2O$ spectra external TMS was employed as reference. All the 90 MHz proton spectra and $^{13}C$ spectra were recorded on a Bruker WH-90 spectrometer. For $^{13}C$ spectra, external dioxane in $D_2O$ was used as a standard. For low temperature spectra, external $CDCl_3$ was used as a carbon reference. The IR spectra were recorded as nujol mulls on a Perkin Elmer 221 spectrophotometer.

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# Chapter III

## KINETICS OF OXIDATION OF ASCORBIC ACID, ITS 5, 6 ACETONIDE AND D—ISO—ASCORBIC ACID

Introduction

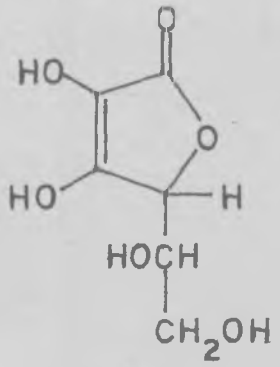
Ascorbic acid (AA) and dehydro-ascorbic acid (DHA) form a redox pair of considerable importance in biological chemistry.[1] The oxidation of AA by molecular oxygen catalyzed by metals has been the subject of numerous investigations and has been reviewed recently.[2] However, there have been, in comparison, very few studies of the uncatalyzed reaction which has been alternatively called 'spontaneous oxidation'.[3-9] The few studies that have appeared do not give a consistent picture of the reaction. Nevertheless, some of the reported findings are interesting.

It has been suggested that the reaction takes place in two stages.[10] The thermodynamics of the first stage have been said to be independent of pH while the activation parameters for the second were found to be pH dependent. At pH > 4.5, the reaction proceeded according to the kinetics of the second stage which was suggested to involve the formation of a complex between AA and its mono anion. Dissociation of AA has been suggested as the first stage. A study of the pH dependence of the oxidative decay of AA over the pH range, 3.5 to 7.2, at an ionic strength of 0.4 at 67°C indicated a rate maximum at a pH of 4.25 which is equal to the pKa of the acid.[11] This finding would imply that the rate determining step involves both AA and its anion. The occurence of a rate maximum at a pH of around 4 seems to be supported in another study.[12] There has been

a report to the effect that a rate maximum could be observed at a pH of 3.5 and a minimum at 7.[13] This study also showed that the reaction was inhibited by ethanol. In a more recent study of the spontaneous and metal catalyzed oxidations of AA, it has been shown that in the pH range 2 to 5.5, the species involved in oxidation is the mono anion.[9] The specific reaction rates were found to depend on pH. No rate maximum was observed in this study. The reaction appeared to be first order with respect to $O_2$ as well as the mono anion. The activation parameters for the reaction have been determined. The rate determining step of the reaction has been suggested to be a hydrogen atom transfer from the ascorbate to give an anion radical. It has not been made clear how the mechanism that has been suggested would account for the very large negative entropy of activation observed for the reaction. There have also been reports to the effect that AA undergoes oxidative decay only above a pH of 8.[4,6,7] Evidence obtained from studies for AA alone or in the presence of one of its stereoisomers seemed to show that the pseudo first order rate constants obtained are themselves dependent on the concentration of the reagent.[14] This study has also mentioned the possibility that the reaction involves a complex of AA with its anion.

Thus, the picture that emerges from these studies is a confusing one. It is not clear how different behaviours

can be obtained for something like pH dependence of the rate of oxidative decomposition. The existence of more than one path for the reaction and a lack of information as to which one prevails under what conditions can make the problem of analysis difficult. We have observed in an earlier chapter that there is evidence which points to the existence of ascorbic acid (I) in the tautomeric hetero form (II). It was also pointed out that if this is so, we must also consider the possibility of bicyclic forms (III) and (IV) for the compound in solution. We have already

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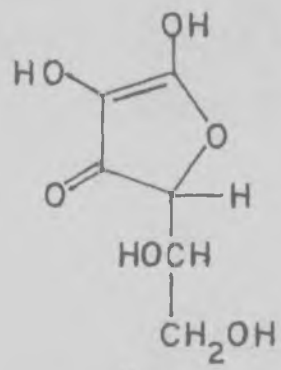


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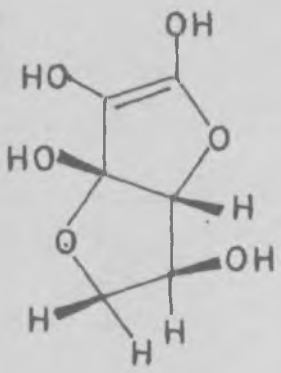
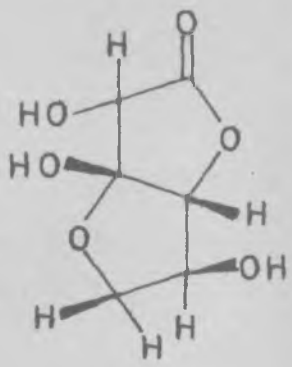



III




IV

dealt with the results obtained in attempts to study this question by spectroscopic means, and found that, although

the possibility that has been visualized cannot be discounted, the populations of possible tautomeric forms are not high enough for spectroscopic observation. However, the nature of the species in question, is such that it can have important implications for chemical reactivity. Form (III) should be highly reactive since it is an ene-diol with an extra oxygen substituent on the double bond and this bond is not deactivated by conjugation to a carbonyl group. The possibility that this might be the species involved in the 'spontaneous oxidation' had to be considered. The activation parameters reported by Khan and Martel[9] were a further pointer in the same direction. It was therefore decided that it would be useful to compare the 'spontaneous oxidation' rates of AA with those for its 5,6-acetonide and one of its stereo isomers, namely D-iso-ascorbic acid in some simple experiments. It was proposed to see how these changed with concentration and with progressive neutralization of the acid function. An attempt was also made to compare the reducing powers of these ene-diol systems in their reactions with some other reagents including $Fe^{+++}$ and $Cu^{++}$ ions.

## Materials, Procedures and Methods

### I. Materials

i) Ascorbic acid : GR grade sample of 99.9% purity (Sarabhai Merck) was recrystallized twice from double distilled water.

ii) Distilled water : Water obtained from a laboratory electric still had a pH of 5.4. This was distilled twice from an all glass assembly to get a water of pH 5.8. This was then passed through a bed of mixed ion exchange resins in a column. The first 10 litres of water was discarded. About 50 litres of water was collected in a clean glass vessel which was cleaned with chromic acid and washed well with water and finally again several times with double distilled deionised water. This water was boiled for 15 minutes to expell any $CO_2$ that might be present in it, cooled under a alkali guard tube and then stoppered well with a ground glass stopper. While drawing water for each experiment an alkali guard tube was connected to prevent $CO_2$ absorption.

iii) Air : Air which was to be passed into the solution for bringing about AA oxidation was first passed through a strong (50%) solution of sodium hydroxide and then through distilled water.

iv) AA acetonide and benzoate were prepared according to procedures described in chapter VI.

v) Fehling's reagent[16] Fehling solution A was prepared in the usual manner and B with 1/3rd amount of alkali recommended.

vi) Standard $Fe^{2+}$ solution: A 0.001 M solution of ferrous ammonium sulfate was prepared in double distilled water.

vii) Ammonium citrate: A 5% solution was prepared in double distilled water.

viii) AA solution: A 10% aqueous solution was used.

ix) o-Phenonthroline solution: A 0.25% solution of the material in double distilled water was used.

x) 0.1 M Phosphate buffer of pH 6.8: 1 M Stock solutions of $K_2HPO_4$ and $KH_2PO_4$ were mixed in the appropriate proportion to get pH 6.8 and diluted with double distilled water to get 0.1 M solution.

II. Procedures

A. Oxidation with molecular oxygen

All the glass vessels used were washed first with chromic acid, then with water and finally with double distilled deionised water.

The decay of AA was followed by measuring the residual AA by titrating an aliquot of the solution against 0.001 N iodine solution using starch as indicator. The iodine solution was prepared by diluting the required amount of standard iodine solution of 0.1 N strength with double distilled water. The exact normality of 0.1 N iodine solution was determined by titrating it against standard sodium thiosulfate solution. The more dilute

solutions required larger amounts of KI to keep the iodine vapour pressure above the solutions low enough.

Actual measurements of oxidative decay were done in the following manner. 100 Mg of AA was accurately weighed and was dissolved in 100 ml of stock water maintained at 30°C. An aliquot of this solution containing the required amount of AA was pipetted out into a clean 250 ml volumetric flask and was diluted upto the mark with stock water at 30°C. For making partially or completely neutralised (25%, 50% ... 100%) solutions, the required amount of 0.01 N KOH was added and the solution diluted to 250 ml. 10 Ml of the diluted solution was titrated against standard $I_2$ solution. The remaining solution was then transferred to the reaction vessel and the air current was started at the rate of 90 to 100 cc/min. The typical experimental set up is shown in Fig. 1.

An aliquot of solution was withdrawn after every 10 minutes and titrated against standard $I_2$ solution. The percentage of unoxidised AA was plotted against time (t) of reaction to get the decay rate curves. The initial slopes of log [AA] vs t plots were multiplied by a factor 2.303 to get the "specific" reaction rates. Only the slope of the initial 40 minute period was taken into consideration. Each set of experiments were repeated thrice to check the reproducibility.

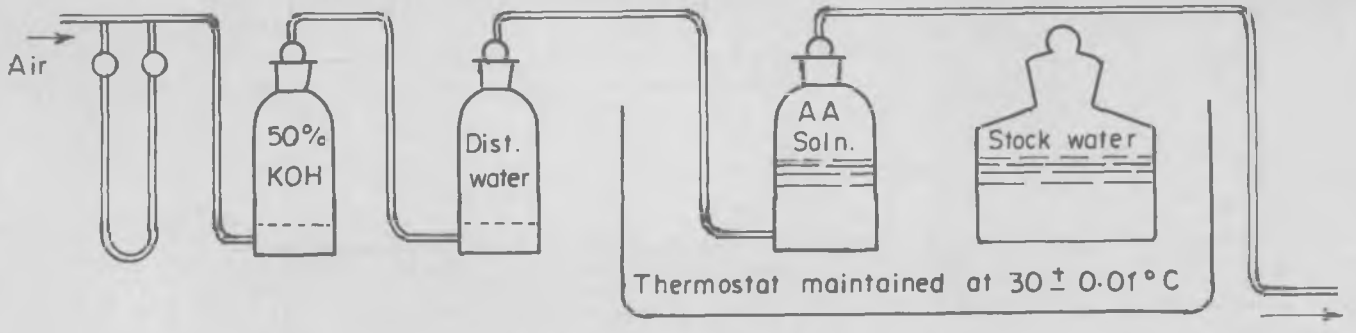



FIG. 1.

III. Methods

A. Oxidation of AA by air

The procedure employed for the preparation of the water used in this study ensured the exclusion of trace amounts of metals which can affect the rate of reaction of ascorbic acid with oxygen. The oxidative decay rates in ordinary distilled water were found to be much higher than the ones obtained in double distilled deionised water. Since we were working at very low concentrations where AA decay was very sensitive, the quality of water and air used had to be very consistent. About 50 litres of water was prepared by the procedure described and the entire study was done using only this stock water.

Initial oxidations done by drawing air directly into the AA solutions gave erratic results. The composition of laboratory air was not the same all the time, and also the $CO_2$ present in air was likely to affect the pH of the solution. So, to avoid any uncertainty, a purified stream of air was used as described earlier. Passage of air through strong alkali solution made it free of $CO_2$ and other acidic vapours and the subsequent passage through distilled water made it free of any alkali spray and got it saturated with water vapour . Results obtained with this kind of purification did show good reproducibility.

Preliminary experiments indicated that the more interesting of the observations to be made were in the very dilute region (for instance, 5 to 50 mg per 100 ml of water) in case of Aa. It was also in such extremely dilute solutions that the anomalous disappearance of UV absorption of ascorbic acid in conductivity water had been studied[15]. In connection with identification of the nature of the reactive species involved in the autoxidation reaction, there was need to do it under varying pH conditions. Instead of employing buffer systems to maintain constant pH, it was considered more important to maintain purity of the reacting system and the alternative course of working with different proportions of the acidic ene-diols and their potassium salts was chosen. These mixtures were obtained by partial neutralisation (25, 50 and 75%) and the pure salts by full neutralisation of the acid forms.

The decay rates were obtained under the constant oxygen concentration that could be obtained by the air saturation method. From the decay curves obtained, initial rates were calculated so that the values would correspond to the initial pH conditions. From these initial rates, it would, of course, be more meaningful to calculate a "specific rate" in the case of the experiments with pure acids or the pure salts, on the assumption of a given order of involvement of either species. Since the exact mechanism of the oxidation appears

uncertain in the context of the conflicting reports mentioned, an empirical approach was adopted here for the analysis of rate data. When kinetics is of a clean type, the implications are usually quite straight forward. But when it is not so, a step-wise approach has to be adopted to bring out the features of the reaction. For the experiments with pure acids and their salts, some provisional "specific rate" constants have been calculated on the basis of an assumed unimolecularity with respect to the species involved since the rates appear to depend on their concentrations. These so-called "specific rates" are in most cases not true velocity constants. They are, however, useful for discussion purposes. In all cases, only the change in total ene-diol content, i.e. the sum total of the acid and the salt, has been followed. For experiments with mixtures of acids and salts, it is therefore much less justifiable to talk of "specific rates" although these numbers can provide some useful information. As will be seen shortly, true unimolecularity has been seen only for the acetonide salt.

Residual AA or analogous ene-diol was followed by titration of an aliquot against standard $I_2$ solution. As a verification for the correctness of this procedure, a reaction employing a concentration of 5.0 mg/100 ml was followed by UV spectrometry also, where an aliquot was suitably diluted and the absorption intensity immediately measured. The agreement was satisfactory. The decay rate

at the concentration of 1 mg/100 ml was, however, followed only by UV spectrophotometry.

"Specific reaction rates" as qualified earlier were measured from the initial slopes of each decay curve (first 40 minutes only). Since the reactions involved are known to become complex in extended reactions, the portions of the curves for the later periods were neglected.

In the case of the acetonide, the oxidation experiments were restricted to the sodium salt, in view of the hydrolysis of the acid form under autocatalysis. The behaviour of AA salt under comparable conditions was also studied.

The reproducibility in the experiments with acid forms were excellent. In the experiments at higher pH, the reproducibility was better for the more concentrated solutions. For reaction periods away from the initial, the reproducibility problem became often quite bad. But, these are sections which were not made use of since only initial rates have been considered for discussion purposes.

The reactions were followed by estimation of total ene-diols (acid and salt forms) at various time intervals. In almost all experiments sample were withdrawn at the same set of intervals, namely, 0, 10, 20, 30, 40, 50,60,70,80 and 90 minutes. The estimated percentages at these intervals are given below for the various experiments along with other particulars. In a few cases where the

intervals mentioned above were not adhered to, the time of reaction is indicated in brackets.

1) 1 mg of AA/100 ml, neutralisation nil.

   100, 94.30(8), 90.40(15), 81.3(27), 712(40), 1.92(110).

2) 5 mg of AA/100 ml, neutralisation nil.

   100, 98.33, 96.66, 94.16, 91.66, 89.16, 86.66, 82.5, 80.00, 76.66.

3) 5 mg of AA/100 ml, 25% neutralisation.

   100, 96.66, 93.33, 90.83, 87.75, 83.33, 80.83, 78.33, 75.00, 71.66.

4) 5 mg of AA/100 ml, 50% neutralisation.

   100, 95.83, 92.5, 89.16, 85.83, 82.50, 80.00, 77.50, 73.33, 70.83.

5) 5 mg of AA/100 ml, 75% neutralisation.

   100, 95.00, 91.66, 87.50, 84.16, 81.66, 78.33, 75.00, 73.33, 70.83.

6) 5 mg of AA/100 ml, 100% neutralisation.

   100, 90.83, 85.83, 81.66, 79.16, 76.66, 73.33, 71.66, 70.83, 70.00.

7) 10 mg of AA/100 ml, neutralisation nil.

   100, 98.34, 97.52, 96.64, 94.62(45), 92.97(55), 90.90(65), 89.25(75), 86.77(85).

8) 10 mg of AA/100 ml, 25% neutralisation.

   100, 97.93, 96.69, 95.86, 94.21, 92.56, 90.90, 89.25, 87.60, 85.95.

9) 10 mg of AA/100 ml, 50% neutralisation.

   100, 97.52, 96.28, 95.45, 93.38, 91.73, 90.08, 88.42, 86.77, 85.95.

10) 10 mg of AA/100 ml, 75% neutralisation.

100, 96.69, 95.45, 93.80, 92.97, 90.90, 89.66, 88.42, 86.77, 86.77.

11) 10 mg of AA/100 ml, 100% neutralisation.

100, 95.86, 92.97, 90.90, 89.66, 88.42, 87.60, 86.77,85.95.

12) 20 mg of AA/100 ml, neutralisation nil.

100, 99.37, 98.54, 97.70, 97.08, 96.66, 95.83, 95.00, 93.75, 92.91.

13) 20 mg of AA/100 ml, 25% neutralisation

100, 98.75, 97.91, 97.08, 96.25, 95.41, 94.58, 93.75, 92.91, 92.08.

14) 20 mg of AA/100 ml, 50% neutralisation.

100, 98.75, 97.71, 96.88, 96.05, 95.02, 94.19, 92.94, 92.11, 91.28,

15) 20 mg of AA/100 ml, 75% neutralisation.

100, 98.54, 97.70, 96.87, 95.62, 94.58, 93.33, 92.70, 91.87, 90.83.

16) 20 mg of AA/100 ml, 100% neutralisation.

100, 98.33, 97.05, 96.25, 95.41, 94.58, 93.33, 92.50, 91.66, 90.83.

17) 50 mg of AA/100 ml, neutralisation nil.

100, 99.66, 99.49, 99.32, 99.15, 98.82, 98.65, 98.48, 98.31, 97.97.

18) 50 mg of AA/100 ml, 25% neutralisation.

100, 99.66, 99.32, 98.98, 98.31(45), 97.97(55), 97.81(65), 97.47(75), 97.13(85).

19) 50 mg of AA/100 ml, 50% neutralisation.

100, 99.32, 98.82, 98.49, 97.98, 97.57, 97.31, 96.81, 96.48, 96.14.

20) 50 mg of AA/100 ml, 75% neutralisation.

100, 99.32, 98.48(25), 97.89(35), 97.30(45), 96.46(55), 95.95(65), 95.62(75), 93.93(105).

21) 50 mg of AA/100 ml, 100% neutralisation.

100, 98.81, 97.97, 97.47, 96.79, 96.20, 95.78, 95.19, 94.60, 94.09.

22) 10 mg of D-iso-AA/100 ml, neutralisation nil.

100, 99.57, 99.15, 99.15, 98.31, 97.47, 96.63, 95.79, 94.95, 93.27.

23) 10 mg of D-iso-AA/100 ml, 25% neutralisation.

100, 98.36, 97.54, 96.72, 95.90, 95.08, 94.26, 92.62, 91.80, 90.98.

24) 10 mg of D-iso-AA/100 ml, 50% neutralisation.

100, 98.36, 96.72, 95.08, 94.26, 93.44, 92.21, 91.39, 90.98, 90.16.

25) 10 mg of D-iso-AA/100 ml, 75% neutralisation

100, 98.37, 96.74, 95.93, 94.30, 92.68(55), 91.46(65), 90.24(75), 89.02(85).

26) 10 mg of D-iso-AA/100 ml, 100% neutralisation.

100, 97.15, 95.93, 95.12, 94.30, 93.49, 92.68, 91.05, 89.43, 89.43.

27) 50 mg of D-iso-AA/100 ml, neutralisation nil.

100, 99.83, 99.66, 99.41(35), 99.16(45), 98.82(55), 98.65(65), 98.32(75), 98.23(85).

28) 50 mg of D-iso-AA/100 ml, 25% neutralisation.

100, 99.57, 99.24, 98.82, 98.48, 98.15, 97.81, 97.31, 96.80, 96.30.

29) 50 mg of D-iso-AA/100 ml, 50% neutralisation.

100, 99.49, 99.15, 98.82, 98.14, 97.81, 97.30, 96.80, 96.46.

30) 50 mg of D-iso-AA/100 ml, 75% neutralisation.

100, 99.32, 98.64, 98.30, 97.79, 97.13, 96.43(65), 95.92, 95.58.

31) 50 mg of D-iso-AA/100 ml, 100% neutralisation.

100, 99.15, 98.48, 97.81, 97.22, 96.63, 96.04, 95.70, 95.03, 94.36.

32) 20 mg of D-iso-AA/100 ml, neutralisation nil.

100, 99.79,99.16(25), 98.75(35), 97.50(55), 96.66(65), 96.25(75), 95.62(85), 95.20(95).

33) 5,6-Acetonide equivalent to 5 mg of AA/100 ml, 100% neutralisation.

100, 100, 99.19, 98.38, 96.77, 96.77, 96.77, 95.96, 95.16.

34) 5,6-Acetonide equivalent to 20 mg of AA/100 ml, 100% neutralisation.

100. 99.59, 98.97, 98.36, 98.15, 97.54, 96.92, 96.31, 95.90(85).

35) 5,6-Acetonnide equivalent to 50 mg of AA/100 ml, 100% neutralisation.

100, 99.83, 99.57, 98.98, 98.30(45), 98.05(55), 97.28(65), 96.44(80), 95.42(90).

B. Reducibility of $Cu^{++}$ to Cu

Fehling's solutions A and B were mixed in equal proportions just before use. 20 mg of AA, D-iso-AA and equivalent amounts of acetonide and benzoate (i.e. 24.5 mg and 31.8 mg respectively) were dissolved separately in 5.0 ml of

0.1 M phosphate buffer of pH 6.8. To these solutions 0.74 ml portions of the mixture of Fehling's reagent were added. The changes in colour and the nature of the precipitates formed were noted as a function of reaction time for the three cases.

C. Reducibility of $Fe^{+++}$ to $Fe^{++}$

The reductions of $Fe^{+++}$ to $Fe^{++}$ were followed by the complexation of the latter with o-phenanthroline and spectrophotometric measurement of the blood red colour of the complex formed. In the context of our interest in Udenfriend hydroxylations, the reductions were done in 6.8 pH phosphate buffer of 0.1 M strength which was the medium used in these hydroxylations. Moreover, in order to get meaningful results for the acetonide, there was need to work under pH conditions where it would be stable to hydrolysis.

The calibration curve to measure the amount of $Fe^{++}$ formed was prepared in the following manner. To an aliquot of standard solution ferrous ammonium sulfate solution was added 4.0 ml of 10% solution of AA, 4.0 ml of o-phenanthroline and 1.0 ml of ammonium citrate solution. This mixture was diluted to 50.0 ml with 0.1 M, 6.8 pH phosphate buffer. The red colour developed was measured at 512 nm. The instrument was set to zero with the help of a blank solution, i.e. the solution of all the above said components except the standard $Fe^{++}$ solution. The calibration data are presented in Table I.

Table I

Comparison of reaction mixtures

| No. | Solution of ferrous ammonium sulfate ml | $NH_4$ citrate solution ml | AA solution ml | o-phenanthroline solution ml | Total volume made up with buffer ml | O.D. |
|---|---|---|---|---|---|---|
| 1. | 0.0 | 1.0 | 4.0 | 4.0 | 50.0 | 0.0 |
| 2. | 0.20 | 1.0 | 4.0 | 4.0 | 50.0 | 0.212 |
| 3. | 0.40 | 1.0 | 4.0 | 4.0 | 50.0 | 0.440 |
| 4. | 0.60 | 1.0 | 4.0 | 4.0 | 50.0 | 0.640 |
| 5. | 0.80 | 1.0 | 4.0 | 4.0 | 50.0 | 0.865 |
| 6. | 1.00 | 1.0 | 4.0 | 4.0 | 50.0 | 1.07 |
| 7. | 1.20 | 1.0 | 4.0 | 4.0 | 50.0 | 1.29 |

The ammonium citrate used served to keep all $Fe^{+++}$ present in solution, which otherwise had a tendency to precipitate from the 6.8 pH buffer. For uniformity, 1.0 ml of $NH_4$ citrate solution was added to all the solutions of the calibration set.

The reduction of the $Fe^{+++}$ by ene-diol was measured in the following manner. Into a 50 ml volumetric flask was added 4.0 ml of ammonium citrate solution, 4.0 ml of o-phenanthroline solution and a weighed amount of the ene-diol whose reducing power was to be measured. Equivalent amounts of ene-diols were used. The contents were then diluted to

49.0 ml with buffer. 0.25 Ml of standard ferric ammonium sulfate (1 ml containing $55.85 \times 10^{-5}$ gms of $Fe^{3+}$) was added and the contents were shaken thoroughly. Optical density was recorded soon thereafter. The change in the optical density was recorded with time.

Table II

Optical density with time

| Compound | 1.0 min | 5.0 min | 10 min | 20 min | 50 min |
|---|---|---|---|---|---|
| AA (125 mg) | 0.315 | 0.340 | 0.371 | 0.420 | 0.428 |
| Acetonide (153.37 mg) | 0.320 | 0.345 | 0.383 | 0.440 | 0.455 |
| Benzoate (198.7 mg) | 0.498 | 0.518 | 0.541 | 0.595 | 0.605 |

The high optical density observed in the cases of benzoate and acetonide did not appear to be entirely due to the ferrous complex. Even if all the $Fe^{3+}$ added were to completely get reduced to $Fe^{2+}$, the expected optical density was 0.53. However, in the case of the benzoate, it was 0.605. The presence of a masking colour was thus obvious. The colours of solutions of the acetonide and the benzoate even before addition of $Fe^{++}$ were not satisfactory. The nature of the interference observed could not be made out. Although these experiments did not serve the intended purpose, the experience has been related here for purposes of record.

Results and discussion

The variation of oxidative decay rates of AA as a function of concentration in water, kept saturated with clean scrubbled air, is presented in Fig.2. The procedure employed ensures constancy of oxygen concentration. The initial decay was of the first order with respect to concentration. Fig. 2 also shows that, at higher concentration, the decay curves are almost straight lines, and, as one goes to lower concentrations, they take the shape of curves. The reproducibility of the decay curves was good only at the higher concentrations. At the lower concentrations, there was more of erratic behaviour. Fig. 2 also shows that the decay rate increases with increasing dilution. Since the first product of the oxidation is known to undergo further degradative transformations, the kinetic behaviour observed was bound to get more complex as the reaction time increased. So, the first order constants or rather "specific rates", were obtained from the initial decay rates. These were themselves found to be dependent on the concentration of substrate employed. Table III gives the variation of specific rates with changing concentration of AA, which is also depicted in Fig.3. The curve has roughly the shape of a hyperbola. It is clear from Fig. 3 that the decay rates rise very steeply at low concentrations and change very slowly at high concentrations and it might be recalled here that a concentration dependence of "specific" reaction rates has been indicated

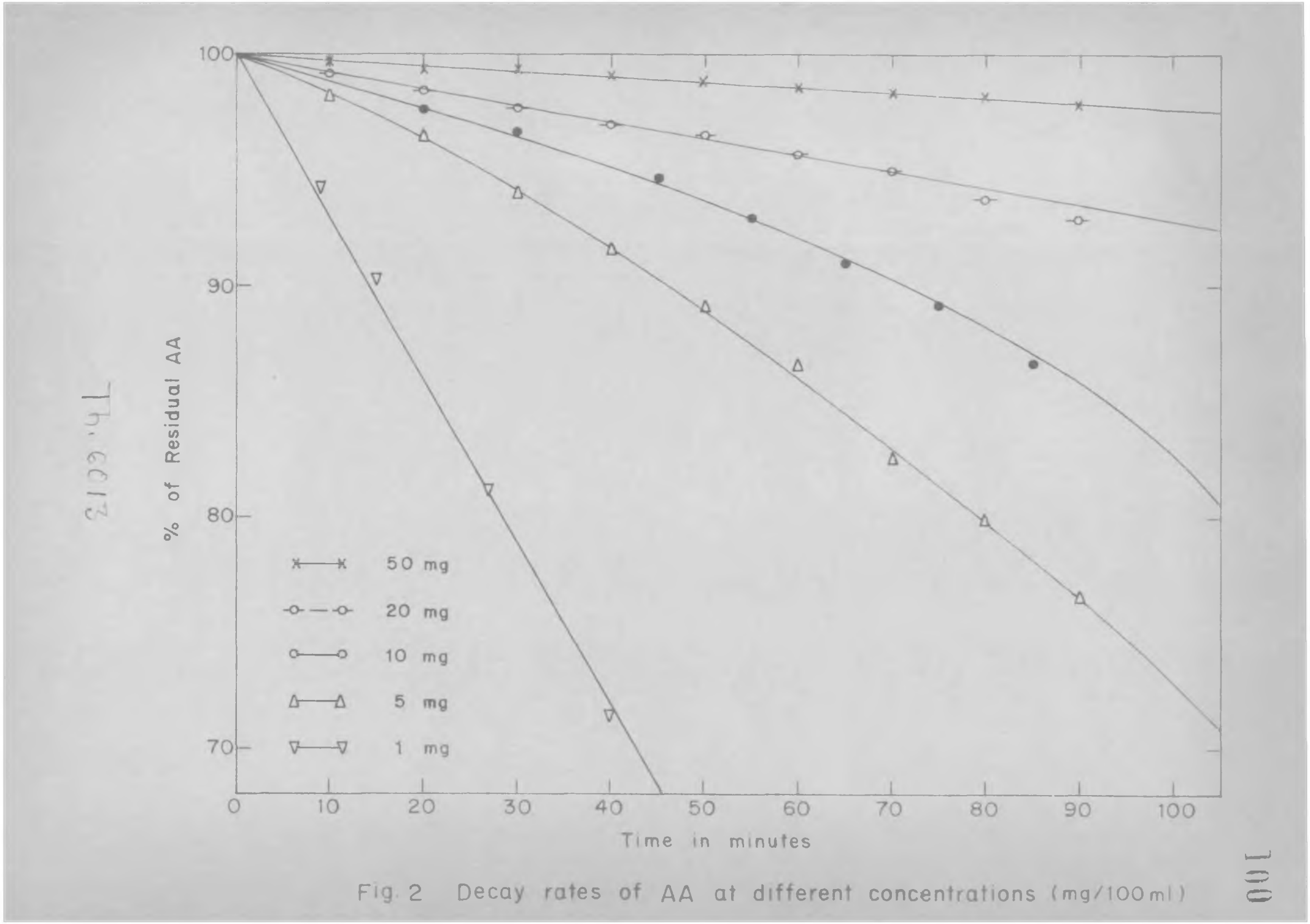



Fig. 2 Decay rates of AA at different concentrations (mg/100 ml)

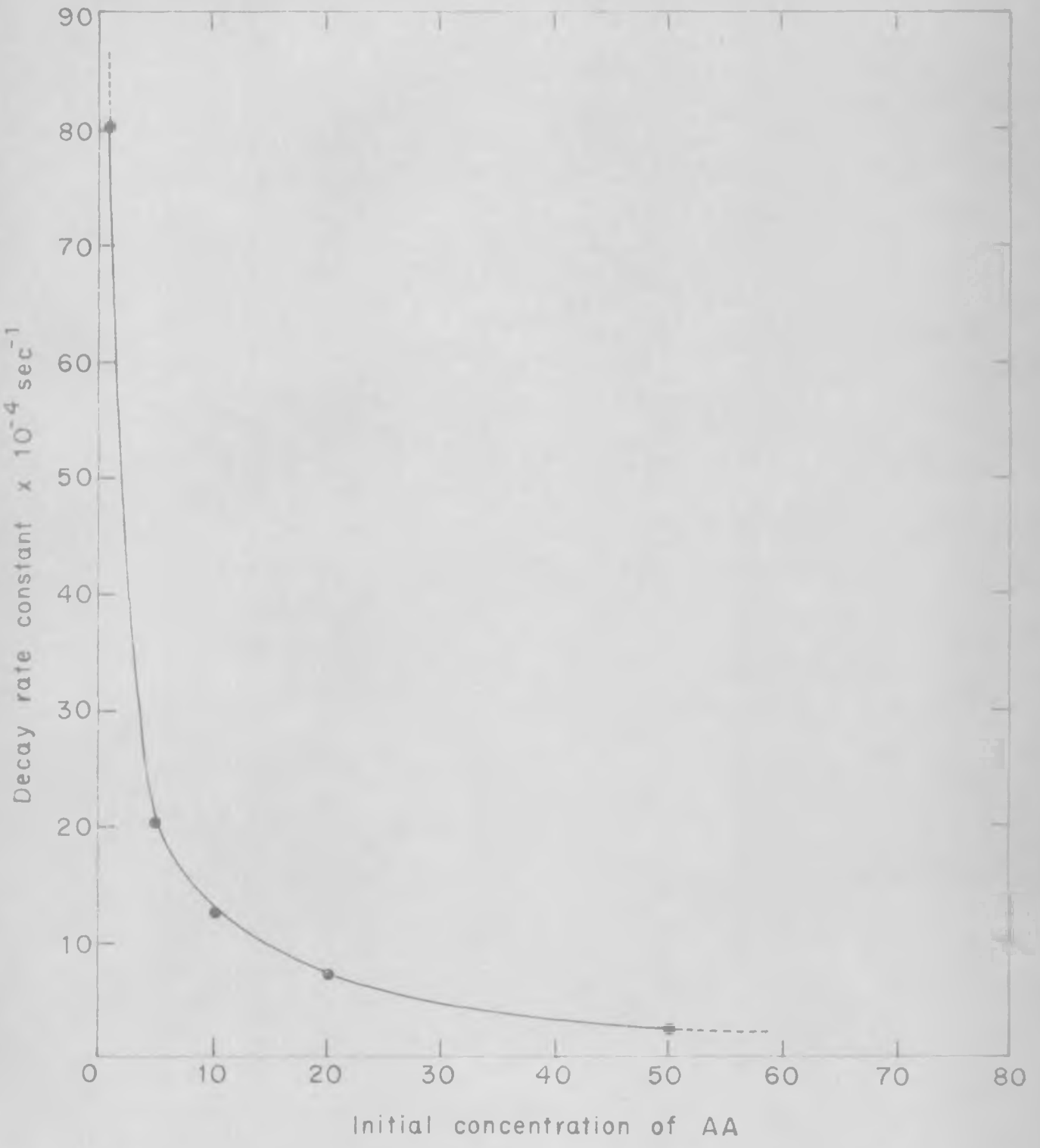



Fig.3 Dependence of 'specific' decay rate constant on initial concentration of AA

in one of the earlier studies.

Table III

| AA concentration (mg/100 ml) | "Specific" decay rates $\times 10^{4}$ $min^{-1}$. |
|---|---|
| *1.0 | 80.6 |
| 5.0 | 20.7 |
| 10.0 | 12.9 |
| 20.0 | 7.1 |
| 50.0 | 2.3 |

[* The decay rate at this dilution was followed by the fall in the absorption of UV maxima while at other concentrations, it was studied by iodometric titrations described in experimental section].

In some of the earlier studies reported in literature, the AA decay was shown to be independent of initial concentration. This was so apparently because many of these studies were done at a higher concentration ranges where such dependence does not seem to be observable. A concentration range of 5-50 mg/100 ml is best suited if one wants to see the dependence of decay rate on concentration. AA being a weak acid, the only thing that can happen on dilution is the increase of dissociation. The behaviour observed suggests that

dissociation of the acid is helpful for the oxidation. It is thus clear that the anion of the acid is involved in the reaction. At high concentrations of AA, the degree of dissociation being less, the concentration of the anion in solution is relatively low and thus the decay is also very slow.

It is of interest to mention here that it is not possible to prepare DHA by oxidation of a strong aqueous solution of AA by a current of air unaided by anything else.

Since ionization was found to help oxidation of AA, it was natural to ask whether a pH increase would also not result in an enhancement of the oxidation rate. The rates were actually measured as a function of pH, not by employing buffers, but by using mixtures of AA and its potassium salt in varying proportions keeping total concentration of AA in solution constant. The decay that is spoken of here is for the total of AA and its salt. It was found that the decay rates were higher when more of the salt was present, i.e. at a higher pH. Actually, the experimental solutions were obtained by partial neutralisation of the same amounts of acid. Decay rate data for two sets of 'total' concentrations are shown in Table IV. An increase in decay rate with increasing neutralisation was obtained for every one of the four sets of data. Table VI shows the variation for the other two concentrations studied along with other information. The rates were maximum for ascorbate solutions, i.e. at the highest pH.

Table IV

| Concentration of AA mg AA/100 ml | Percentage neutralisation | "Specific" decay rates* x $10^4$ $min^{-1}$. |
|---|---|---|
| 5.0 | 0.0 | 20.7 |
| | 25.0 | 33.5 |
| | 50.0 | 38.6 |
| | 75.0 | 40.3 |
| | 100.0 | 43.7 |
| 20.0 | 0.0 | 7.1 |
| | 25.0 | 9.7 |
| | 50.0 | 9.7 |
| | 75.0 | 10.5 |
| | 100.0 | 10.6 |

* See Experimental

Figs. 4 and 5 show the increase in decay rates with increasing neutralisation at 5, 10, 20 and 50 mg of AA per 100 ml. These figures also show that, the variation was less at higher concentrations. The curves are more smooth at higher concentrations.

At higher neutralisations, the reproducibility was not good. This behaviour is comparable to that at low

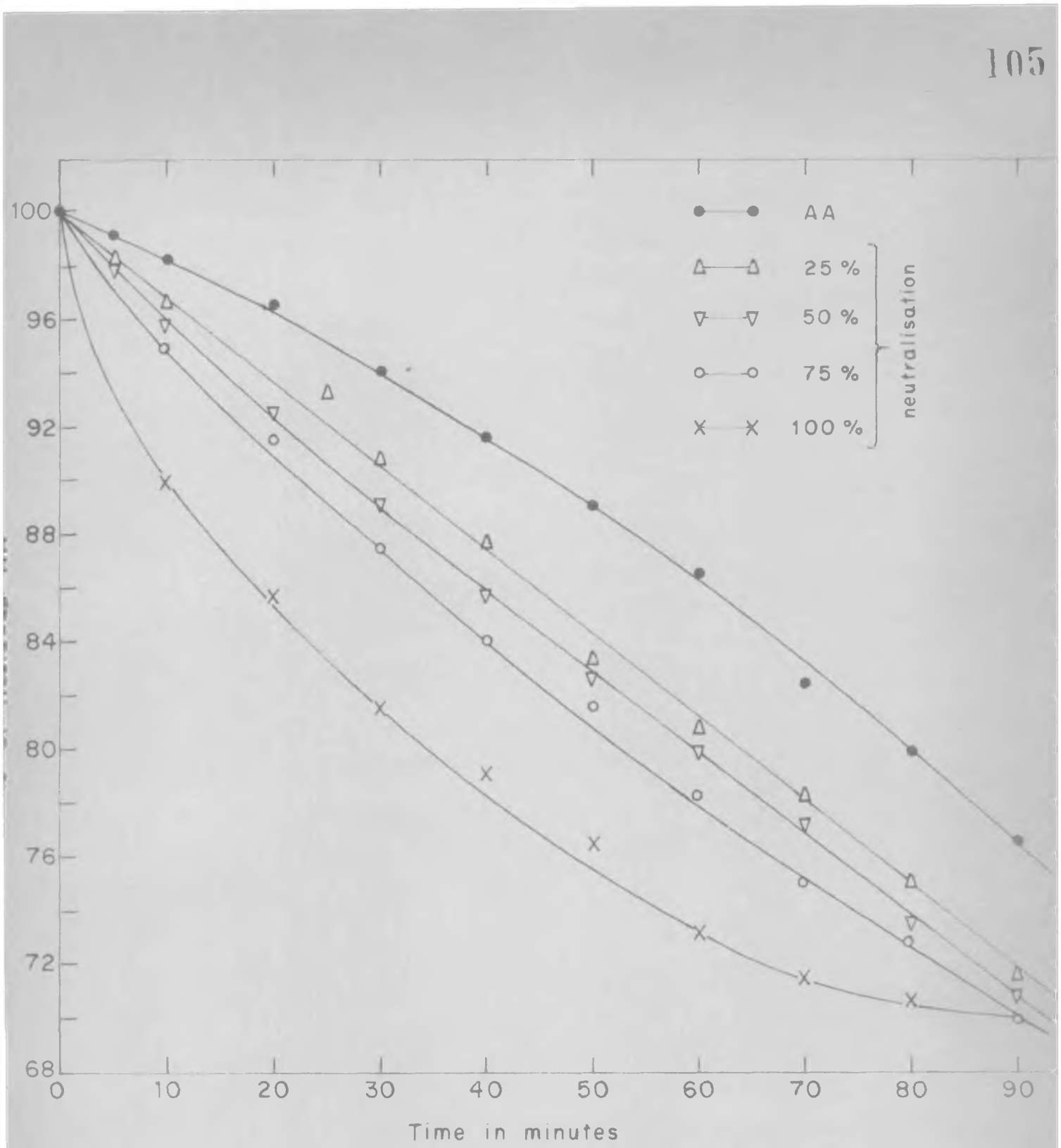



Fig 4 Decay rates of partially neutralised AA solutions (5 mg/100 ml)

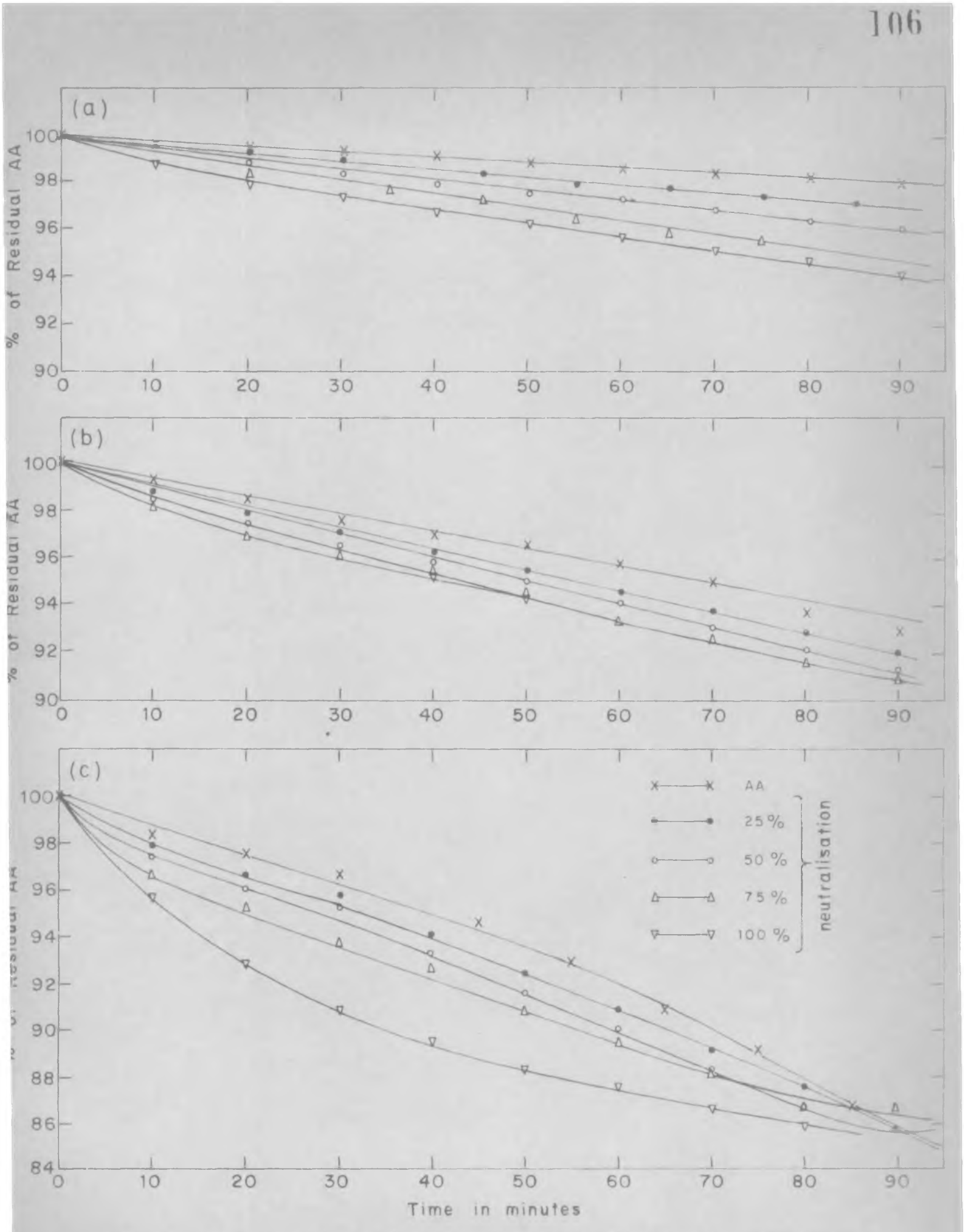



Fig. 5 Decay rates of partially neutralised AA solutions
(a) 50 mg/100 ml (b) 20 mg/100 ml (c) 10 mg/100 ml

concentration of pure AA solutions. This means that when the proportion of anion in the solution increases there will be more uncertainties in the oxidation reaction. The reproducibility of partially neutralised solutions at all concentrations was not as good as that obtainable for experiments involving the pure acid. However, they were not bad enough to affect the conclusions drawn.

The observed behaviour is not consistent with any simple reaction mechanism involving only the anion of AA and the undissociated acid in the slow step. If this were the case, the rate of variation should have shown a maximum for 50% neutralisation. This is apparently not the case.

The decay rates of D(-)-iso-AA were also studied under varying conditions employed for AA. The behaviour obtained for the oxidation of D(-)-iso-AA was similar to that for AA, but, the variation of the first order "specific" decay rates with concentration was much less steep. Table V shows a selected "specific" decay rates for AA and D(-)-iso-AA at different concentrations. Fig. 6 depicts a graphic representation of the whole set of data. The higher the concentration, the closer was its behaviour to that of AA both qualitatively and quantitatively. Experiments with partial neutralisation of the acid gave results similar to those for the parallel ones with AA (Fig. 7). Samples of the exact figures obtained are shown in Table VI. The data

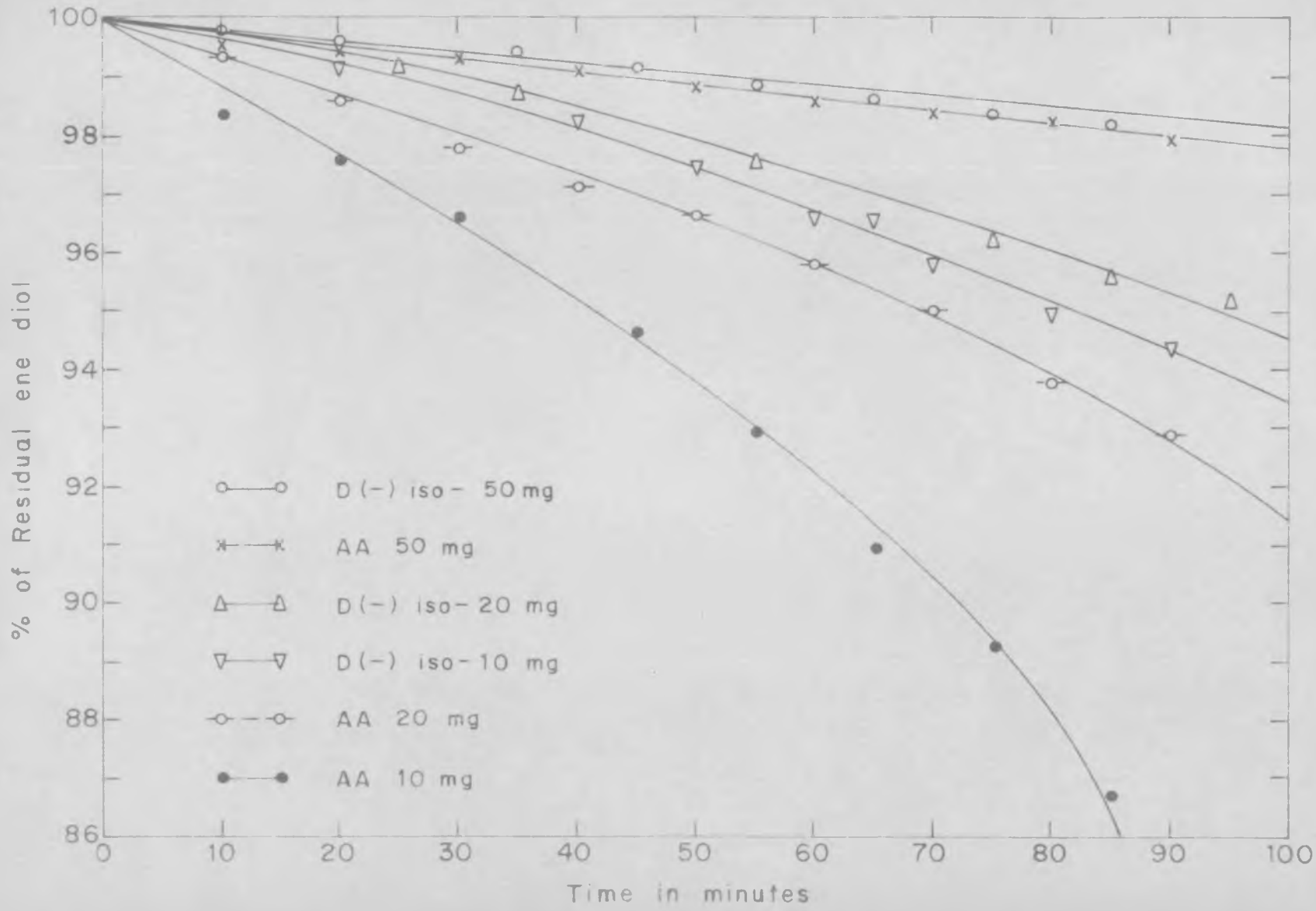



Fig.6 Decay rates of solutions of AA and D (−) iso − AA at different concentrations (mg / 100 ml)

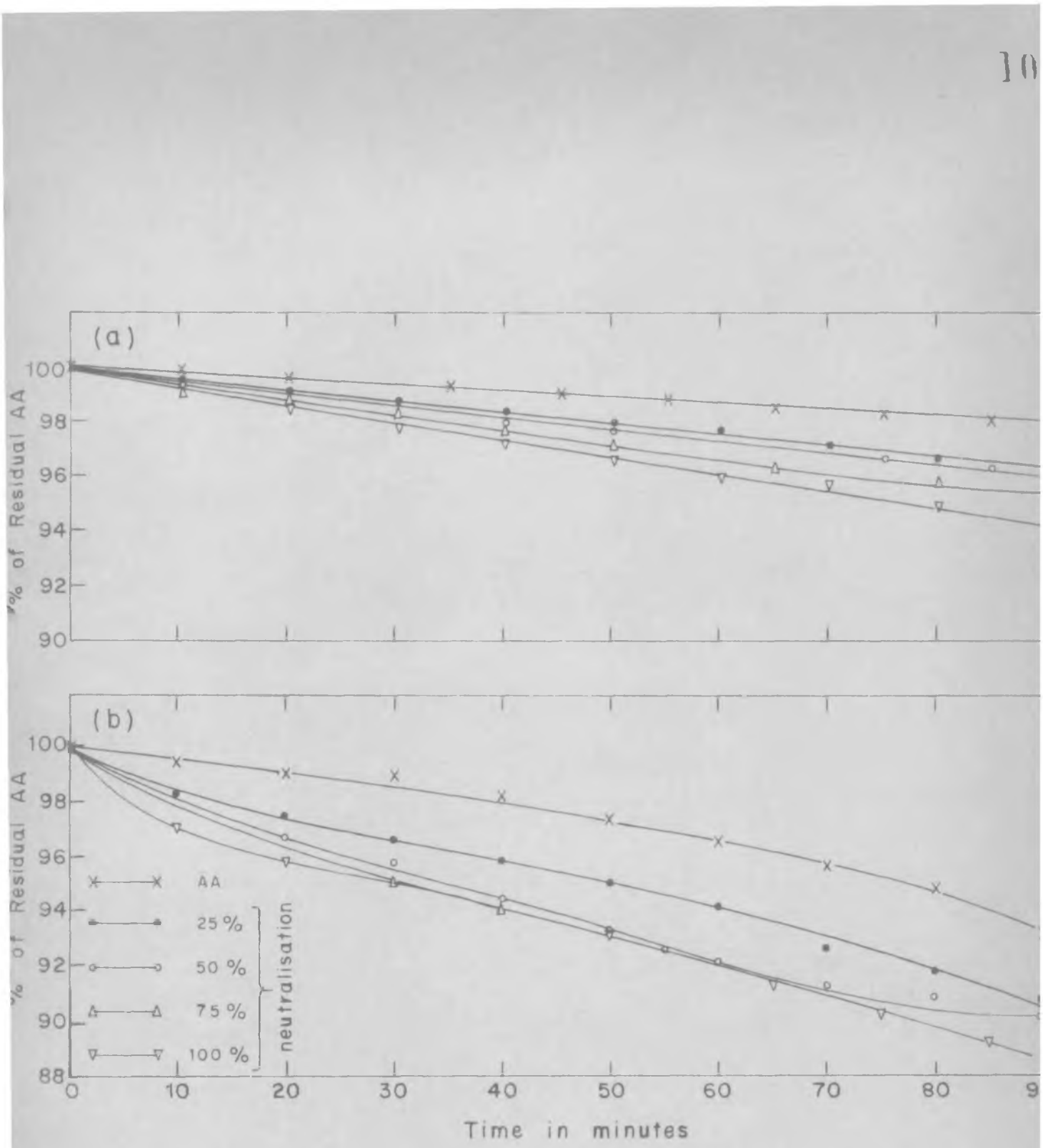



Fig. 7 Decay rates of partially neutralised solutions of D(–)-iso-A
(a) 50 mg/100ml (b) 20 mg/100ml

Table V

| * Concentration mg/100 ml | "Specific" decay rates x10$^{4}$ min$^{-1}$. | |
|---|---|---|
| | *dl*-A | D(-)-iso-*dl*-A |
| 10.0 | 12.9 | 4.1 |
| 20.0 | 7.1 | 2.9 |
| 50.0 | 2.3 | 1.8 |

clearly show that the variations observed with increasing neutralisation in the case of D(-)-iso-*dl*-A were less as compared with those obtained for *dl*-A under similar conditions. This was true even for the free acids.

Table VI

| | Concentration mg/100 ml | % neutralisation | "Specific" decay rates x $10^{-4}$.min$^{-1}$. | |
|---|---|---|---|---|
| | | | *dl*-A | D(-)-iso-*dl*-A |
| * | 10.0 | 0.0 (pure acid) | 12.9 | 4.1 |
| | | 25 | 15.2 | 8.3 |
| | | 50 | 16.1 | 12.4 |
| | | 75 | 15.5 | 14.4 |
| | | 100 | 25.3 | - |
| | 50.0 | 0.0 (pure acid) | 2.3 | 1.8 |
| | | 25 | 3.6 | 4.0 |
| | | 50 | 4.8 | 4.5 |
| | | 75 | 6.0 | 4.6 |
| | | 100 | 7.2 | 5.5 |

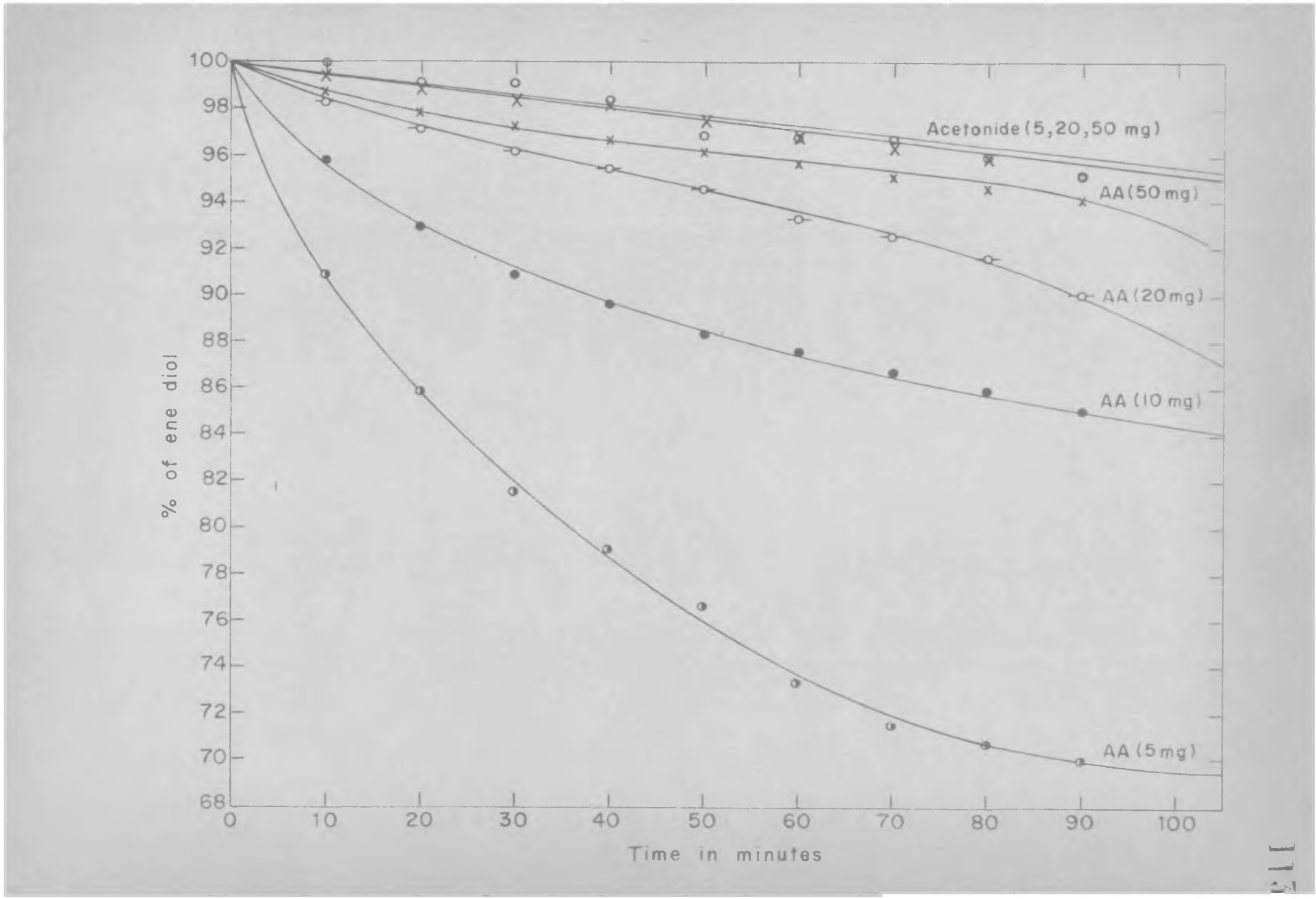
We now come to the behaviours of the sodium salts of AA and its acetonide. The acid forms could not be compared on account of self catalysed hydrolysis of the acetonide in aqueous solution. Though, this problem did not arise for the 6-benzoate of AA, it had a low solubility and so could not be studied. The behaviours of the two salts studied were highly interesting. The decay rate constants for the salts of AA and its acetonide at the same concentrations are as shown in Table VII.

Table VII

| Concentration of enediol present as K-salt mg/100 ml. | "Specific" decay rates x $10^{-4}$.$min^{-1}$. | |
|---|---|---|
| | AA salt | Acetonide salt |
| 5 mg of AA and equivalent of acetonide | 43.8 | 3.7 |
| 20.0 mg of AA and equivalent of acetonide | 10.1 | 5.0 |
| 50 mg of AA and equivalent of acetonide | 7.2 | 4.0 |

Oxidations with these salts were done in exactly the same manner as before, and the concentration dependence of reactivity was studied. The first order "specific" decay rates obtained for the sodium salt of the acetonide were

roughly the same (within $\pm$ 0.8 x $10^{-4}$) for all concentrations within the experimental errors, i.e. they were true first order reactions and the reaction rates were low. But, in the case of AA salt, the apparent first order constants were themselves concentration dependent. The decay rates for both cases are presented in Fig. 8. The percentage decrease in substrate concentration is shown on the y-axis. The top curve represents the change for all three concentrations of the acetonide salt. In a given time the same percentage is decomposed irrespective of concentration. However, there is a dramatic change in the behaviour of the corresponding salt of AA itself. The rate of oxidation increases steeply with dilution as shown by the remaining curves in Fig. 8. Apparently, the species involved in oxidation is not of the same type as in the case of the acetonide, and the reaction with this species is obviously more facile. Something that happens with greater ease in the more dilute solution is producing a more readily oxidisable species in the ascorbate reaction. It appears that the more readily oxidisable species is getting formed via hydrolysis, which is more pronounced in more dilute solutions. Hydrolysis of the sodium salt of the acetonide should also be more pronounced in the more dilute solutions. But apparently, there is no way of getting to the more susceptible species in this case from the hydrolysis products which are the acetonide molecule and the hydroxyl ion. In the case of sodium

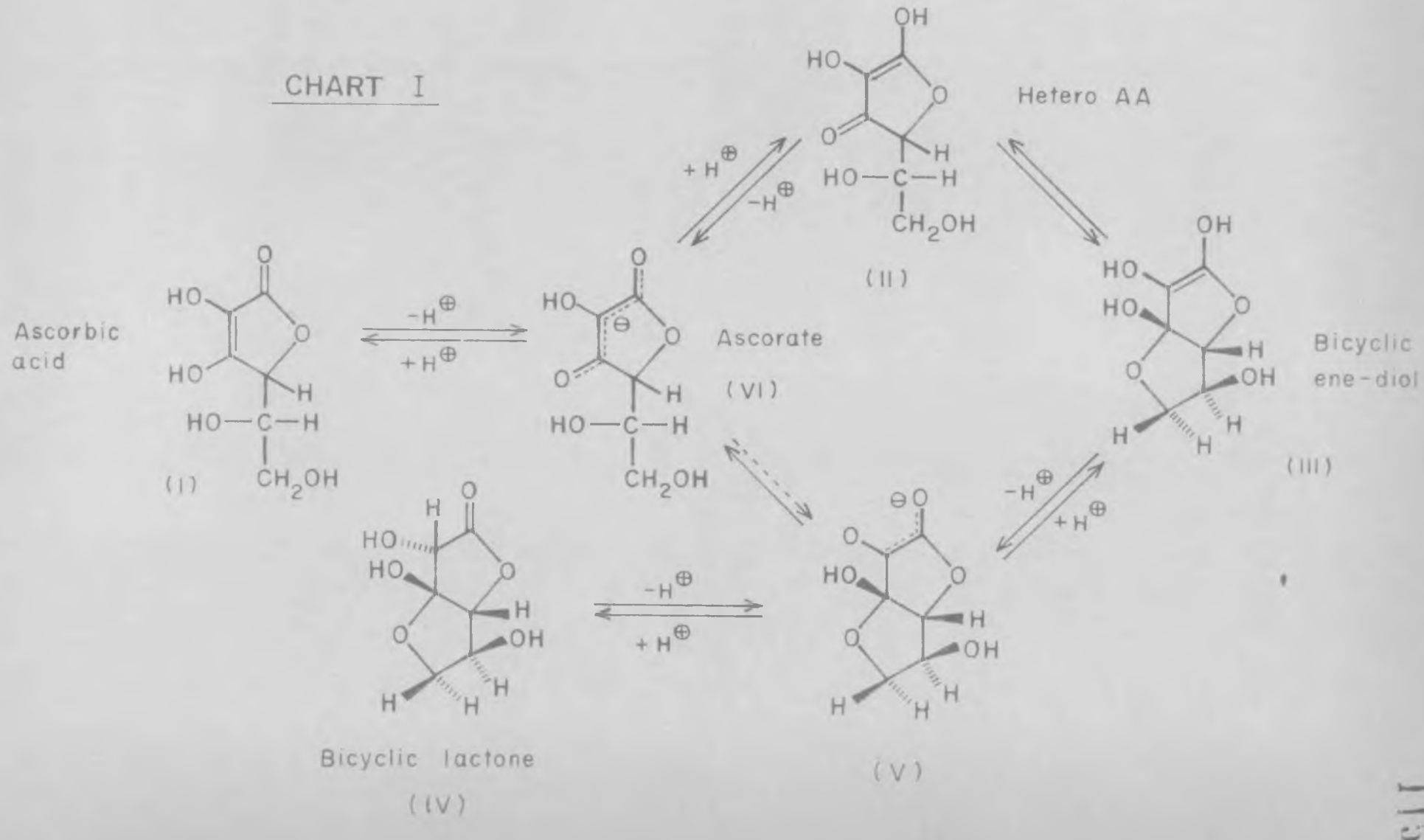

100
98
96
94
92
90
88
86
84
82
80
78
76
74
72
70
68
% of ene diol
0
10
20
30
40
50
60
70
80
90
100
Time in minutes
Acetonide (5,20,50 mg)
AA (50 mg)
AA (20 mg)
AA (10 mg)
AA (5 mg)

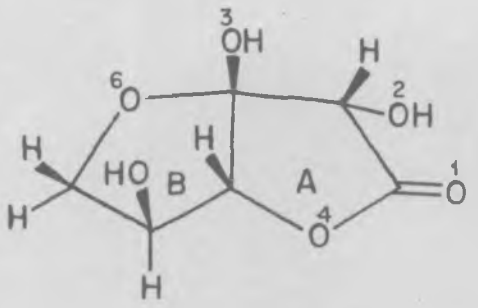
ascorbate the hydrolysis products are AA itself and the hydroxyl ion. It is very clear that AA is able to go over to a more susceptible form which its acetonide is unable to do. The results thus suggest that the formation of the more susceptible species is dependent on the availability of a free hydroxyl at the 6-position.

We have been able to bring out from these experiments some very interesting facts about the nature of AA. There is need to tie up all these observations into one mechanistic scheme which accounts for every thing. What is suggested by the oxidation results is the intervention of the side chain and formation of a more reactive bicyclic intermediate, which we had first considered in connection with the spectroscopic studies. The complexity of the situation that arises can be seen from the following schematic representation of possibilities that can exist (Chart 1).

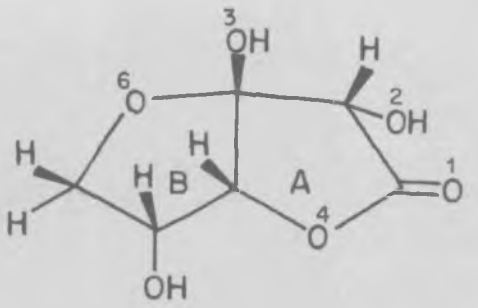
The reactive species involved is probably the anion of the bicyclic ene-diol ( III ). It is not yet clear what further assumptions are required in order to account for observed dependences. But, it appears likely that a suitable set can be found. The manner in which the present experiments have been done may turn out to be not particularly well suited for analysis. But they have been able to bring out some of the more important features involved.

CHART I


Ascorbic acid
(I)
Ascorate
(VI)
(II)
Hetero AA
Bicyclic ene-diol
(III)
Bicyclic lactone
(IV)
(V)



IV



VII

It is of interest here to note that a quantitative difference is detectable between the behaviours of D(-)-iso- and AA which are qualitatively similar. Apparently the formation of the bicyclic intermediate is not quite facile in the case of D(-)-iso-AA. The change of configuration at $C_{(5)}$ puts the 5-hydroxyl cis to the $C_{(4)}$ oxygen (VII) (i.e. the lactone oxygen). In the bicyclic structure, these will almost eclipse each other and will therefore give a structure of relatively higher energy.

The conclusions regarding the reactions of these ene-diols with molecular oxygen were further confirmed by their behaviour towards cupric copper. Actually three oxidising agents including $I_2$, $Cu^{++}$ and $Fe^{+++}$ were tried. The reactions of these substrates with 0.1 M iodine in a phosphate buffer of 6.8 pH were too fast for any difference to become noticeable. Those with $Cu^{++}$ gave nice results and it was expected that the $Fe^{+++}$ reduction experiments might t

out to be even better. However, the expectation for the last mentioned reagent was not realised because of experimental difficulties.

The results of the reactions of AA, its 5,6-acetonide and 6-benzoate with Fehling's reagent are shown in Table VIII. Comparable amounts of these substrates (20.0 mg of AA and its equivalents) and the reagent (0.74 ml of a mixture of Fehling's solutions A and B) were used in the reactions. The table clearly shows that reduction of $Cu^{++}$ was more rapid with AA and D-iso-AA. The reactions with the acetonide and the benzoate were visibly slower. The colour change from blue to green took appreciably longer time for the second pair of compounds. Practically no difference in rate could be made out in the experiments with AA and D-iso-AA. The same was true for the second pair. However, we know that the acetonide is stable under the alkaline conditions of the reaction mixture while the benzoate is not. The latter would hydrolyse at quite a fair rate to give the more reactive ascorbate ion. It was to reduce this hydrolysis that alkali of the Fehling's reagent was cut down. The results may therefore be taken to imply that the reactivity order is AA $\simeq$ DHA > benzoate > acetonide. The same order of reactivity was found even in the Udenfriend hydroxylations described in Chapter VI.

It was hoped that similar experiments on the reduction

Table VIII

| | Compound | Change of colour and other particulars with time in minutes | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0.0 | 0.25 | 0.5 | 1.0 | 2.0 | 3.0 | 5.0 | 10.0 |
| 1. | A | Blue | * Yellowish green | Yellow | Bright yellow | Dark yellow | orange | Orange | Dark orange precipitate in colourless solution. |
| 2. | D-iso-AA | Blue | -do- | Yellow | -do- | -do- | Orange | Orange | Dark orange precipitate in colourless solution |
| * 3. | 5,6-Acetonide of AA | Blue | Blue | Green | Light green | Greenish yellow | Yellow with light green tinge | Dirty Yellow | Light orange precipitate in light green solution |
| 4. | 6-Benzoate of AA | Blue | Blue green | Green with yellow tinge | Yello-ish green | Greenish yellow | Dirty yellow | Dirty yellow | Orange precipitate in colourless solution |

* The reaction mixture were clear just after mixing but, very soon they become hazy due to precipitation and the colours indicated actually refer to the colours of the suspensions.

of $Fe^{+++}$ to $Fe^{++}$ would give even better results and that they would be specially useful in the context of experiments on Udenfriend hydroxylation. The actual optical density data obtained at the wavelength of the ortho-phenanthroline complex (512 nm) were, however, found to be not true measures of ferrous ion formation in the case of the acetonide and the benzoate. The values for the comparative experiments are however, shown in Table II, for purposes of record. The optical density figure (0.605) obtained for the benzoate exceeded the theoretical value (0.53) calculated from the calibration plot. The data showed that there was obviously a masking colour in the cases of the acetohide and the benzoate. The difficulty here was not investigated further, since the results with cupric copper were so clear and could be readily reproduced any number of times.

Conclusions

The experiments on the relative ease of oxidation of AA, its acetonide and D(-)-iso-AA under a variety of conditions brought out the important fact that the side chain of ascorbic acid has a role in determining the reactivity of the ene-diol system of the molecule. The difference in behaviour obtained for the potassium ascorbate and the potassium salt of the acetonide was striking. The concentration dependence of the ease of oxidation was more pronounced for AA than for D(-)-iso-AA. Visual support for the conclusions

of the autoxidation studies could be obtained by the $Cu^{++}$ reduction experiments, which were very easy to carry out. It has been suggested that the side chain intervention is through the formation of a more readily oxidisable bicyclic species.

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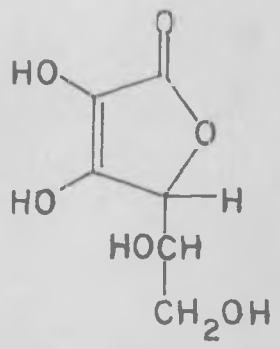
# Chapter IV

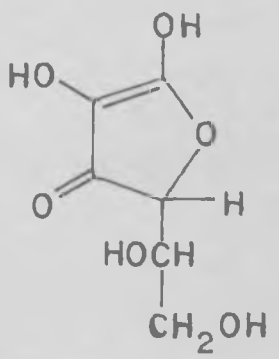
## METHYLATION AND METHYL ETHERS OF ASCORBIC ACID

Introduction

The properties of the methyl ethers of ascorbic acid (AA) were investigated at some length soon after the constitution of the vitamin (I) was established[1]. The 1-, 2- and 3-O-methyl derivatives (III, IV and V) were prepared[1-11] and as noted earlier the first of these was called 1-O-methyl-hetero-ascorbic acid, a derivative of an isomeric form (II) of AA which was presumably present in solution in very minor amounts[2,4], but was not isolable. The 2,3-dimethyl ether (VI)




I

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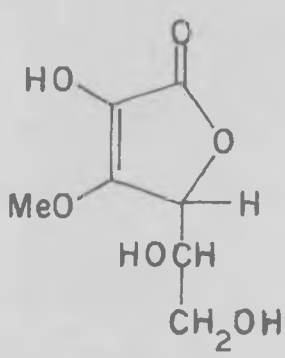


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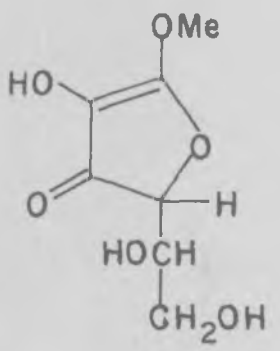
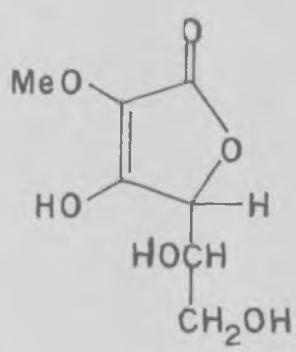


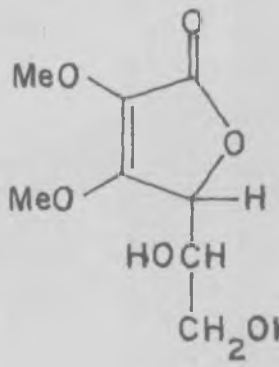

III



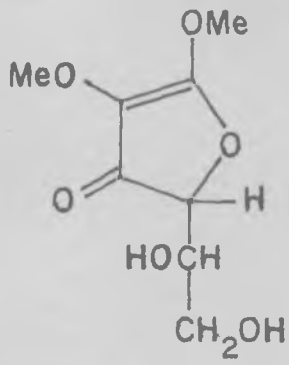

IV

of AA and the 1,2-dimethyl ether (VII) of its hetero isomer were also obtained[2,3,7]. The 2,3-dimethyl ether was further

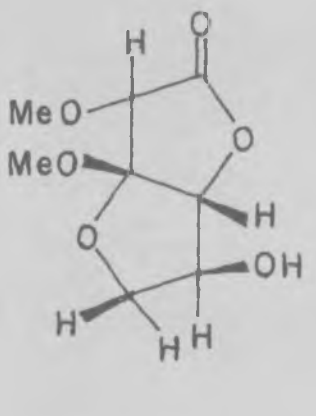
converted into several other ether derivatives in methylation and tritylation reactions[2,3,9-11]. One of the most remarkable properties of these compounds was their capacity to go over into isomeric forms on treatment with alkali and re-acidification[3,8-10]. Thus the 2,3-dimethyl ether could be isomerized into iso-di-O-methyl ascorbic acid for which a bicyclic structure (X) was suggested. The substance did not




VI

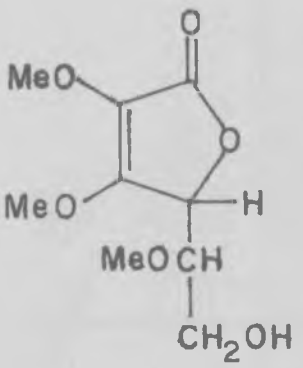



VII

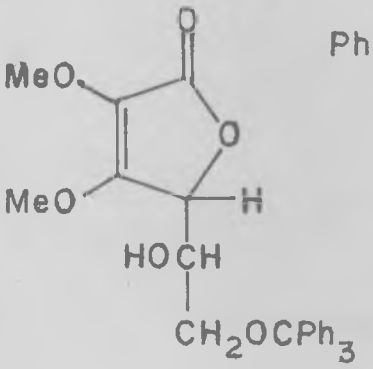



X

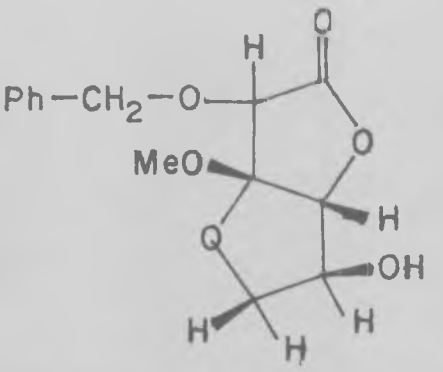
have a UV absorption and had surprising stability. It was found to be unaffected by $KMnO_4$ in acetone[3]. Similarly, it was found that 2,3,5-tri-O-methyl and 2,3-di-O-methyl-6-trityl derivatives (VIII and IX) of AA could also be isomerized by similar procedures[3]. For these cases, no structures were suggested. 3-O-Methyl-2-O-benzyl AA has been reported to be isomerized to a mixture of epimers (XI) which do not seem to have been separated[12]. Also, the methylation of AA by $BF_3$ in methanol has been shown to




VIII

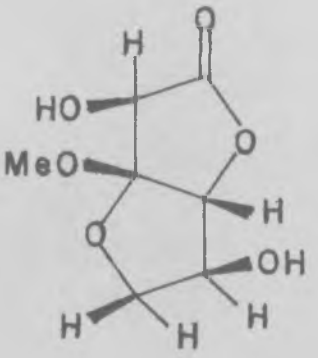



IX

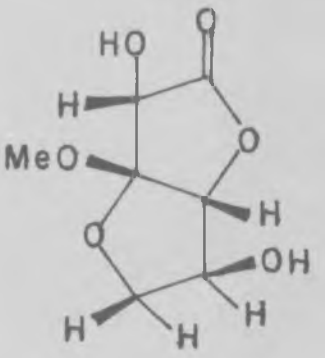



XI

give two bicyclic 3-O-methyl derivatives[13] (XII, and XIII)




XII




XIII

The isolation of only one isomeric bicyclic form for the 2,3-dimethyl ether suggested that its epimer, which should normally have been formed, was unstable. In the case of the bicyclic forms of the 3-O-methyl derivative, one has been

shown to be less stable than the other[13]. The existence of bicyclic forms for the ethers suggested the possibility of similar forms for not only some other derivatives but also for the parent compound itself.

The stereochemistry at the 2-position for iso-dimethyl-AA has not so far been studied and the structures of the other isomeric derivatives have not been investigated. It has already been pointed out that the possibility of occurence of bicyclic forms for AA itself can have great significance for its reactivity and the mechanisms of its action. It was therefore thought that more information should be obtained on the known bicyclic derivatives and the present study was undertaken in this context. It was decided to compare the spectra of iso-dimethyl AA with those of some of its close analogues for an assignment of stereochemistry. In the effort to make this compound and other bicyclic derivatives, improved procedures for making several of these materials were worked out.

Results and discussion

Since we were interested in getting iso-dimethyl AA in good quantities, a fair amount of effort was made to improve the procedures for making 3-O-methyl and 2,3-di-O-methyl derivatives of AA and also the isomer of the latter. A good many experiments had to be done in this regard. In the methylation of 6% solutions of AA in methanol at $0^{\circ}$ with one mole equivalent of diazomethane, although the yields obtained in solution were usually very high (97-98%), the amount of pure product that could be isolated was often poor because of the difficulty encountered in crystallisation. On removal of the solvent, one normally obtained an oil from which crystallisation of the product was extremely slow and did not give attractive amounts. The time required for crystallisation was uncertain and was of the order of 2 weeks or more. But, on removal of impurities by chromatography over silica gel, one could obtain the 3-O-methyl derivative in crystalline form in high yield (80 to 85%) within a matter of several hours.

However, by this procedure only one product could be obtained. Formation of isomeric products in very small yields was reported both by Herbert, Hirst et al.[2] and by Reichstein, Grussner and Oppenauer[4]. However, the compounds isolated by the two groups were different. Attempts to make these isomeric compounds did not give consistent results. Earlier workers in this laboratory[14] had obtained a product that

corresponded well with the one (m.p. 162°C) reported by Herbert, Hirst *et al.*[2] and also by Vestling and Rebestock[6]. But, later attempts to reproduce the results gave only a material that seemed to resemble the one (m.p. 152-153°C) reported by Reichstein *et al.* The sample of the isomeric compound, which was obtained in very small quantities in this study, did not show the characteristic strong absorption of a lactone carbonyl in the IR. Instead, it showed heavy absorption around $\nu$ 1560 $cm^{-1}$ which is shown in Fig.1. But, on dissolution in water, it showed a UV absorption with a $\lambda_{max}$ at 281 nm shifting to 325 nm on addition of alkali. Its PMR spectrum in DMSO (Fig.2) was similar to that (Fig.3) of the 3-O-methyl compound. But mass spectral fragmentation showed an intensity pattern quite different from that of the 3-O-methyl derivative. There were also appreciable differences in the fragmentation patterns although many peaks were common. The mass spectra are shown in Fig.4. The PMR spectrum in DMSO did not change with time. This behaviour was very much unlike that obtained for the 1-O-methyl derivative (III) which seemed to correspond with that obtained by Herbert, Hirst *et al.* In this case, the methoxyl shifted upfield to 192 Hz from its original position at 244.5 Hz in 5 days (Fig.5), suggesting the addition of the 6-hydroxyl to the 3-carbonyl leading to a bicyclic form (XIV). It looked likely from the data that the material of m.p. 152° isolated in this study was a hydrated form of 1-O-methyl hetero-ascorbic acid (XV).

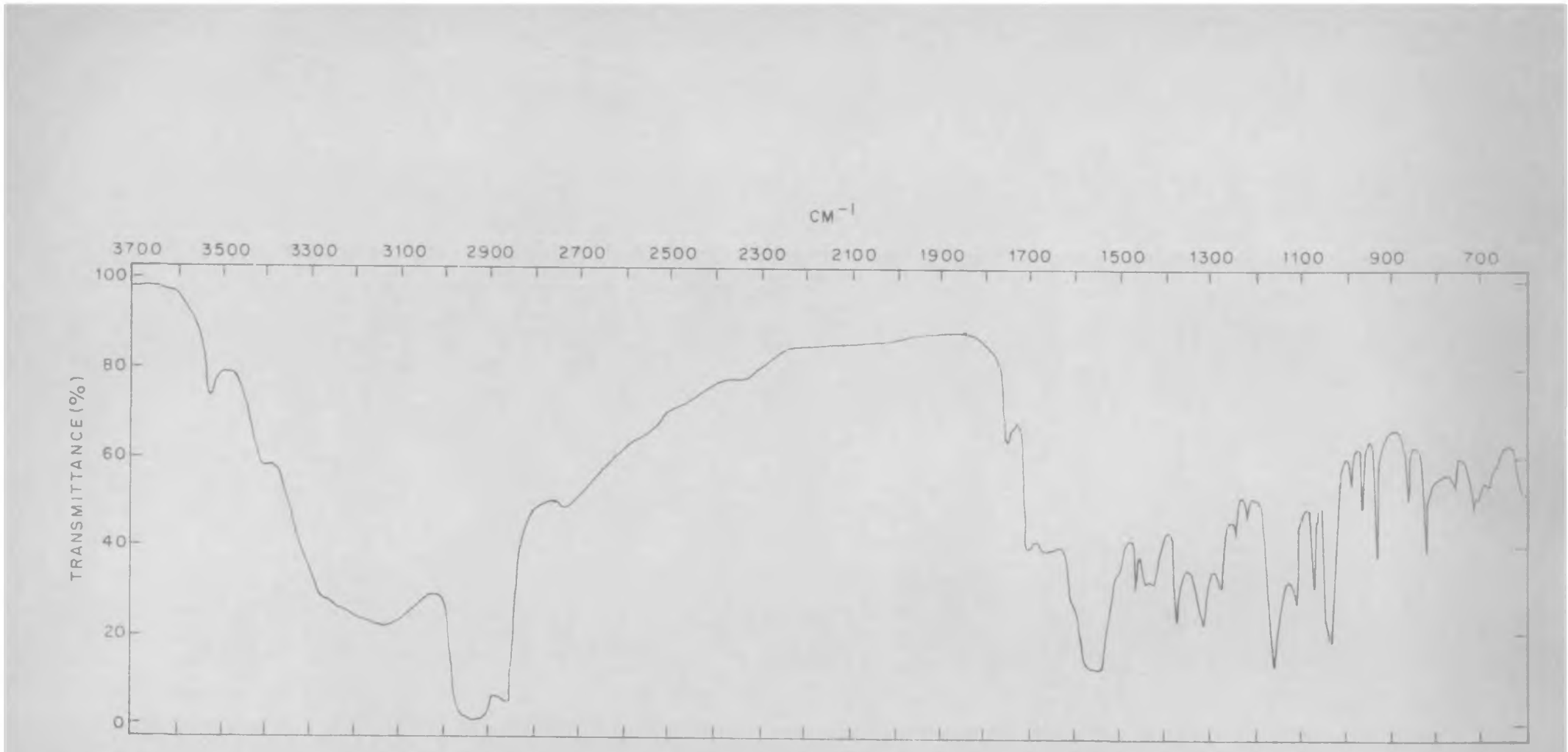



Fig. 1. IR Spectrum of 1—O- methyl hetero AA (m.p. 152 ℃)

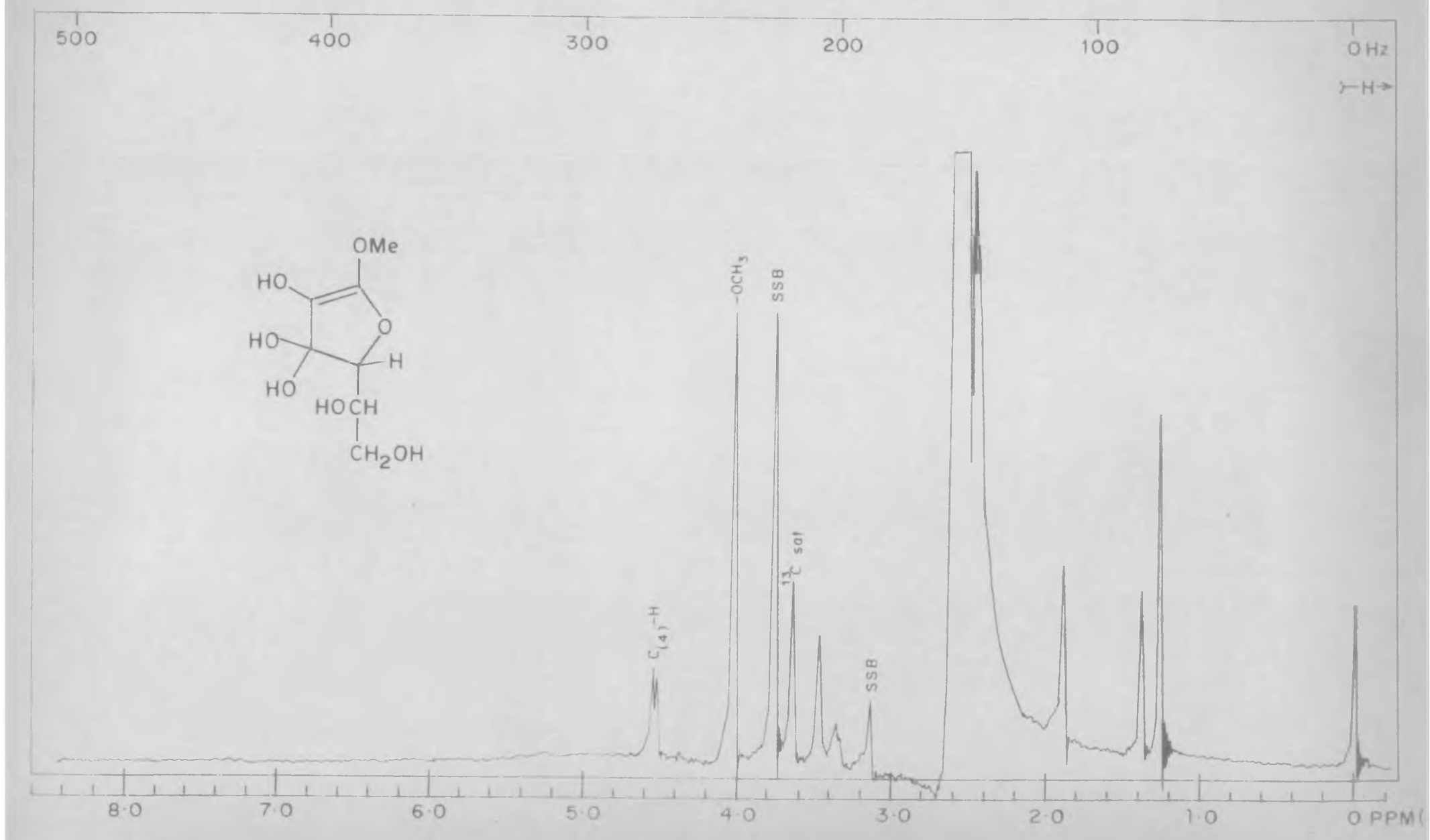
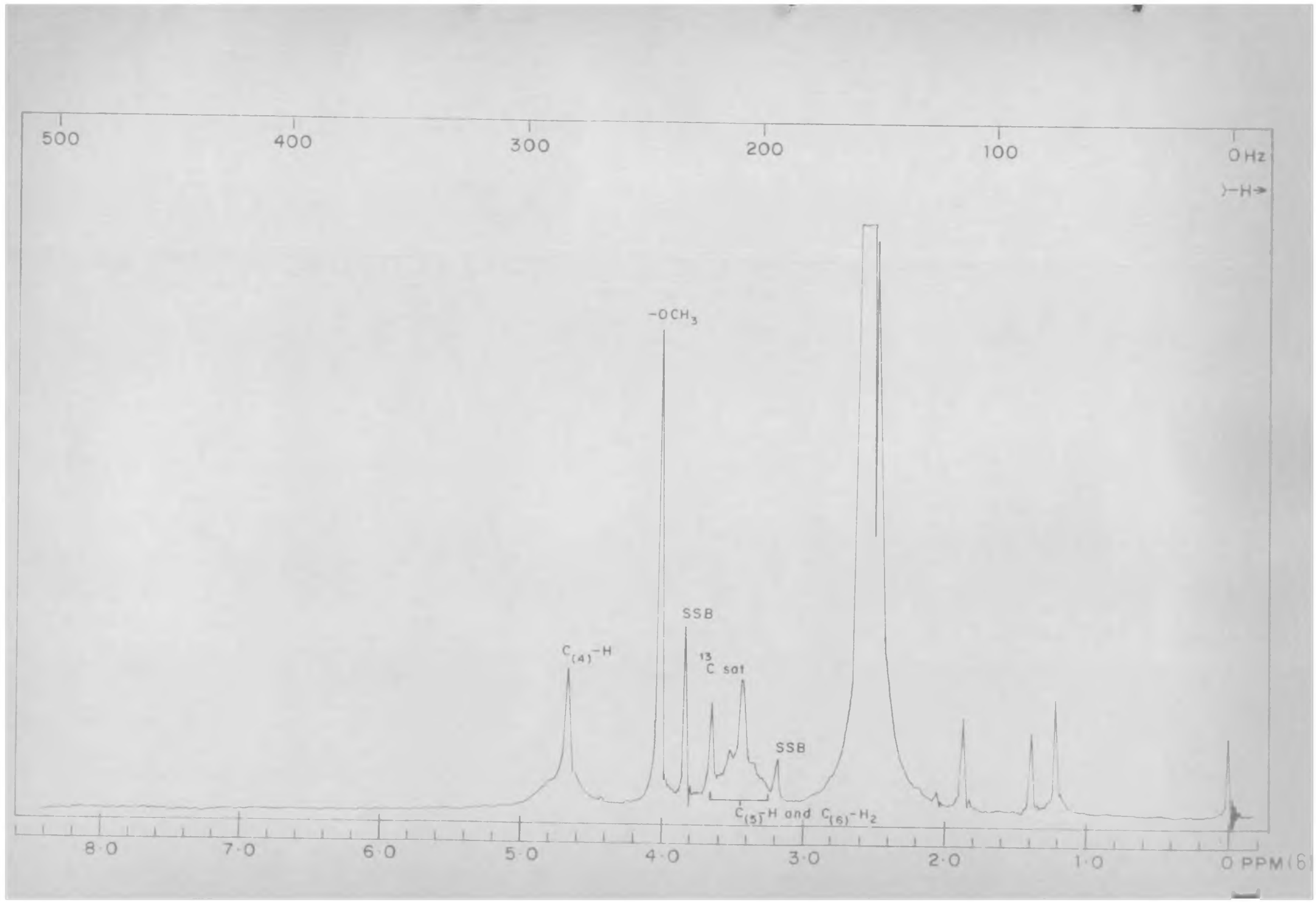



Fig. 2. 60 MHz PMR Spectrum of 1-O-Methyl AA (m.p. 152° C) in DMSO


500
400
300
200
100
0 Hz
)–H→
–OCH3
SSB
C(4)–H
13C sat
SSB
C(5)–H and C(6)–H2
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0 PPM (δ)

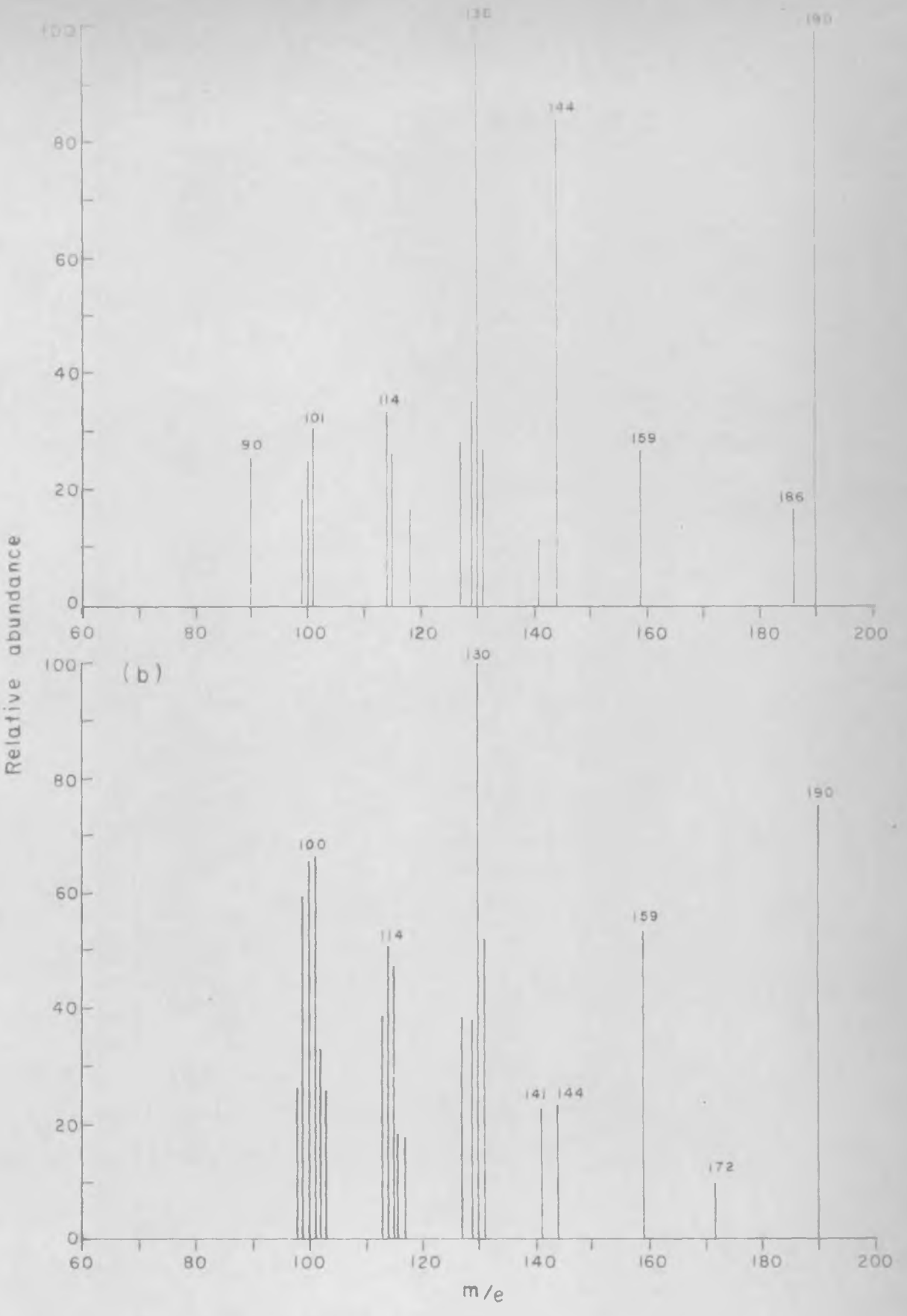



Fig. 4 Mass spectral fragmentations of : (a) 3-O-Methyl-AA (b) 1-O-Methyl hetero-AA (m.p. 152°C)

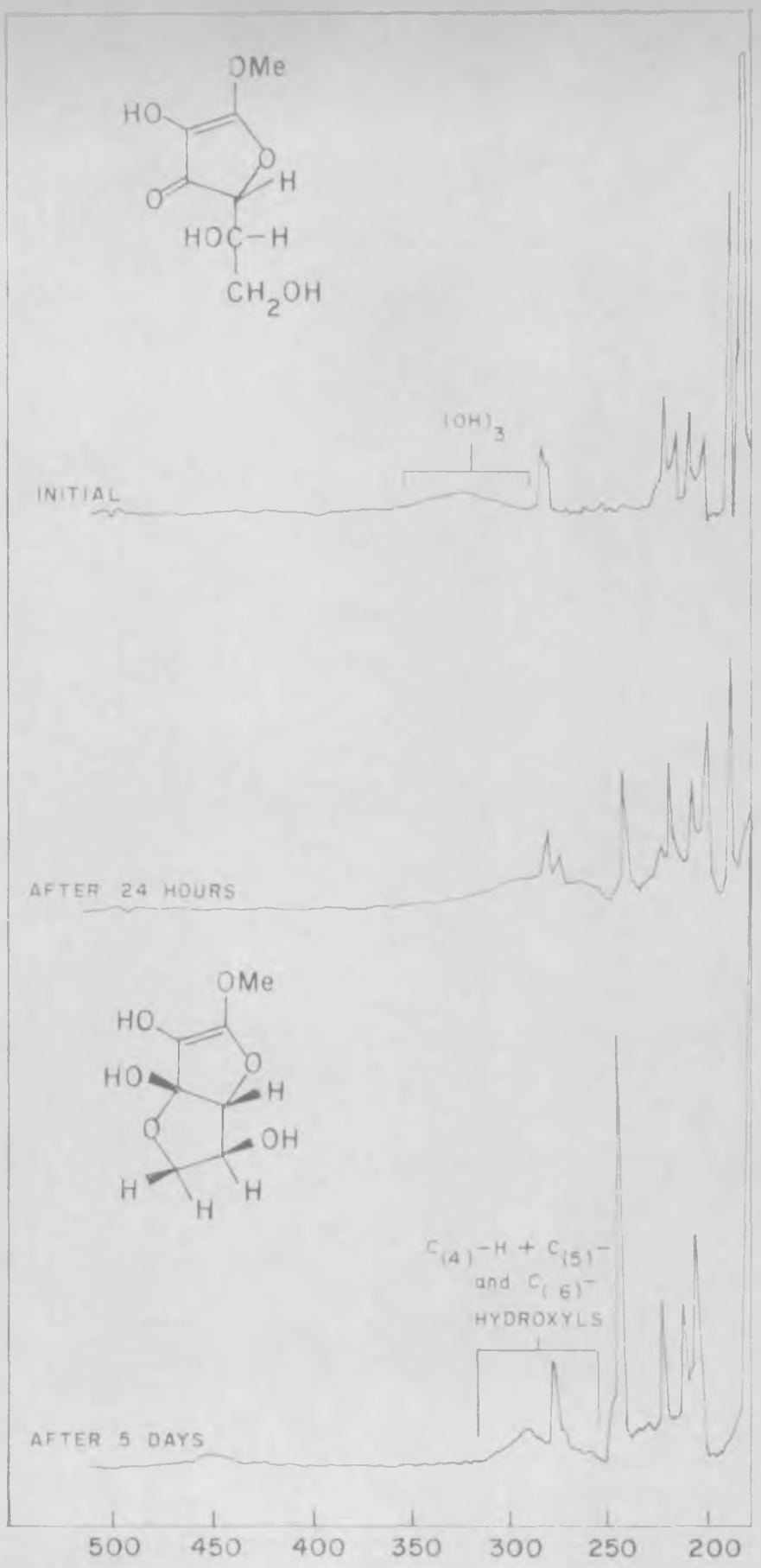
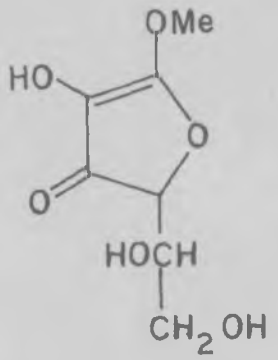
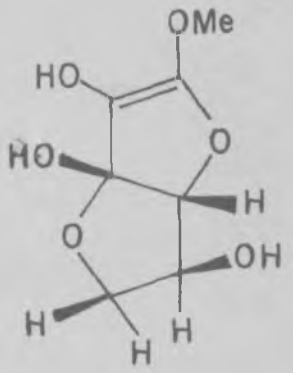


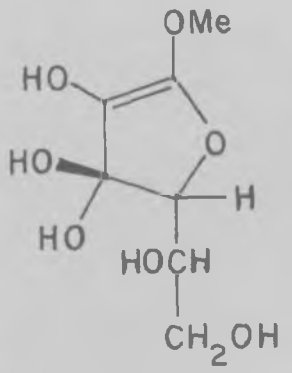

Fig. 5 NMR Spectra of 1-O- Methyl-hetero-Ascorbic acid (m.p.163°C) in DMSO




III




XIV




XV

The properties of the mono-methyl ethers are compared in Table I.

In an attempt to see if trace amounts of alkali would induce formation of the 1-O-methyl derivative in the methylation experiment, a monomethyl derivative of extreme sensitivity to oxygen was isolated in some experiments. While working with 6 g lots of AA in 100 ml of MeOH, a couple of drops of 20% aqueous KOH were added to the AA solution and the methylation done in the usual manner at low temperature using diazomethane in ether. On addition of the diazomethane solution, a curdy white material precipitated out quite frequently, but not invariably in several trials that were made. The formation of this material tailed off with the progressive addition of the diazomethane solution. After the addition of two equivalents of diazomethane was complete, an attempt was made to separate out the precipitated solid by filtration under suction. The precipitate was washed first

with methanol and then with ether. The material began turning brown at strikingly rapid rate. It was quickly dissolved in $D_2O$ and a PMR spectrum obtained immediately. The spectrum is shown in Fig.6. It was clear that the material was a mono methyl derivative, whose OMe absorption was at high field. The behaviour that was seen could not be attributed to any of the known forms for AA

Table I

| | Reaction | Methyl ether | | |
|---|---|---|---|---|
| | | 3-O-Methyl AA | 1-O-Methyl hetero AA (m.p. 162°) | 1-O-Methyl hetero AA (m.p. 152°C) |
| 1 | Reaction with Iodine | Reacts with acidified $I_2$ solution | Reacts with acidified $I_2$ very slowly | Reacts with acidified $I_2$ very slowly. |
| 2 | Fehling solution | Reduces Fehling solution on boiling | Readily reduces hot Fehling solution | Readily reduces hot Fehling solution. |
| 3 | Colour with $FeCl_3$ | Gives a stable intense blue colour (The first drop gives a colour that disappears immediately, the next few drops give a stable blue colour). | Gives a transient blue colour | Gives a transient blue colour |

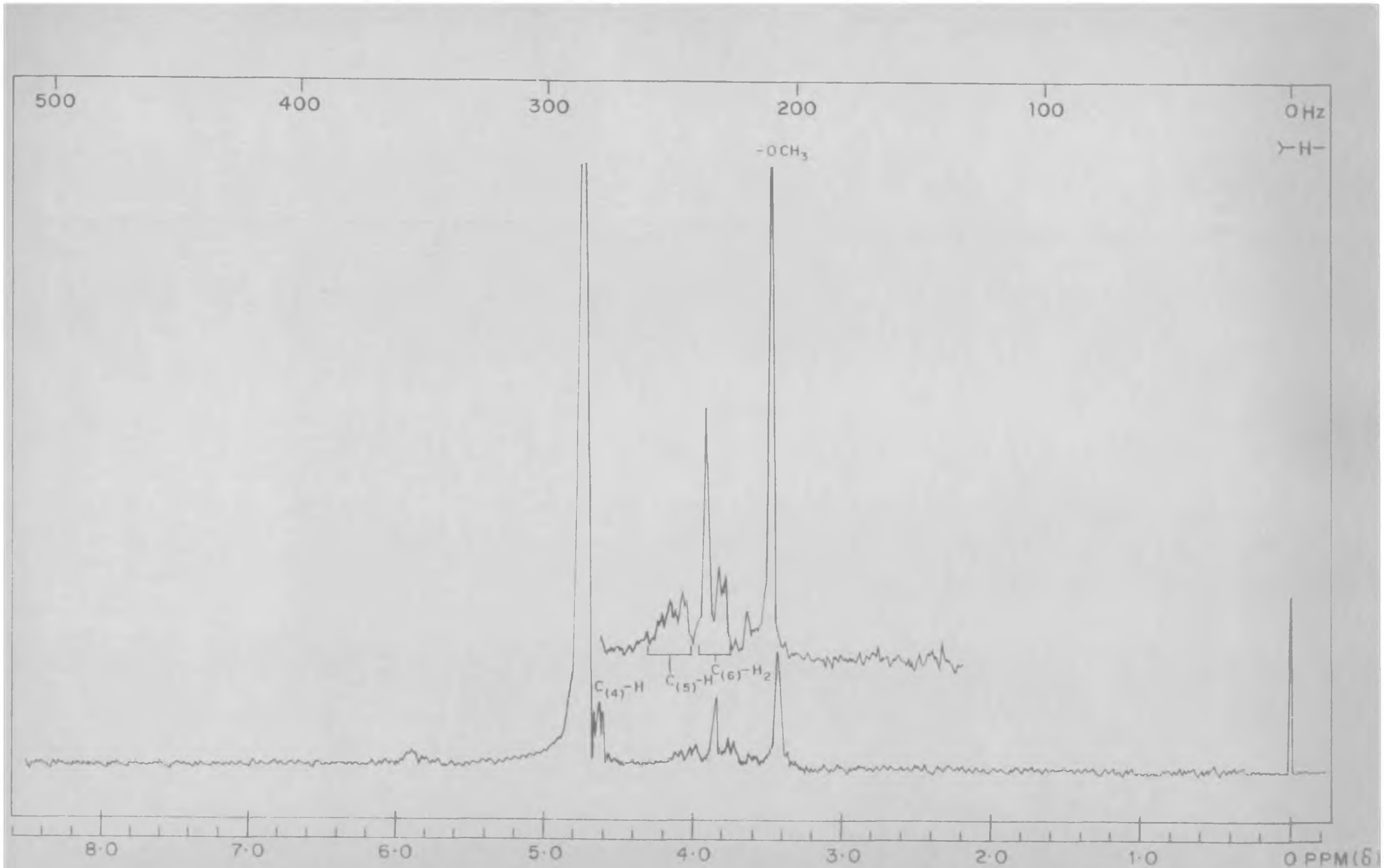


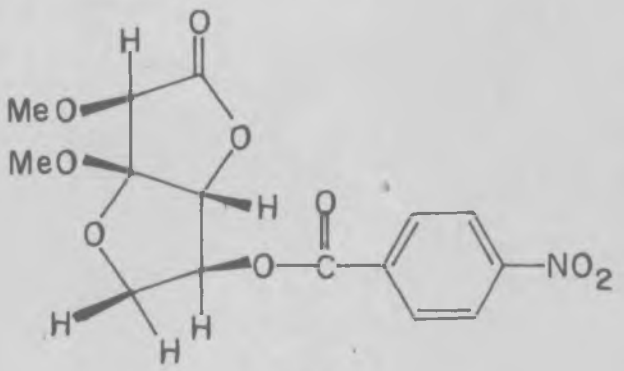

Fig. 6 60 MHz PMR Spectrum of the unstable monomethyl derivative of AA in $D_2O$

and its derivatives. The material was stable so long as it was covered with solvent. Its extreme susceptibility to oxygen made it difficult to get further evidence relating to its structure.

In the preparation of the 2,3-di-O-methyl derivative also, the employment of chromatography greatly facilitated the isolation of the compound in very good yields (90%). This was done on a cellulose column with ether as the eluting solvent. Isomerization of the compound in a manner similar to that described earlier in the literature gave iso-dimethyl [illegible]. The conditions for isomerization were studied in some detail. It was found that employment of dilute solutions gave better results. There was need to use alkali in about 50% excess and a minimum period of 10 hrs was required for the alkali treatment to give good results. In the acid treatment, the amount of acid used was equivalent to the alkali used, and, again, the sample was allowed to stand for some 10 hrs. After removal of solvent water, the isomerized derivative was obtained in pure form from the resulting oil by chromatography over cellulose. Employing the modifications mentioned, yields as high as 85-90% could be obtained in the isomerization and only one epimer was obtained. If enough time was not allowed in the alkali and acid treatments, the yields obtained were very poor. It should also be mentioned here that Hirst et al. had employed distillation for obtaining the pure isomer. It was found in this study that this procedure

was not satisfactory. The quantities that could be recovered were low and the purity of the sample obtained was poor. The 3-O-methyl-AA did not undergo any isomerization under these conditions.

The iso-dimethyl ether formed only a mono p-nitro-benzoate (XVI) on esterification with p-nitro-benzoyl chloride.




XVI

The PMR spectrum (Fig.7) of the product showed that it was the 5-OH that was esterified. A new singlet CH absorption was seen at 254 Hz and the $C_6$ proton shifts (280 Hz and 252 Hz) showed that their environments were appreciably different. The $C_6$-hydroxyl had disappeared in the isomerization. This confirmed the suggested bicyclic structure for the derivative. The properties of the compound agreed with those anticipated from the earlier report. The PMR and CMR data for the p-nitrobenzoate and the parent compound are shown in Table II.

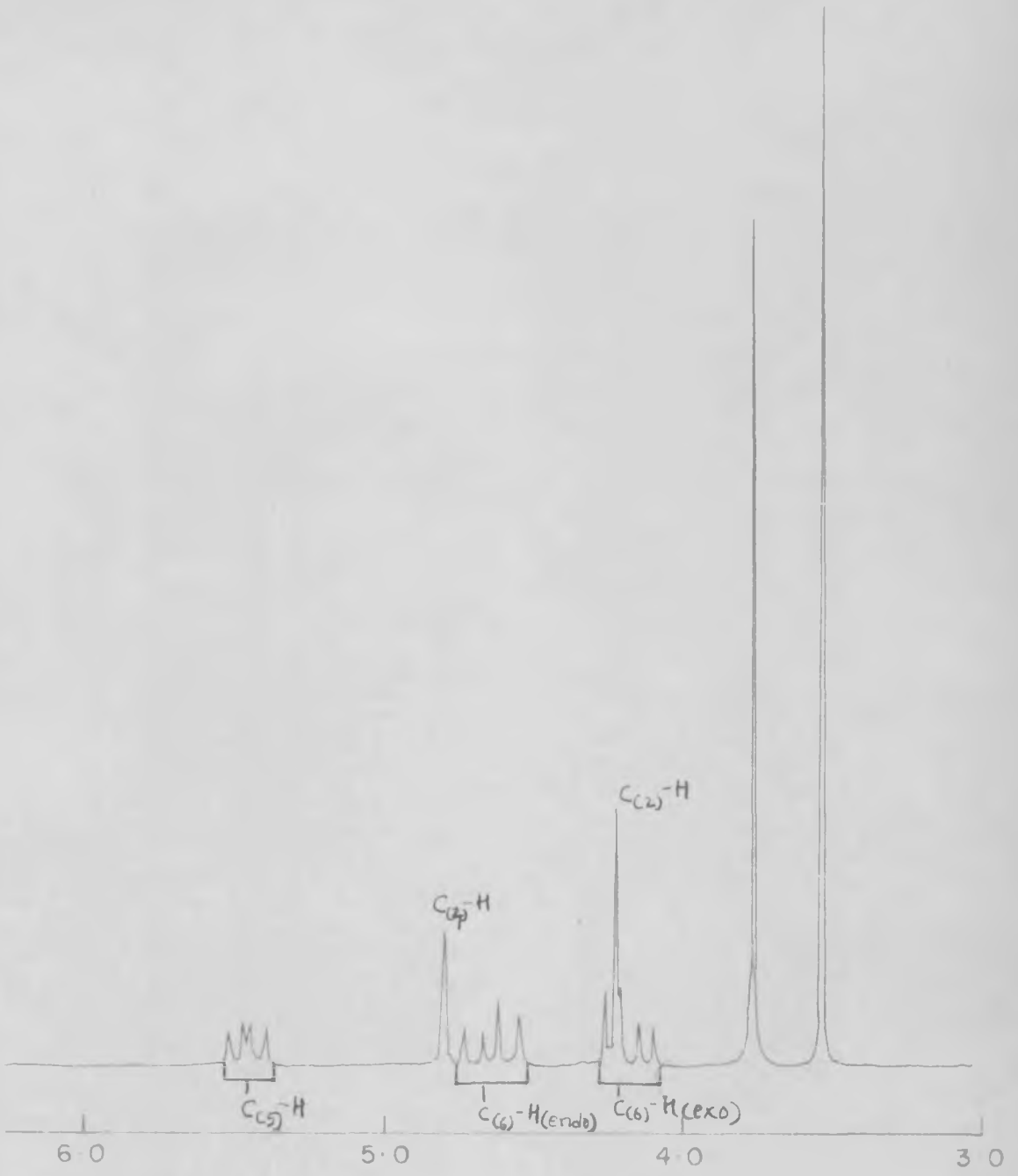



Fig. 7. 90 MHz PMR Spectrum of Isodimethyl-p-nitrobenzoate of AA in $CDCl_3$

Table - II

| No. | Compound | Proton chemical shifts in ppm | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | $C_{(4)}$-H | $C_{(5)}$-H | $C_{(2)}$-H | $C_{(6)}$-$H_2$ | $C_{(2)}$-OMe | $C_{(3)}$-OMe | Others |
| 1. | Iso-dimethyl AA | 4.59 | 4.46 | 4.21 | $H_{(endo)}$ 4.34<br>$H_{(exo)}$ 4.07<br>$J_{5,6(endo)}$ 5.8<br>$J_{5,6(exo)}$ 3.2<br>$J_{6(exo),6(endo)}$ 10 | 3.73 | 3.52 | |
| 2. | Iso-2-O-benzyl-3-O-methyl AA | 4.82 | 5.49 | 4.24 | $H_{(endo)}$ 4.67<br>$H_{(exo)}$ 4.200<br>$J_{5,6(endo)}$ 6.3<br>$J_{5,6(exo)}$ 4.5<br>$J_{6(exo),6(endo)}$ 10.5 | 3.79 | 3.56 | Phenyl protons = 8.28 |

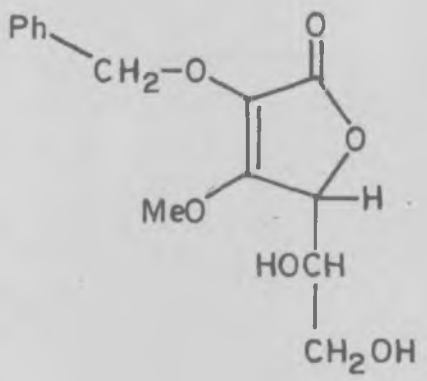
contd...

Table - II (contd.)

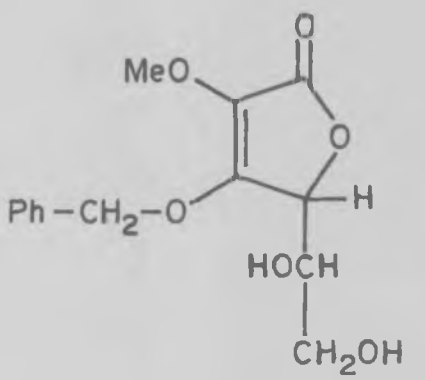
| No. | Compound | $^{13}C$ Chemical shifts* in ppm | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | $C_{(1)}$ | $C_{(2)}$ | $C_{(3)}$ | $C_{(4)}$ | $C_{(5)}$ | $C_{(6)}$ | $C_{(2)}$-OMe | $C_{(3)}$-OMe |
| 1. | 2,3-di-O-methyl-AA | 169.4 | 123.2 | 158.2 | 75.0 | 69.7 | 62.5 | 58.9 | 59.8 |
| 2. | Iso-dimethyl-AA | 171.7 | 79.9 | 109.8 | 88.3 | 74.5 | 75.4 | 51.1 | 58.4 |
| 3. | Iso-dimethyl-AA p-nitrobenzoate | 163.9 | 79.8 | 121.4 | 85.1 | 77.0 | 72.8 | 51.3 | 58.3 |

* The $^{13}C$ shifts are all expressed in $\delta$ ppm downfield from TMS. The spectra were recorded at 22.63 MHz using dioxane as an intermediate external standard with a value of 67.4.

For the assignment of the stereochemistry of this product, it was decided that it would be helpful to compare its PMR spectrum (Fig.8) with those of the iso derivatives of the mono-methyl monobenzyl ethers. Both 2-O-benzyl-3-O-methyl (XVII) and 2-O-methyl-3-O-benzyl ethers (XVIII) were therefore prepared.




XVII




XVIII

The former was prepared by reaction of the sodium salt of the 5,6-acetonide of 3-O-methyl-AA with benzyl chloride. The latter was obtained by the diazomethane methylation of the 3-O-benzyl derivative obtained in benzylation experiments on AA which are to be dealt with in the next chapter. Both these ethers were isomerized by procedures analogous to that described for the 2,3-dimethyl ether to get the corresponding isomeric compounds (XI and XIX). The PMR spectra for these two bicyclic derivatives are shown in Fig. 9 and 10 respectively. The yields obtained were 85 to 90% in the isomerization and the pure compounds were obtained by chromatography over cellulose.

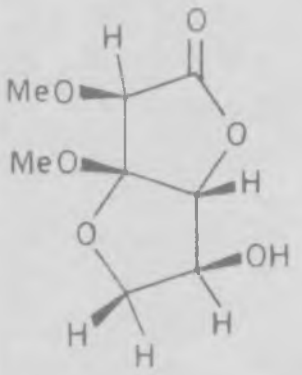






Fig. 8. 270 MHz PMR Spectrum of isodimethyl-AA in $CDCl_3$

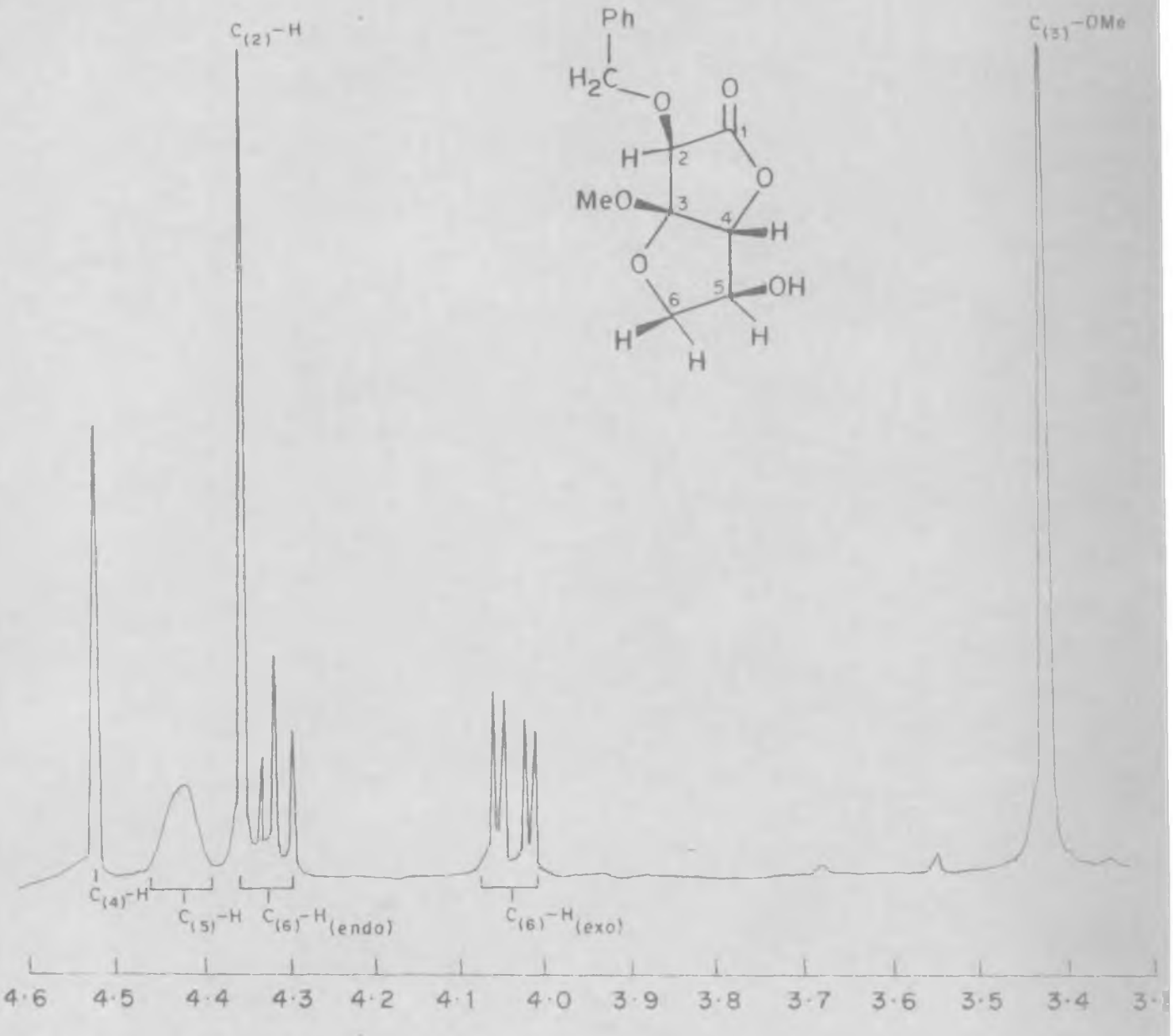



Fig. 9. 270 MHz PMR Spectrum of 2-O-benzyl, 3-O-Methyl AA-cyclic in $CDCl_3$

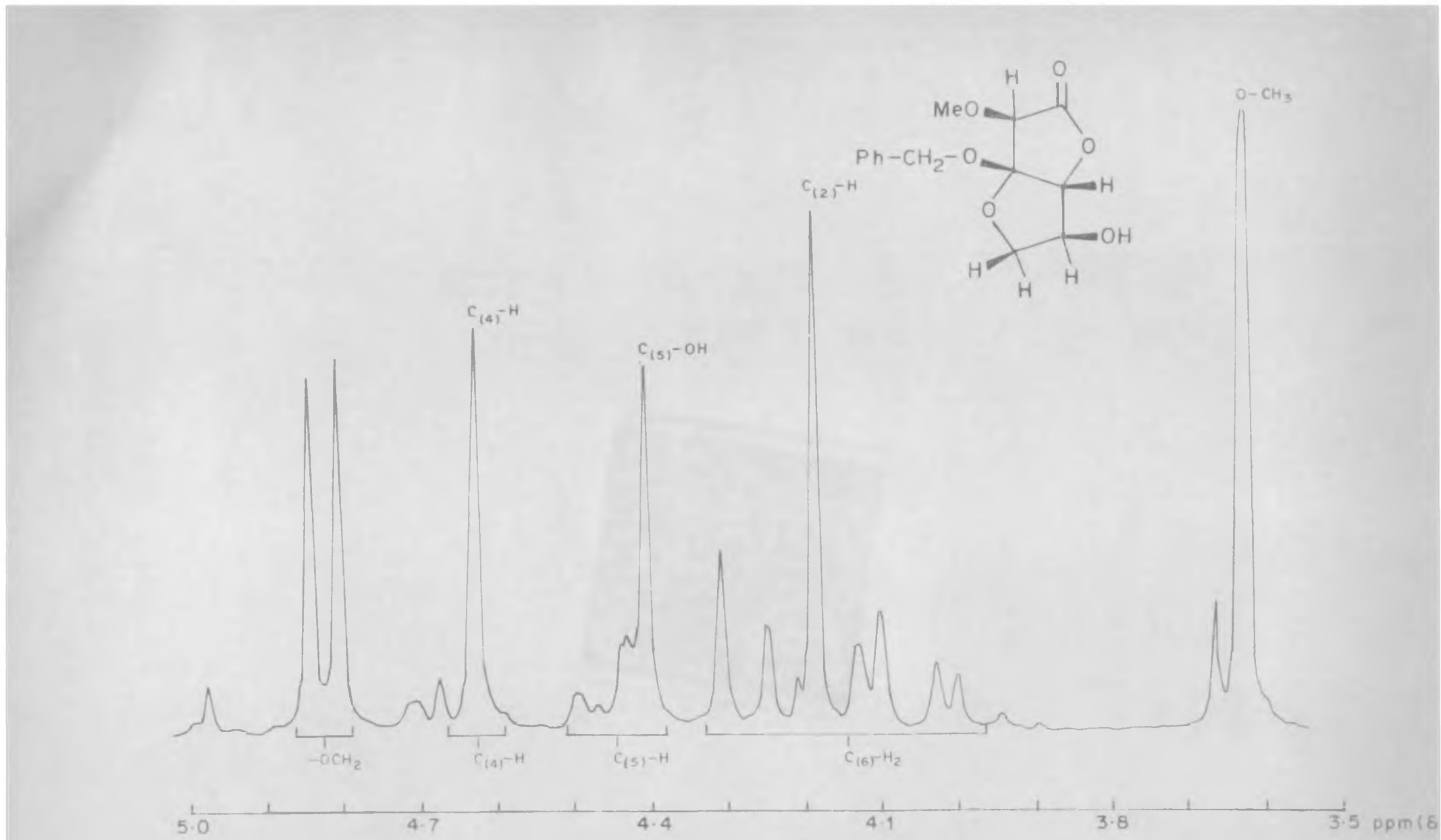
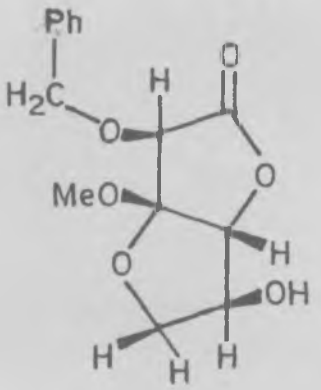
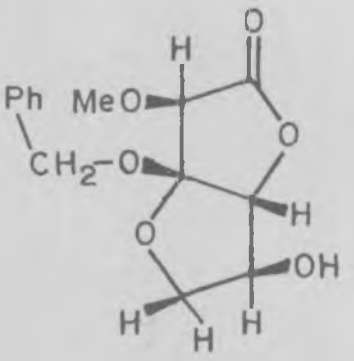



Fig. 10. 90 MHz PMR Spectrum of iso 2-O-methyl-3-O-benzyl-AA in $CDCl_3$




XI




XIX

The PMR spectral data for the three dialkyl ethers and their isomers are shown in Table III. The stereochemical assignment for iso-dimethyl ascorbic acid and its analogues depends on the assignments for OMe absorption and the effect of replacement of the 2-OMe group by a benzyloxy group ($-OCH_2Ph$), on the 3-OMe and other proton absorptions of the compound. In the conversion of the dimethyl to the iso-dimethyl derivative, the methoxyl signals of the former were shifted upfield[15]. In the spectrum of the open chain compound (Fig.11), there was a very substantial difference of shifts for the two methoxyls. The lower field signal could be readily assigned to the 3-OMe and the one at higher field to the 2-OMe, since the former was at the β-position in the α,β-unsaturated carbonyl system. In the iso-derivative, an upfield shift could be anticipated not only for the 3-OMe, but also for the 2-OMe.

Table - III

| No. | Compound | Solvent | Proton chemical shifts in ppm down field from internal TMS | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | $C_{(4)}$-H | $C_{(5)}$-H | $C_{(6)}$-$H_2$ | $C_{(2)}$-H | $C_{(2)}$-OMe | $C_{(3)}$-OMe | Other protons |
| 1. | 2,3-di-O-methyl AA | $CDCl_3$ | 4.70 | 3.83 | 3.83 | - | 3.83 | 4.17 | - |
| 2. | Iso-di-methyl AA* (270 MHz) | $CDCl_3$ | 4.50 | 4.42 | $H_{(endo)}$ -4.337 $H_{(exo)}$ -4.070 | 4.211 | 3.518 | 3.733 | - |
| 3. | 2-O-Benzyl-3-O-methyl-AA | $CDCl_3$ | 4.77 | 3.70 | 3.70 | - | - | 3.97 | $-OCH_2$ 5.05 phenyl protons 7.37 |
| 4. | Iso-2-O-benzyl-3-O-methyl AA* (270 MHz) | $CDCl_3$ | 4.53 | 4.42 | $H_{(endo)}$ 4.330 $H_{(exo)}$ 4.037 | 4.355 | - | 3.42 | $-OCH_2$ 5.55 and 5.293 Phenyl protons 7.30 |
| 5. | 2-O-methyl-3-O-benzyl-AA | $CDCl_3$ | 4.63 | 3.67 | 3.67 | - | 3.67 | - | $-OCH_2$ 5.40 Phenyl protons 7.27 |
| 6. | Iso-2-O-methyl-3-O-benzyl-AA | $CDCl_3$ | 4.63 | 4.41 | $H_{(endo)}$ 4.36 $H_{(exo)}$ 4.07 | 4.19 | 3.62 | - | $-OCH_2$ 4.83 Phenyl protons 7.03 |
| 7. | Cyclic-3-O-methyl-AA (stable epimer) | $CDCl_3$ | 4.48 | 4.27 | $H_{(endo)}$ 4.21 $H_{(exo)}$ 3.85 | 4.48 | - | 3.43 | - |

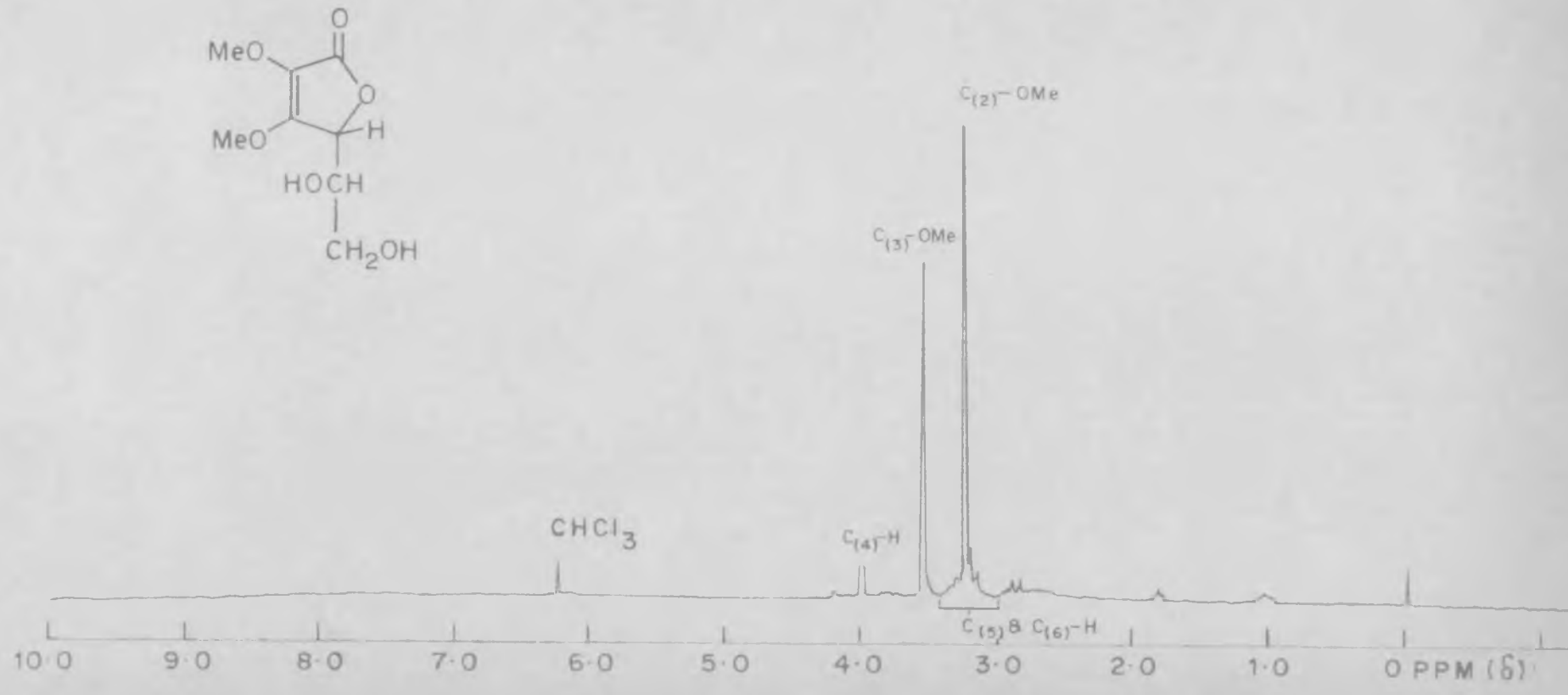
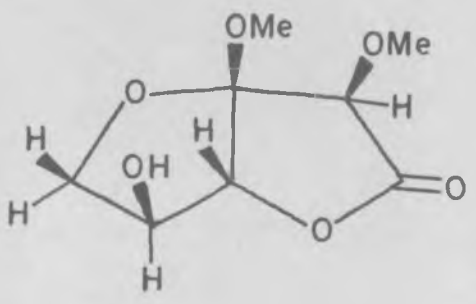



Fig 11. 90 MHz PMR Spectrum of 2,3-dimethyl ether of AA in $CDCl_3$

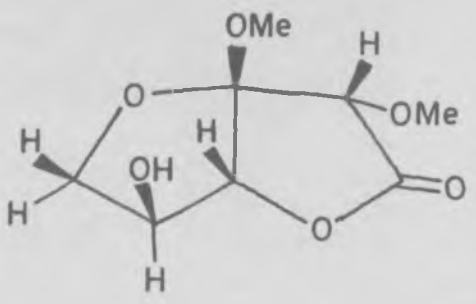
The 3-OMe, an enol ether function subject to the electron withdrawing action of the lactone carbonyl in the starting compound, was going over to a ketal form in the isomeric compound. A relatively large upfield shift could therefore be anticipated for this change. In comparison, the 2-OMe, which was an enol ether function, was changing over to an ordinary ether and the shielding change associated with it was smaller, but in the same direction. The methoxyls of the iso derivatives were assigned on this basis, the lower field one being assigned to the 3-OMe. This was consistent with its ketal character. In the spectrum of the analogous derivative, where the 2-OMe was replaced by a 2-O-benzyl group, the only signals, in addition to the that of the $C_{(2)}H$, whose absorption positions were affected were those of the 3-OMe and $C_{(4)}$-H. The 3-OMe signal suffered an appreciable shift of 0.33 ppm and the $C_{(4)}$-H signal, one of around 0.067 ppm. The $C_{(5)}$- and $C_{(6)}$-proton absorptions were practically unaffected. This pattern of effects was consistent only with an assignment where the 2-O-benzyl and the 3-O-methyl are cis to each other i.e. the 2-O-benzyl had to be exo and the proton attached to $C_{(2)}$ endo in the bicyclic system. In the spectrum of the epimer of this derivative, substantial high field shifts would have been expected for the $C_{(5)}$- and $C_{(6)}$-protons, since the rotation of the O-$CH_2$-Ph group could bring the phenyl close to these protons in the required conformation.

The data for the iso-derivative of the 2-O-methyl-3-O-

benzyl (XIX) were also obtained to get some idea of the benzyl substitution effects, although in this case the benzyloxy group was at the ring junction and its steric effects could be appreciably different from those of the 2-benzyloxy group. For the 2-benzyloxy case the motion of the benzyl group would be restricted by ring A and 3-OMe at the ring junction. But, in the 3-benzyloxy case the movement of the benzyl group would be restricted mostly by the 2-OMe group and only a little by the ring system. The cis effect of the 3-benzyloxy group on the 2-OMe was therefore less than that of the similar effect obtained when the positions of this functionalities were exchanged. A Dreiding model of the iso-dimethyl derivative showed that the ring system had only a little conformational flexibility and that the $C_{(2)}$-H of this compound was in quasi equatorial position with respect to the lactone function. The $C_{(2)}$-O linkage was similarly in a quasi axial situation. This geometry apparently accounts for the difficulty in its enolization and consequent resistance to oxidation. In contrast, its epimeric derivative (XX) should be expected to



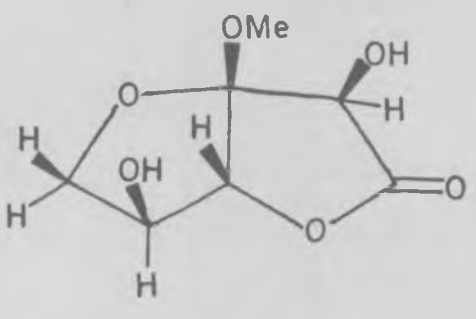

X



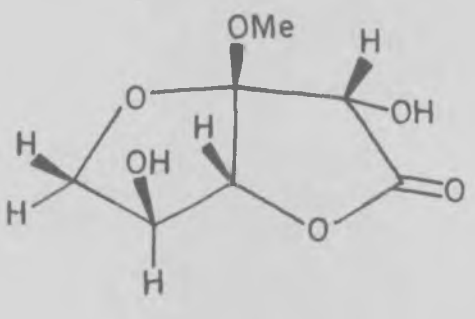

XX

enolize readily and would be more susceptible to oxidation and ring opening. These properties are of considerable interest in connection with the question of the existence of bicyclic forms for AA itself.

The endo stereochemistry for the $C_{(2)}$-proton assigned for the iso-derivatives is supported by the reported properties of bicyclic forms of 3-O-methyl-AA[13] (XII and XIII)




XII




XIII

which refused to isomerize under the conditions employed for the di-ethers we have discussed. Methylation of AA with methanol in the presence of $BF_3$ gave a set of two mono-methyl derivatives, one of which was found to be more stable than the other. These were shown to be bicyclic in nature and the Cotton effects in the circular dichroism of these derivatives as well as IR data for the compounds and their acetates were shown to suggest that the less stable one had

an endo hydroxyl group and the more stable one an exo-hydroxyl in a 3,6-anhydro-structure. In an attempt to repeat this work, we could obtain only the more stable iso-compound.

The $^{13}C$ chemical shifts for several of the ether derivatives, including the 2,3-di-O-methyl derivative, its bicyclic isomer and its p-nitro-benzoate, have been measured. The shifts for the latter two were consistent with bicyclic structures. The data for these two compounds have been already presented in Table II. The assignments and related matters are discussed elsewhere.

2,3-Di-O-methyl-6-trityl AA (XXI) was prepared by a modification of the procedure described in the literature. The preparation never came through satisfactorily when the procedure from literature was adopted. When the material was employed here, isomerized by the sequence of operations, a new substance was, of course, obtained. But, the PMR spectrum (Fig.12) of the product showed that it was a binary mixture. Considerable difficulty was encountered in the separation of the components and no satisfactory procedure has so far been worked out.

One of the derivatives that we were specially interested in was the 6-methyl ether (XXII) of AA which does not seem to have been prepared by any one so far. It was thought that it might be possible to obtain this by demethylation of the 2,3,6-trimethyl ether of AA whose preparation has been described. The 2,3-dimethyl ether could be converted into

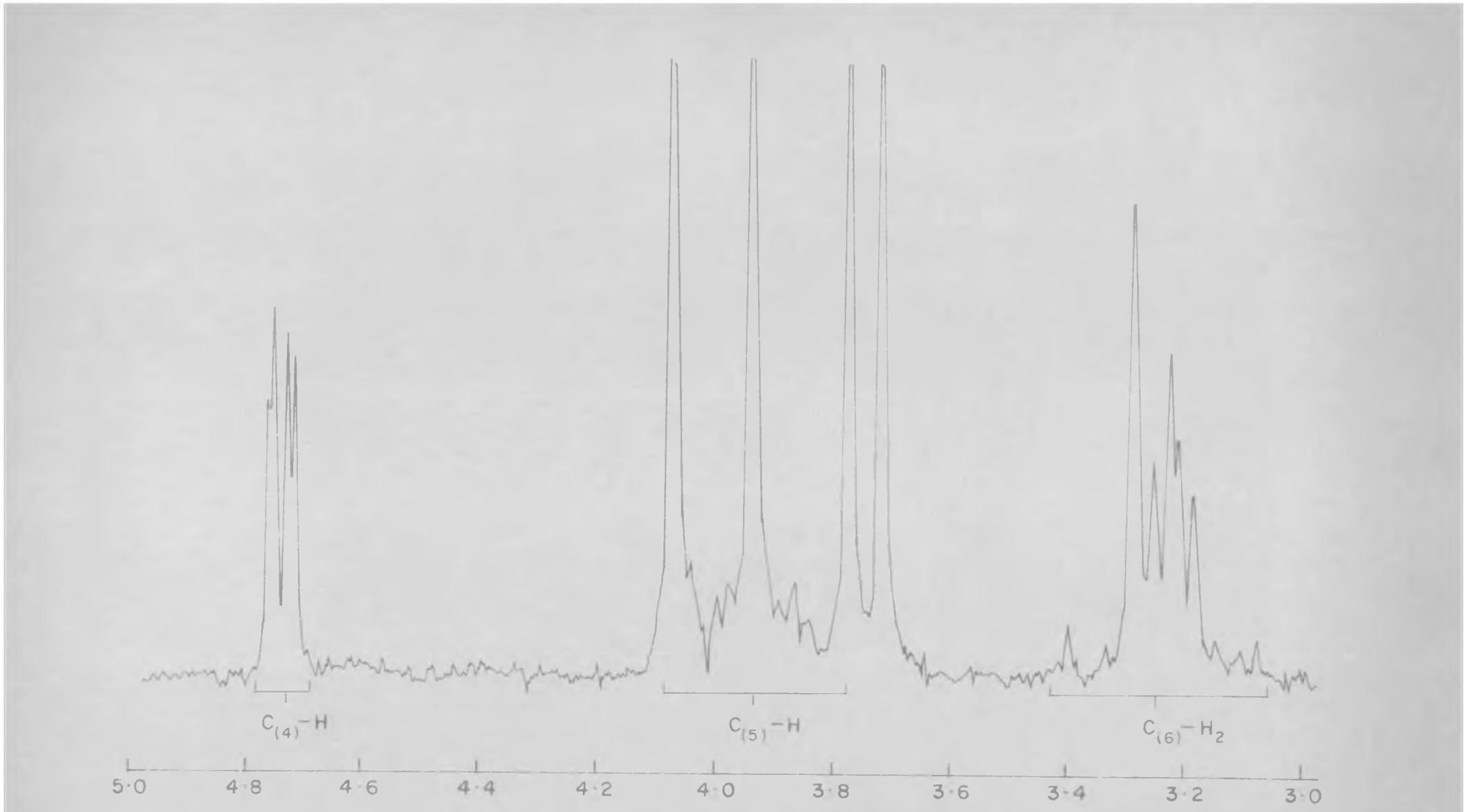
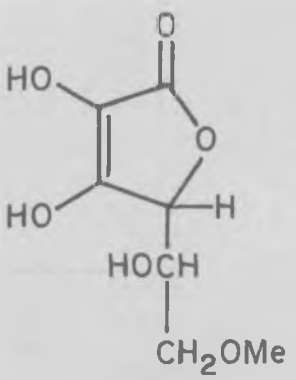
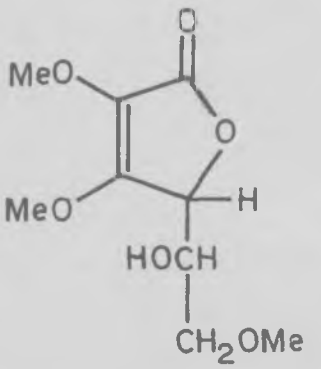



Fig. 12 90 MHz PMR Spectrum of the isomerised product of 2,3-di-O-Methyl, 6-O-trityl-AA in $CDCl_3$




XXII




XXIII

the 2,3,6-trimethyl ether (XXIII) by treatment with diazomethane in the presence of fluoboric acid. But attempts to remove the enol ether methyls did not succeed. Even with strong hydriodic acid only the 3-OMe could be demethylated. Employment of elevated temperatures and $AlCl_3$ did not help. In the preparation of 2,3,6-trimethyl ether, it was first obtained as an oil, and, again, chromatography over cellulose had to be resorted to in order to get the pure material.

Conclusion

The stereochemistry of iso-dimethyl ascorbic acid has been established with the help of a couple of its mono-benzyl analogues using PMR spectral data. The $C_2$-proton has been shown to be endo in the bicyclic structures for these derivatives. The instability of the epimeric form is obviously related to the difficulty of formation of this

type of form in any appreciable amount for ascorbic acid itself. Chromatographic procedures have been worked out for the purification of several ether derivatives including the 3-methyl, 2,3-dimethyl, 3-methyl-2-benzyl, 3-benzyl-2-methyl, 2,3,6-trimethyl and 2,3-dimethyl-6-trityl ethers as well as several isomerization products that could be obtained from these.

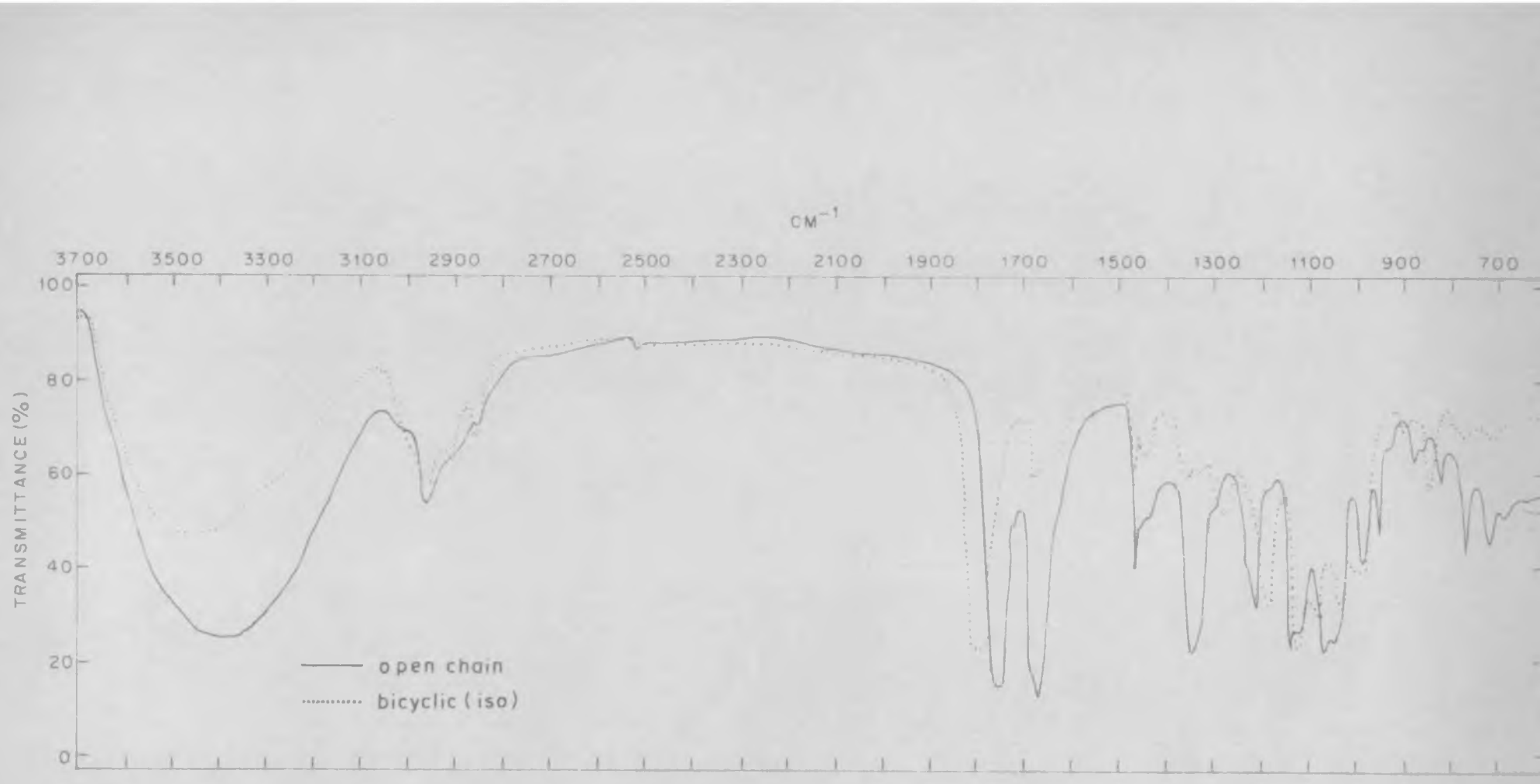



Fig. 13 IR Spectrum of 2,3-dimethyl ether of AA & iso dimethyl AA

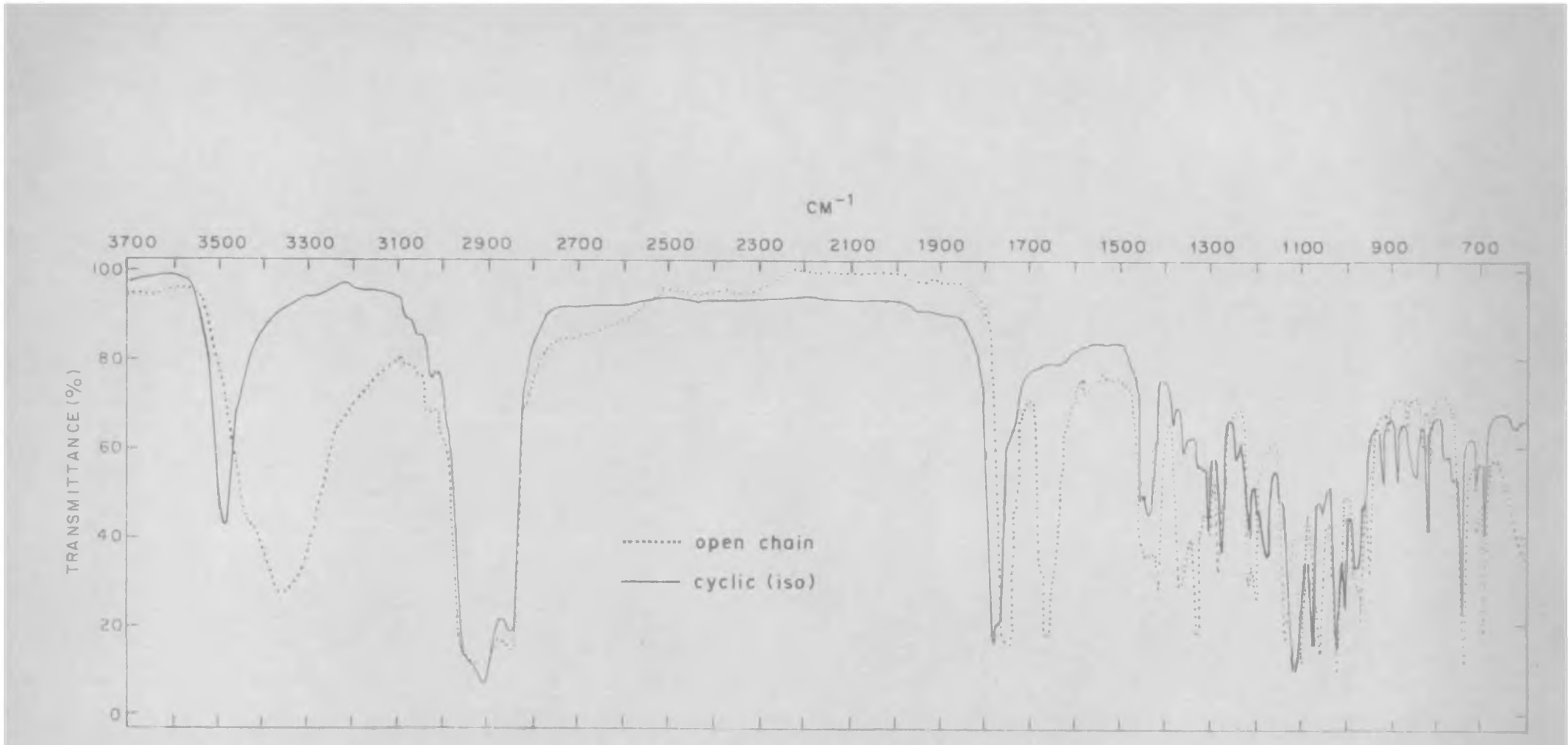



Fig. 14. IR Spectrum of 2-O-benzyl-3-O-methyl-AA and its iso derivative

Experimental

Diazomethane was generated from nitrosomethyl urea in the usual manner in ether solution[16]. The urea was washed with ether to make it free from any ether soluble impurities. The ether used for the preparation was totally free of peroxides. The solution of diazomethane was standardised against ascorbic acid, two equivalents being required for conversion to the dimethyl derivative. Persistence of the colour of diazomethane beyond 10 minutes was taken as the criterion for the end point.

TLC of the reaction mixture was done on silica gel plates. The developing solvent was prepared by mixing methanol, acetone and benzene in the ratio of 15:15:70. Spots were identified using an iodine chamber. In all the experiments described here, the solvents were removed from reaction mixtures at a temperature of about 15-25°C in a rotary evaporator under suction. In no case was the temperature allowed to exceed 30°C.

1) 3-O-Methyl-AA

AA (3.0 g) was dissolved in dry methanol (50.0 ml) and the solution was cooled to the required temperature (methylations at -40, -10, 0°C were tried). Diazomethane solution was also cooled to the same temperature. A calculated quantity (one molar equivalent) of the diazomethane solution was slowly added to the AA solution, so that the temperature rise was not more than 5°C. At the end of the reaction, the decolorisation of added diazomethane solution became very slow. But, in no

case could we observe the white precipitate described earlier by Vestling and Rebstock[6]. The average methylation was 97-98% as judged from PMR spectra of the oils obtained on removal of solvent. (The $C_{(4)}$-H to OMe integration ratio should be 1:3 in the pure compound). The TLC of the material indicated three distinct spots. The middle spot was very high in intensity and was due to 3-O-Me-AA. The very small fast moving spot was probably 1-O-methyl-hetero-AA. The slow moving spot was that of unmethylated AA.

Commercial chromatographic silica gel was found unsuitable for chromatography of the methyl ether, since it contained small amounts of unwashed mineral acid. However, it could be made neutral by the following procedure. It was stirred with excess of saturated sodium bicarbonate solution for 3 to 4 hrs and left overnight in the same medium. The material was then washed several times with distilled water, till the washings were completely neutral. This was then activated at 700-800°C for 4 to 5 hrs. From the material thus obtained, grade-II silica gel was made in the usual way by treatment with the requisite quantity of water. The grades of silica gel obtained was checked with standard dyes[17].

The crude oil from the reaction was loaded with a little acetone onto a one inch silica gel column, prepared from 200 g of silica gel grade-II in pet.ether (60-80°C) slurry. It was first eluted with acetone-pet.ether (20:80). Some unidentified

impurity was obtained in this fraction. The elution was continued until no further material come out. The pure 3-O-methyl derivative was then eluted out with 30 to 45% (v/v) acetone in pet.ether. This fraction was concentrated by removing the solvent under suction. The turbid solution was allowed to stand overnight which deposited 3-O-methyl AA as beautiful needles, m.p. 121-122°C, yield 80-85% UV $\lambda_{max}$ 245 nm, $\epsilon$ = 13050; IR $\nu_{max}$ 1740 and 1675 $cm^{-1}$; $[\alpha]_D$ + 28.9° in water; [Found: C, 43.9; H, 5.0; Cal. C, 44.2, H, 5.3%].

The chromatographic procedure however failed to give 1-O-methyl-hetero-AA which is formed only in small amounts as indicated by the TLC of the crude methylation products. For getting 1-O-methyl-AA, a different set of methylations were done under the conditions described. The crude oils obtained were seeded with the chromatographed 3-O-methyl AA. The oils on standing for long did crystallise. The solid mass was extracted with dry acetone to get an acetone insoluble portion. (The fresh oils however did not give any acetone insoluble portion). In many experiments, it turned out to be only unreacted AA. However, four of the methylations done at -40°C gave an acetone insoluble portion which contained mainly 1-O-methyl AA. This was crystallised 5 times from acetone-methanol-pet-ether (60-80°) mixture to get shining flakes melting at 152-153°C.

2) 2,3,di-O-Methyl ascorbic acid

Ascorbic acid (3 g) was dissolved in dry MeOH (50 ml) and

and cooled to 0°C and the diazomethane solution, similarly cooled to 0°, was added slowly, not allowing the temperature to rise above 5°C, until a yellow colour persisted. The mixture was allowed to stand over-night at 10-15°C after addition of a 5% excess of diazomethane. The solvent was then stripped at 50-60°C under suction in a rotary evaporator to get a light yellow oil.

The 3-O-methyl ether could also be further methylated to get 2,3-di-O-methyl ether by a similar procedure. The oil obtained in this case was easier to crystallise.

Even if the crude oil obtained from methylation of AA eventually crystallised, only half the material came out in crystalline form. Chromatography had to be resorted to get good recovery. The oil was dissolved in a small quantity of acetone and loaded on to a one inch column of cellulose (200 g) which was packed in a ether slurry. The dimethyl ether was eluted out neatly with peroxide free ether. Nearly all impurities remained on the column. On concentration of the ether solution and allowing it to stand, the dimethyl ether separated out as sugar-like crystals m.p. 59°C. This was the hydrated form, which, on drying at room temperature in a vacuum desiccator over $P_2O_5$ and recrystallisation from chloroform, gave pure dimethyl ether m.p. 62-63°C. Yield 85%, UV $\lambda_{max}$ in water 232.5 nm ($\epsilon$ = 11,300), IR, 1740 and 1650 $cm^{-1}$. $[\alpha]_D$ = + 30° in water.

3) Isomerisation of 2,3-di-O-methyl-AA

Crystalline dimethyl ether of AA (4.0 g) was dissolved in distilled water (20.0 ml), to which 300 ml of 0.1 N NaOH solution was added. The solution was allowed to stand overnight in darkness. This was then treated with dilute $H_2SO_4$ equivalent to the alkali used and was again allowed to stand overnight. The pH on addition of acid was about 2-3 which gradually rose to 7. The solution was then concentrated under vacuum at 30°C, to get a light yellow oil. This oil was dissolved in chloroform and the solution dried over anhydrous sodium sulfate. Chloroform was then removed under vacuum to get an oil. The extent of isomerisation could be judged from the OMe signals from open chain and isomerised dimethyl ether which indicated a conversion of 90-95%.

2.0 G of this oil was loaded on a one-inch cellulose column which was prepared from 200 g of cellulose in pet.ether (60-80°C) slurry. The elution was started with pet.ether and then the polarity was increased with 5,10 ... 30% of added chloroform. Solvents used had to be absolutely free of moisture. Initial fractions contained some impurities. The pure isodimethyl ether came out in the 20 to 40% chloroform fractions. All the column fractions were checked by TLC and the PMR spectra. The later fractions gave the unisomerised dimethyl ether. The yield of isodimethyl AA was 80-85%. The impure samples showed absorption at 235 nm due to the presence of unisomerised dimethyl ether. The pure sample did not have

any UV absorption. IR showed in the double bond region only a C=O band at 1780 $cm^{-1}$. No C=C absorption was seen.

4) 3-O-methyl 5,6-acetonide

AA 5,6-acetonide was prepared according to the procedure described in Chapter VI. The acetonide (3.0 g) was dissolved in dry methanol (50 ml). The solution was cooled to 0°C and diazomethane solution similarly cooled was added slowly not allowing the temperature to rise above 5°C. The methylation was done only to the extent of 80%. Several trials indicated that complete methylation gave a mixture of 3-O-methyl-acetonide along with normal 3-O-methyl AA. The separation of these two was more difficult. Methylation to the extent of only 80% gave a mixture of 3-O-methyl-acetonide and unreacted acetonide which were easy to separate.

After the reaction, the solvent was removed at 30°C under vacuum. The light yellow oil was seeded with the pure acetonide itself. The product solidified within 2-3 days. This was then extracted with chloroform. The unreacted acetonide, being insoluble in chloroform, was filtered off. The filtrate was concentrated and pet-ether was added slowly till the solution became hazy. This solution on standing deposited needle shaped crystals of the 3-O-methyl-AA-5,6-acetonide, m.p. 89-90°C.

If the oil refused to crystallise for a long time, it was loaded on a cellulose column packed in chloroform. The elution was done only with chloroform. The chloroform solution

was concentrated and pet.ether added to make it hazy and then allowed to stand, when the pure material crystallised out.

5) 3-O-methyl-2-O-benzyl-ascorbic acid

3-O-Methyl-5,6-acetonide of AA (5.0 g) was dissolved in methanol (50.0 ml) to which one equivalent of sodium methoxide solution of 1 N strength was added. After standing for 1/2 hr, methanol was completely removed under vacuum at 40°C. The light brown solid obtained was dispersed in benzyl chloride (15.0 ml) and was heated on a waterbath for 2.5 hrs. The reaction mixture was then poured on water and subjected to steam distillation. On complete removal of benzyl chloride, the aqueous solution was allowed to cool when beautiful needles of 3-O-methyl 2-O-benzyl-AA separated out. It was further recrystallised from chloroform to get a pure product m.p. 120-121°C.

6) Iso-2-O-benzyl-3-O-methyl-AA

The isomerisation of 2-O-benzyl-3-O-methyl AA was done by a procedure very similar to that employed for dimethyl ether of AA. Since the isomerised product was not very much soluble in water, it separated out as an oil. This oil was extracted into chloroform. The aqueous layer was also concentrated at 30°C, which gave very small amount of a yellow oil. This oil was dissolved in chloroform and mixed with the $CHCl_3$ extract. The combined $CHCl_3$ extract was dried over anhydrous sodium sulfate and the solvent removed under vacuum. The yellow oil obtained crystallised without much difficulty. The isomerisation

yield was almost 90-95%. The solid mass was recrystallised from $CHCl_3$-pet.ether mixture, m.p.73-75°C. Total yield was about 90-95%. (IR $\nu_{max}$ 1790 $cm^{-1}$ due to C=O).

7) 3-O-benzyl, 2-O-methyl AA and its isomerisation

3-Benzyl ether of AA obtained from the benzylation of ascorbate in 50% aqueous acetone as described in the next chapter was further methylated by diazomethane at 0°C. Concentration of the reaction mixture gave a yellow oil, which did not crystallise even after chromatography over cellulose. But its PMR spectrum improved on chromatography. This oil was isomerised further by the procedure described above. There also, the isomerised product had poor solubility in water. It separated in the form of an oil. The reaction mixture was extracted exhaustively with $CHCl_3$. The $CHCl_3$ layer was dried over anhydrous sodium sulfate. On concentration, this gave a light yellow oil.

This oil was purified by chromatography on a cellulose column. The column was packed in pet.ether (60-80°). The oil to cellulose ratio was 1:100. Elution was started with pet. ether. The pure isomeric compound was eluted only with 5% $CHCl_3$ in pet.ether. The yield of pure product on chromatography was about 70%. The percentage of the unisomerised product in this case was found to be higher than those in the other two isomerisations.

8) Unstable monomethyl ether

AA (6.0 g) was dissolved in methanol (100 ml) and solution

cooled to -5°C. Two drops of 20% aqueous KOH solution was added to this. Ethereal diazomethane cooled to -5°C was slowly added to this to get a curdy white precipitate. This precipitate was centrifuged. The supernatent solution was discarded. The residual white material was dispersed in methanol and again centrifuged. This material was insoluble in most of the organic solvents. Finally, it was washed with acetone and then with ether several times. It was filtered off under suction very rapidly. This material was highly unstable. When exposed to air, it began turning brown very rapidly. It was immediately dissolved in $D_2O$, and a PMR spectrum was recorded. No other study could be done owing to its extreme unstability.

9) Bicyclic 3-O-methyl ether

AA (5.0 g) was dissolved in dry methanol (100.0 ml) to which freshly distilled $BF_3$ ethereate (5.0 ml) was added. The solution was refluxed for 10 hrs and then neutralised with triethylamine (7.5 ml). The solvent was removed and the reaction mixture loaded on to a one inch silica gel column (200 g of silica gel) packed with $CHCl_3$. The elution was started with $CHCl_3$. The polarity was then gradually increased with acetone. The initial fraction contained some decomposition products. The pure compound was eluted with 30% acetone in $CHCl_3$. Later fractions gave only some decomposition products and a large quantity of unreacted AA remained on the column. The fraction containing bicyclic 3-O-methyl AA on concentration gave sugar-like crystals, which had poor solubility in acetone and $CHCl_3$.

This gave a single spot on silica gel TLC with the solvent system, 6:4 v/v acetone-$CHCl_3$, for which the expected epimers are reported to have different $R_f$ values. This crystalline compound had a m.p. of 167-169°C, indicating that it was the stable epimer (yield 500 mg). The PMR spectrum also indicated that it was only one compound. Repeated attempts to isolate the reported unstable epimer were not successful.

10) 2,3,6-Tri-O-methyl ether

Pure dimethyl ether (1.7 g) was dissolved in dry methylene chloride (25.0 ml) to which 0.50 ml of fluoboric acid (FBA) solution was added. This solution was cooled to 0°C and about 15.0 ml of diazomethane in ether solution was added and a further 0.2 ml of FBA solution was added. The following successive portions of diazomethane and FBA solution were then added in 3 sets over a period of 1 hr. The solution was then filtered and the solvent removed at 30°C under suction. The light yellow oil obtained was loaded on to a one-inch cellulose column (150 ml) packed in pet.ether. The elution was started with pet.ether (60-80°). The polarity was gradually increased with ethylacetate. The pure tri-O-methyl compound got eluted out with 30% ethyl acetate in pet.ether (60-80°). On concentration of the fractions, the trimethyl ether crystallised out as colourless needles, m.p. 99-101°C.

Preparation of FBA solution: Fluoboric acid was prepared by mixing boric acid and HF in equimolecular ratio at 0°C.

0.133 Ml of FBA was dissolved in 250 ml of a solution of 3:1 diethyl ether - methylene chloride.

11) Demethylation of 2,3,6-tri-O-methyl AA

Trial demethylation experiments were done on small samples in a NMR sample tube (5.0 mm diameter) and the reaction was followed by PMR spectra.

Demethylations with $H_2SO_4$, HCl, HBr of various strengths and including the concentrated acids were tried. The trimethyl ether (25 mg) was dissolved in 0.5 ml of the acid, and the PMR spectrum was recorded at different time intervals. At room temperature, no demethylation took place with any of the acids. However, at high temperature there appeared to be some decompositions taking place. Temperatures up to 50°C were tried. But in no case did we succeed in demethylation of the 2- and 3-OMe groups. However, with concentrated HI at 40°C, demethylation only at 3 position took place giving a 2,6-di-O-methyl ether which was not isolated.

Refluxing 2,3,6-trimethyl ether in methylene chloride in presence of anhydrous aluminium chloride was also tried. Here also, the reaction was followed by PMR. No demethylation at 2,3 or 6 positions took place.

12) 2,3-Di-O-methyl-6-trityl ether

2,3-Di-O-methyl AA (0.56 g) was dissolved in 3.0 ml of pyridine to which trityl chloride was added (2.218 g). This solution was left in a desiccator and kept in the dark for

about 7 days. About 100.0 ml of $CHCl_3$ was added first to the reaction mixture. Pet.ether (60-80) was then slowly added to this solution when a brown oil precipitated and the supernatent solution was milky. The brown oil which was mostly a mixture of pyridine hydrochloride and some decomposition products, was discarded. The milky solution was concentrated under vacuum to get a light yellow oil. This oil was washed several times with pet.ether. The oil eventually crystallised. The product was recrystallised from methanol to get colourless needle shaped crystals, m.p. 154°C.

13) Isomerisation of 2,3-dimethyl-6-trityl ether

i) 2,3-Dimethyl-6-trityl-AA (1.0 g) was dissolved in dry methanol (5.0 ml). 0.5 N sodium methoxide solution (3.5 moles) was added to this and the solution boiled for 5.0 minutes. This solution was then neutralised completely with methenolic HCl solution. The solution was then poured on ice and the white precipitate obtained was filtered off and washed several times with distilled water. The solid was recrystallised from dry MeOH.

ii) 2,3-Dimethyl-6-trityl ether (1.0 g) was dissolved in MeOH. The solution was saturated with dry ammonia and left for two days. The solvent was removed under suction to get a white solid which was crystallised from methanol (m.p. 176°C).

The isomeric compound obtained by this procedure had the same m.p. (178°C) as reported in literature. The PMR spectrum

clearly showed that the product obtained was not a single isomer but was a mixture of two products. The separation of this mixture into its components was found to be difficult and was not achieved.

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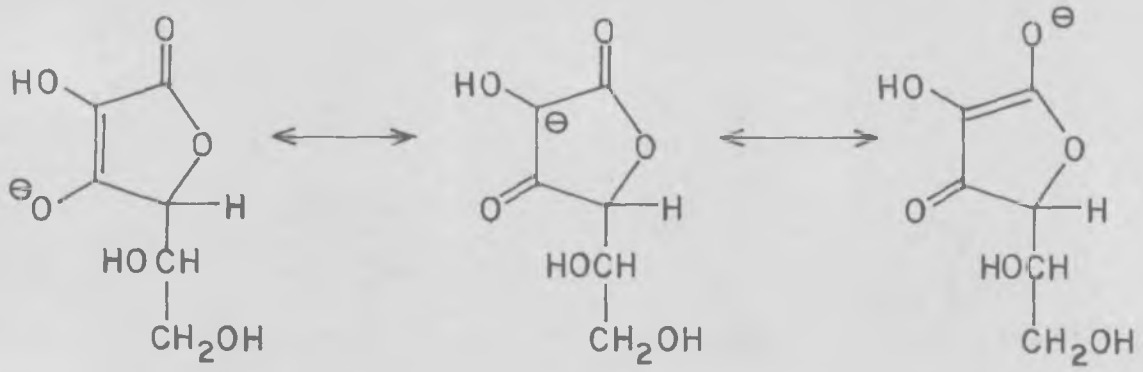
# Chapter V

## BENZYLATION OF ASCORBIC ACID AND ITS MECHANISM

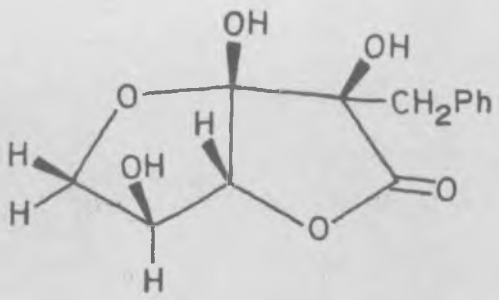
Introduction

Ascorbic acid on treatment with benzyl chloride in aqueous acetone in the presence of 92 % potassium hydroxide was shown to give a 2-C-benzyl derivative in 12 to 15% yield in addition to the 3-O-benzyl derivative whose yield was also 12 to 15%[1,2]. The reactive species involved was the ambident anion (I) of ascorbic acid, and this was presumably the first instance of a reaction of this type in carbohydrate chemistry[1], where benzylation normally gives O-derivatives and has been extensively used for protection of hydroxyl groups[3-8]. A bicyclic form has been suggested for the product and the probable stereochemistry has been indicated as in II[2].




I a I b I c

The γ-lactone ring of ascorbic acid is a planar five-membered ring, and it is unlikely that the side chain at $C_{(4)}$ can have any significant steric effect on a reaction at $C_{(2)}$. Formation of only one product in the benzylation reaction would, therefore, be anomalous since attack by benzyl chloride should be nearly equally facile from either




II a

side of the five-membered ring and two products can normally be expected. At least, there is no good reason for the total exclusion of attack from the side cis to the two carbon side chain if I is the reactive species. However, although a second C-benzylation product was looked for in the product mixture, only one could be found.

No explanation has so far been advanced for this anomaly. Actually, the position is much worse because the assigned stereochemistry for the 2-C-benzylation product requires attack from the side of the ring cis to the side chain. Also, no information is available on the behaviour of ascorbic acid analogues in the same reaction. Such information would be of considerable value in understanding the behaviour of ascorbic acid itself in the reaction. It was, therefore, decided to undertake a detailed study of the benzylation of ascorbic acid and its analogues. The study was to include among other things, a re-examination and the establishment of the stereochemistry of the isolated C-benzyl derivative of ascorbic acid and verification of the presence

or absence of a second (epimeric) C-benzyl derivative among the reaction products. Before a consideration of the results obtained, it is of interest here to survey briefly the available information on alkylation of ambident anions, more particularly those of 1,3-dicarbonyl compounds.

There has been a great deal of interest on the course of alkylation of ambident anions in the past. Particularly intensive studies have been made on phenoxide anions. The main issue in all these studies has been C- vs O-alkylation and the factors affecting the ratio of these alkylations[10-13]. Other anions have been those of nitro alkanes[14-18], substituted phenols[10-12,19-21], β-keto esters[22-27], α,β-unsaturated acids[28,29], α,β-unsaturated ketones[30-36], cyclic ketones (saturated and unsaturated)[37-41], 1,3-diketones[42-45] and other ambident anions[46-50].

Kornblum[11,12,18,20] and co-workers made systematic studies of the various factors affecting the relative extent of oxygen and carbon alkylation that are obtained in reactions of phenolates with alkyl halides. Solvent effects can manifest themselves either through a bulk property like dielectric constant or through specific solvent effects which may cause selective solvation of the species[12]. Other factors involved are the chemical natures of the ambident anionic species, the leaving group[18] and the alkyl moiety[14].

Solvent plays a very important role in deciding the course of the reaction[12]. In aprotic solvents, the dielectric

constant and the capacity for solvating anion are important. Dielectric constant has an effect on the solubility of the anion, the higher the dielectric constant the greater is the solubility. High dielectric constant solvents favour O-alkylation[12a]. The reactions are picturised as taking place between alkyl halide and ion pairs or rather clusters in which anions are associated with more than one metal ion[11]. The dielectric constant of the solvent may be expected and has been found to have an important influence on such reactions. The preference for O-alkylation that is observed in media of high dielectric constant has been rationalised in terms of the relative geometries involved in the transition states for O-attack and C-attack and coulombic attraction terms which can help or hinder alkyl-halogen bond fission. In aprotic solvents, an important additional factor comes into play which can over-ride the influence of dielectric constant. Protic solvents can selectively solvate the anionic oxygen sites through hydrogen bonding and shield these from attack by the alkyl halide, thus promoting C-alkylation[12b,c]. Thus, although water has a high dielectric constant, C-alkylation is favoured in this medium[12a,b]. Non-polar solvents also favour C-alkylation because of the decreased dissociation of the anion and formation of molecular aggregates[10,11]. This view is similar to that of Ingold[51] who had proposed that dissociated ions would give a preponderance of ether formation while aggregates would

give mostly C-alkylation. Consideration of the influence of dielectric constant in terms of its capacity to promote dissociation of the alkyl halide also leads to the same pattern of reactivity. Promotion of an $S_NI$ type of dissociation will result in greater O-attack, that is attack on the more electro negative atom in the anionic part[11,52]. Solvation can be either of the anion or of the leaving group. Heavy solvation however, affects the rate of the reaction and also the yield because it shields the anion from the approach of the alkyl group[12b,c,d,53].

Heterogenity or the homogenity of the reaction mixture also affects the O/C alkylation ratio, heterogenity favouring molecular aggregation and hence C-alkylation and homogenity favouring O-alkylation[11]. Solvents can also affect the orientation of C-alkylation as can be seen in the case of water which gives a mixture of ortho and para C-alkylation[12a,b]. In other solvents ortho alkylation is predominent. Salts of less acidic phenols favour C-alkylation due to their decreased dissociation leading to molecular aggregations[10,46,48,51]. This factor also comes into the picture when concentration of the phenoxide ion is increased, leading to lesser dissociation and consequent greater C-alkylation.

The electro positiveness of the metal cation also affects the course of alkylation, the less electro positive metals giving more of C-alkylation due to the decreased dissociation[10,23,34,40,52,54]. However, in the case of cyclic

β-keto esters like β-carbethoxycyclohexanone one gets more of O-alkylation with less electro positive metal ion[22].

The nature of the alkylating agent also plays an important role specially in molecules which are not planar. The alkylation is always from the least hindered side. In the case of benzylations of 2-nitropropane anions, the course of reaction very much depends on the substitution on the benzyl group and also on the halide atom. p-Nitrobenzyl iodide gives 95% of C-alkylation. Change in halide atom and replacement of $-NO_2$ by other groups affect the reaction very much[18].

Allylic halides which are more reactive than saturated ones favour C-alkylation[46]. The hardness of the leaving group also has an influence on the course of the reaction[18,55].

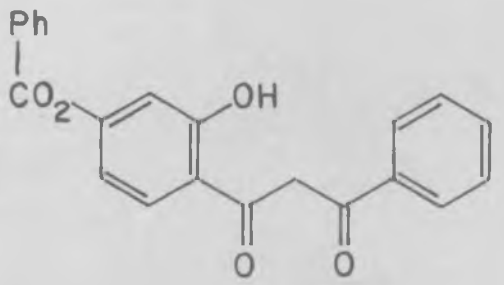
Usually, steric requirements of the alkyl group should be expected to interfere more seriously with carbon rather than oxygen alkylation[48]. However, the case of o-disubstituted phenols provides an interesting exception. Increasing bulk of the alkyl group in this case favours C-alkylation as O-alkylation is sterically more hindered[11,19-21]. The role of the steric factor is also well illustrated in nitrile anions[12c,20].

The nature of the halide ion has significant effects only on the yield of the reaction but not on the selectivity[10,46].

In the alkylation of β-keto esters, the roles played by the above discussed factors have been established[22-24,27]. Some of the alkylations of cyclic ketones have been found to be anomolous[22,24,26], but use of nonpolar solvents and low reaction temperatures have been found to minimise the abnormal reactions in some cases[24]. Halides of unusual reactivity gave more abnormalities[24].

The selective C-alkylation of dienolates, anions derived from α,β-unsaturated acids and esters has been used for many synthetic purposes[28,29]. Here, regioselectivity was found to depend very much on the metal ion. The ketone enolates from α,β-unsaturated ketones were extremely sensitive to solvents and the steric requirements of both enolate and alkylating agents[35,56]. Here also, metal ion had a great influence on regioselectivity[28]. Different solvent systems have been tried for enhancing the carbanion activity[57].

The regioselectivity is quite marked in the case of alkylations of 1,3-diketones (III). Carbon alkylation at α-sites in these compounds is very difficult[42-45]. Even if the alkylation goes at the α-positions, the yields are relatively very poor. Thus, compound IV with powdered NaOH in refluxing pyridine gave a sodium salt which in DMSO with prenyl bromide gave a mixture of C- and O-alkylated products (V) and (VI). But the yield of C-benzylation product was very low[45].



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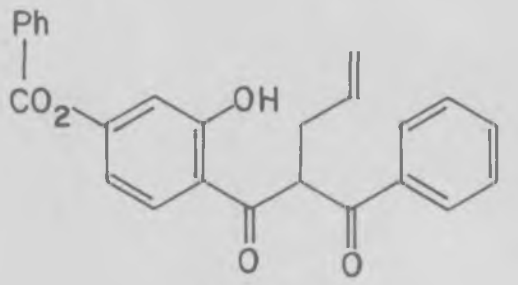

V

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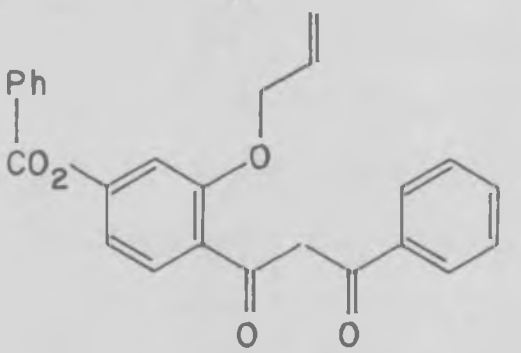
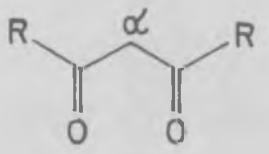


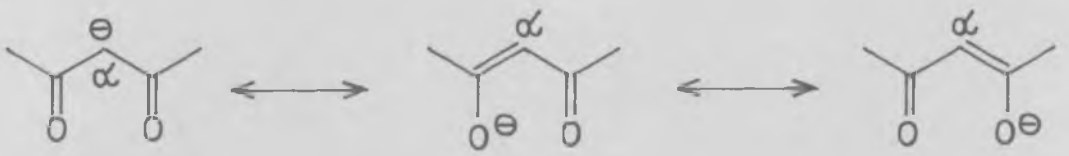
VI



III

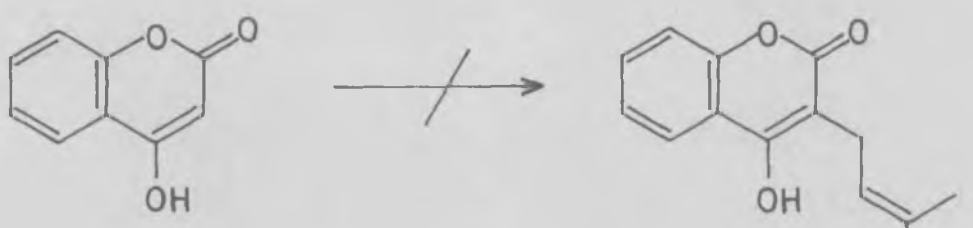
Disubstituted derivatives of ethyl benzoyl-acetate unlike those of ethylacetoacetate can either not be prepared at all or only in very small yields by treating the more substituted ester with sodium ethoxide and an alkyl iodide in alcohol[42]. However, these monosubstituted esters when treated with sodium in toluene and an alkyl halide do give the disubstituted derivatives in good yields. Renfrow and Renfrow[44] have worked out different methods for alkylation of acetoacetic esters at the α-carbon. These alkylations go very poorly in alcoholic media. The reason for not getting C-alkylation in 1,3-diketones is that the negative charge density at the

α-carbon atom is not enough for the alkylation as it is pulled away towards the two adjacent carbonyl oxygens as shown in VII.



VII

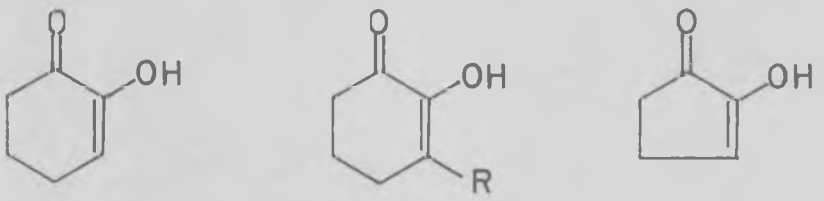
Attempts at the allylation of 4-hydroxy coumarin (VIIIa) to prepare 3-allyl-4-hydroxy coumarins (VIIIb) by different methods have not been successful[45].



VIII a VIII b

This is a situation very similar to that obtained in ascorbate anion. In general, the alkylation of 1,3-diketones at the 2-position is very difficult and the yields are very poor.

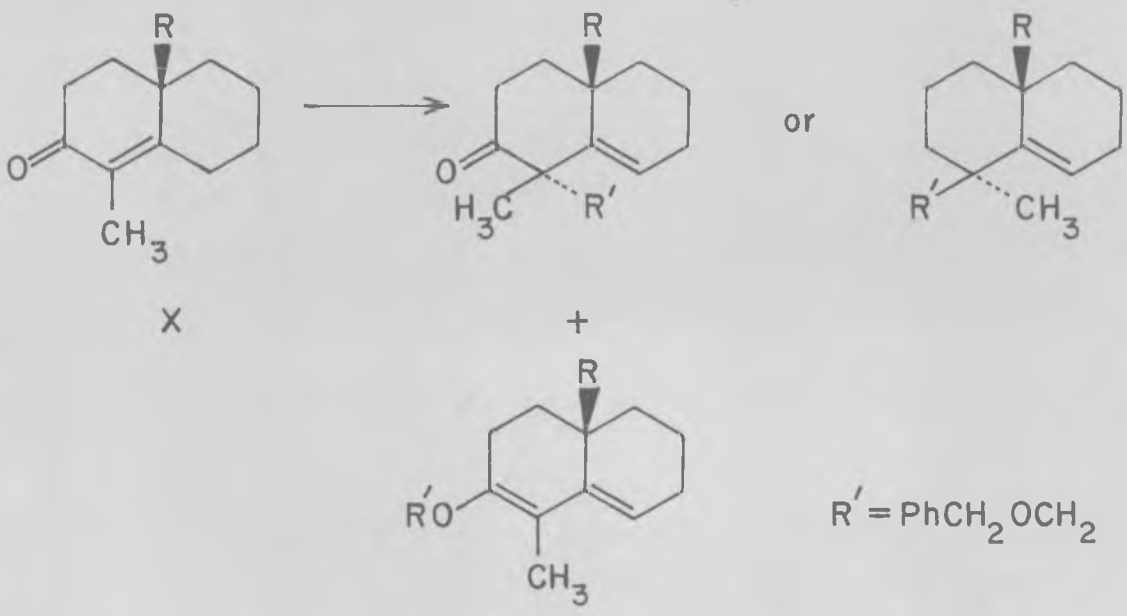
It is interesting to note that cyclic diketones of the type shown in IX, which mainly exist as enols in presence of base give only O-alkylation. Failure to achieve the



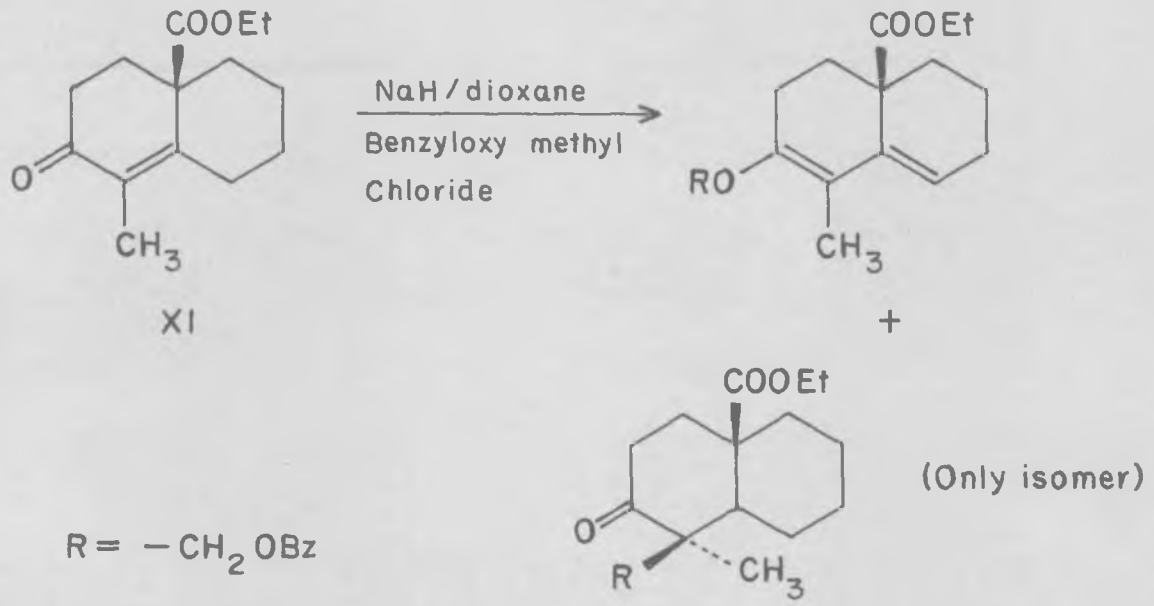
IX

C-alkylation with simple enolates derived from such molecules is consistent with Huckel molecular orbital calculations for corresponding mesomeric anions. These reveal a partial postive charge on the carbon required to act as a nucleophile[59].

Heterogenity of reaction medium, aggregation of ions, metal ion and solvation play a role in deciding O/C-alkylation ratio in the case of α,β-unsaturated cyclic ketones of the type shown below[34] (X).

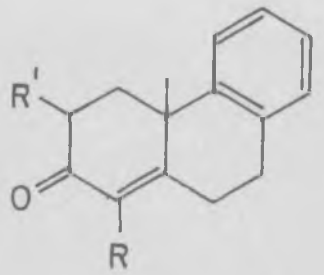


The above mentioned factors affect the ratio of α/β epimers of the C-alkylation, the size of R being the deciding factor. When R = COOEt, it is always the β-isomer that is formed and with R = $CH_3$ the α-isomer is obtained. The principle of least hindered approach is illustrated in the case (XI) with R = Me.




However, when R = COOEt it favours approach from the same side since the dipole of the group helps the polarisation that develops in the transition state because of its proximity[31,32].

Control of the stereochemistry that one can get in alkylation reactions is of considerable synthetic interest. The synthesis of resin acids involve alkylations of cyclic β-keto esters, and α,β-unsaturated ketones (XII)[33,38,40,41,60]. In these reactions, not only the O/C-alkylation ratio but



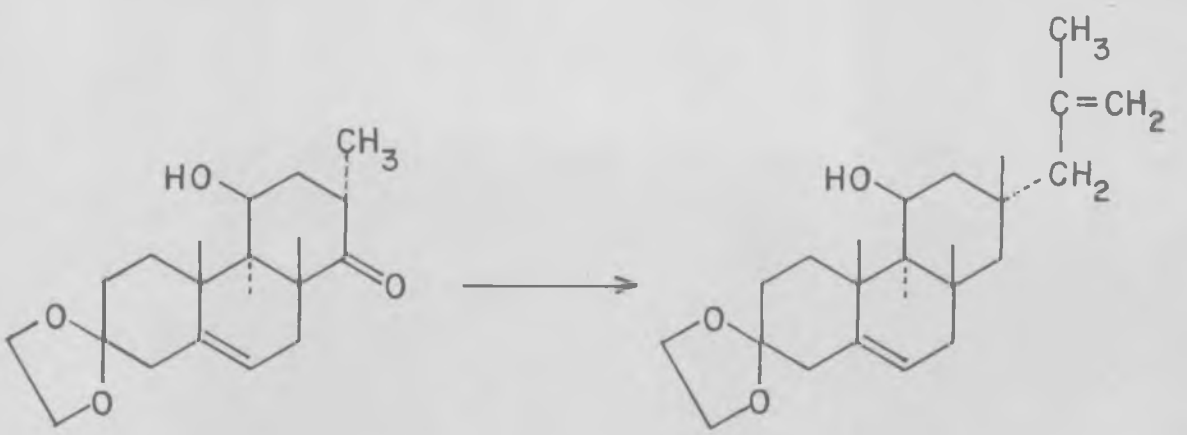

XII

R = H or COOMe

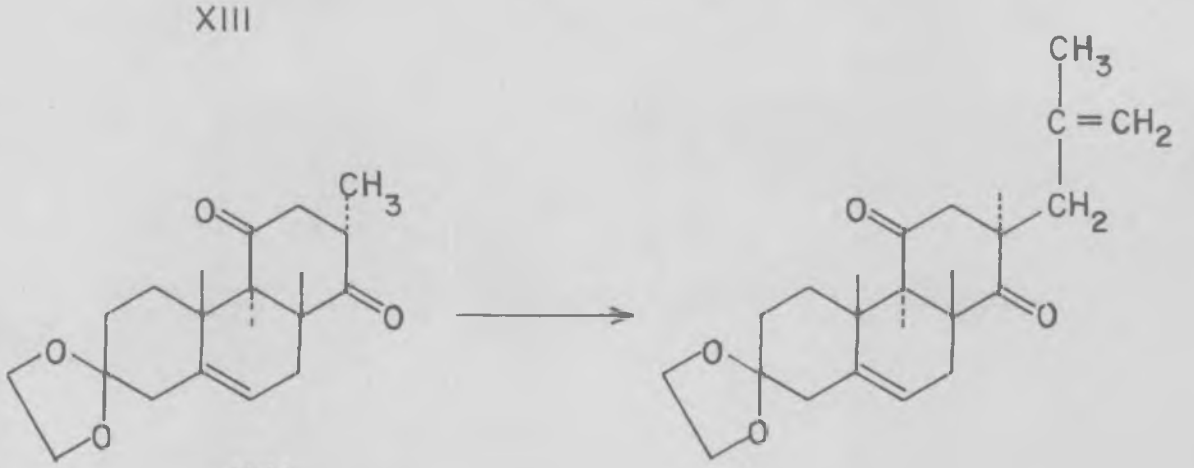
R' = H or COOMe

also the α/β epimer distribution is important from the synthetic stand point. Since the substrates have their own stereochemistry, the epimer distribution will be largely a function of steric interactions involving axial and equatorial substituents. This implies that the size of the alkylating agent will have an influence on the epimer ratio. However, several studies have shown that the nature of the enolate ion is more important than the alkylating agent in determining the stereochemical course of the reaction[33].

Stereospecific alkylations are reported in the case of several steroid molecules[37,39,61]. In adrenal steroids methylation by methallyl iodide in presence of potassium-t-butoxide and t-butanol gives the α-epimer for the monoketo compound and only the β-epimer for the diketo (XIV) compound[37].



XIII



XIV

Alkylation of 3β-acetoxy-9α- and 9β-$\Delta^7$-11-ketones of the ergostane series with methyl iodide and potassium-t-butoxide also gave high yields of products having 9α-methyl groups[39].

The foregoing survey of the nature of the results that are obtained in the behaviour of ambident anions in alkylation reactions does not provide us a ready rationalization of the reported behaviour seen in the C-benzylation of ascorbic acid. It was therefore decided that a verification of the formation of only one C-benzyl derivative in the reaction as well as re-examination of its stereochemistry was essential.

Results

Potassium ascorbate in 50% aqueous acetone was benzylated with benzyl chloride. The procedure was similar to the one followed by Jackson et al.[2] The reaction was carried out for about 80 hrs, at the end of which acetone was removed under suction at 25-30°C. The aqueous layer was washed with pet. ether to remove the unreacted benzyl chloride. It was then extracted with hot ethyl acetate. All the benzylated products got extracted into ethyl acetate. The aqueous layer was also concentrated at 30°C under vacuum. The PMR spectrum (Fig.1) of this concentrated aqueous layer showed that it contained only unreacted potassium ascorbate and no benzylated product. The ethyl acetate layer after concentration gave a light yellow oil (Oil A) which contained the benzylated products. The PMR spectrum (Fig.2) of this oil showed two prominent signals at 187 Hz and 320 Hz assignable to methylenes of C-benzyl and O-benzyl derivatives respectively. The intensities of the signals were almost equal indicating a 1:1 ratio for carbon to oxygen alkylation.

Oil A on standing deposited colourless crystals. While these crystals were insoluble in $CHCl_3$, the residual oil was found to be soluble. The whole mass was, therefore, taken up in chloroform and the crystals were filtered off and washed with chloroform. The filtrate on concentration gave a yellow oil (Oil B). The crystalline compound was identified as 2-C-benzyl-ascorbic acid and it was recrystallized from

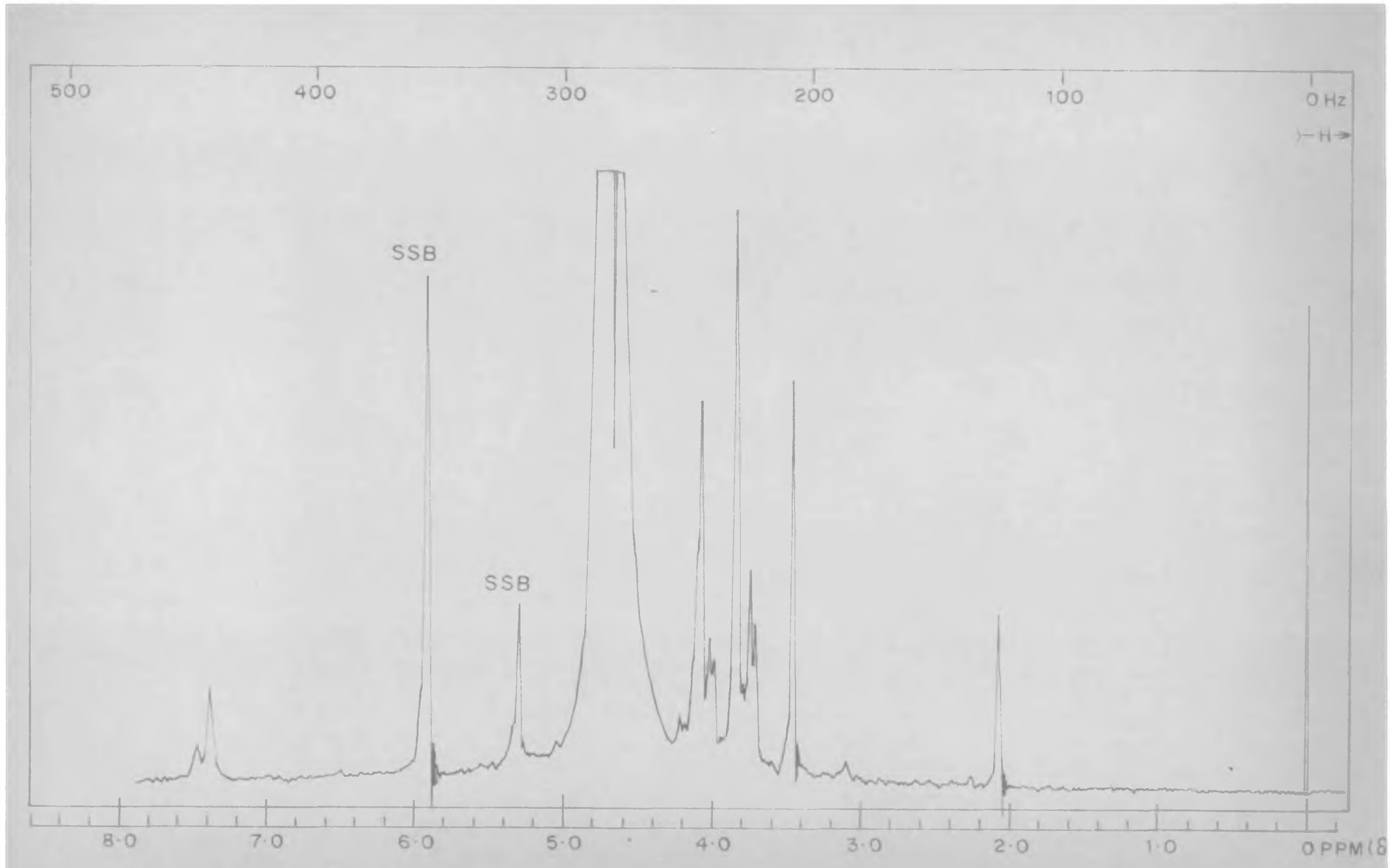



Fig 1 60 MHz PMR Spectrum of aqueous layer of AA benzylation after it was extracted with ethyl acetate

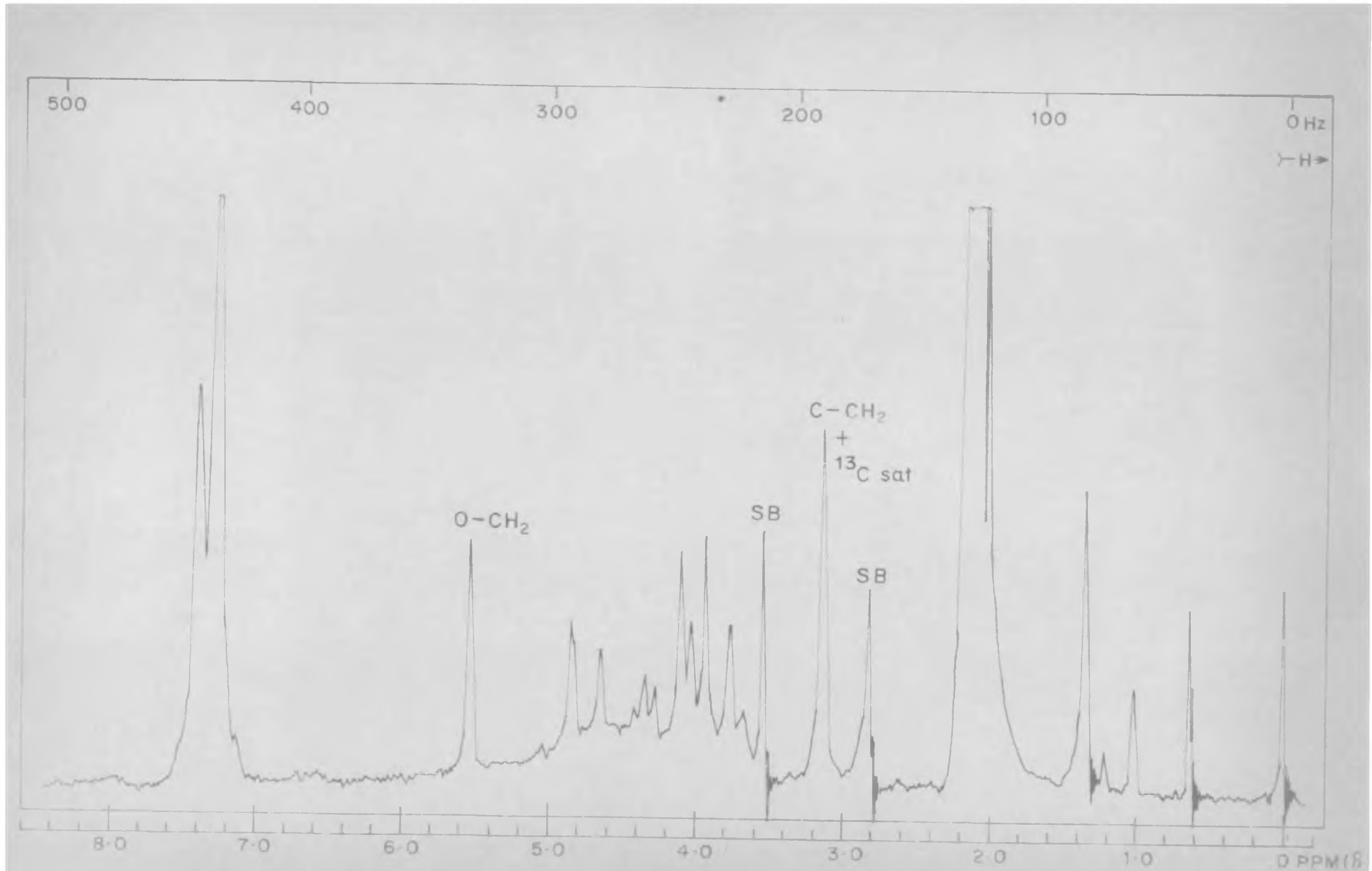



Fig. 2 60 MHz PMR Spectrum of crude product of benzylation of AA in acetone

8:1 chloroform-acetone mixture. The m.p. of the recrystallized product agreed with that reported, and IR spectrum (Fig.3) indicated the absence of a C-C double bond. The PMR spectrum was in general agreement with a bicyclic structure.

The PMR spectrum (Fig.4) of the Oil B showed that it was mostly 3-O-benzyl-ascorbic acid. There was no signal in the region for a benzylic methylene group, indicating that all the C-benzyl derivative had crystallized out completely.

The TLC of the Oil B on silica gel showed that it was a mixture of four compounds. The major one was the 3-benzyl ether as indicated by the PMR spectrum. This oil was separated into the components by chromatography on silica gel. All the fractions were examined by PMR spectra. In all these fractions, we looked for any other C-benzyl derivative that might have been formed; but none was found. The 3-O-benzyl ether accounted for almost 90% of the Oil B. The other small component (about 4-5%) was identified as 2,3-di-O-benzyl ether of ascorbic acid. The remaining two components were present in very small amounts and could not be identified. They were presumably some decomposition products of AA. There was about 2% residue which came out of the column only by methanol elution and this was insoluble in most of the other organic solvents.

Thus, these results clearly indicated that only one C-benzyl derivative was formed and it had the benzyl group

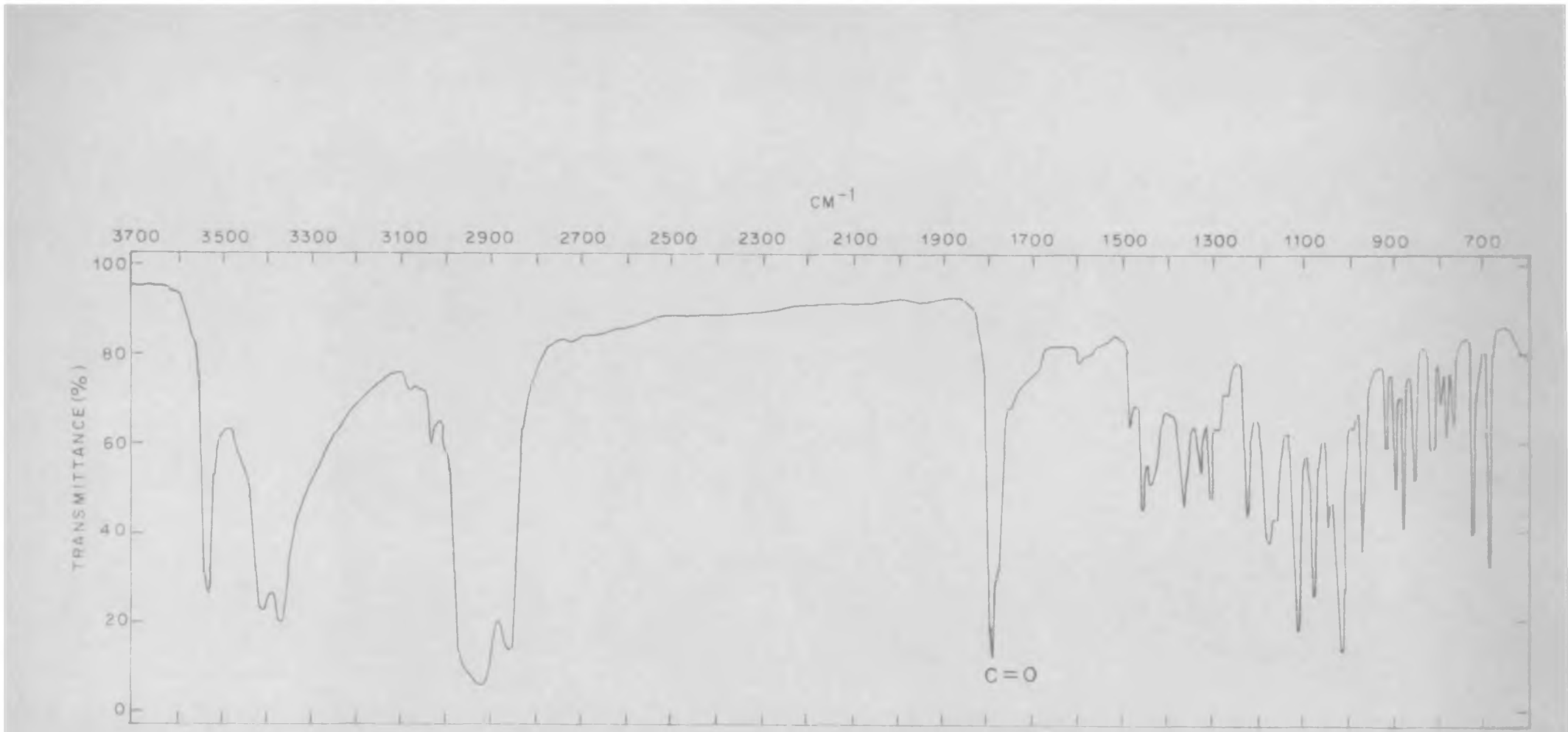
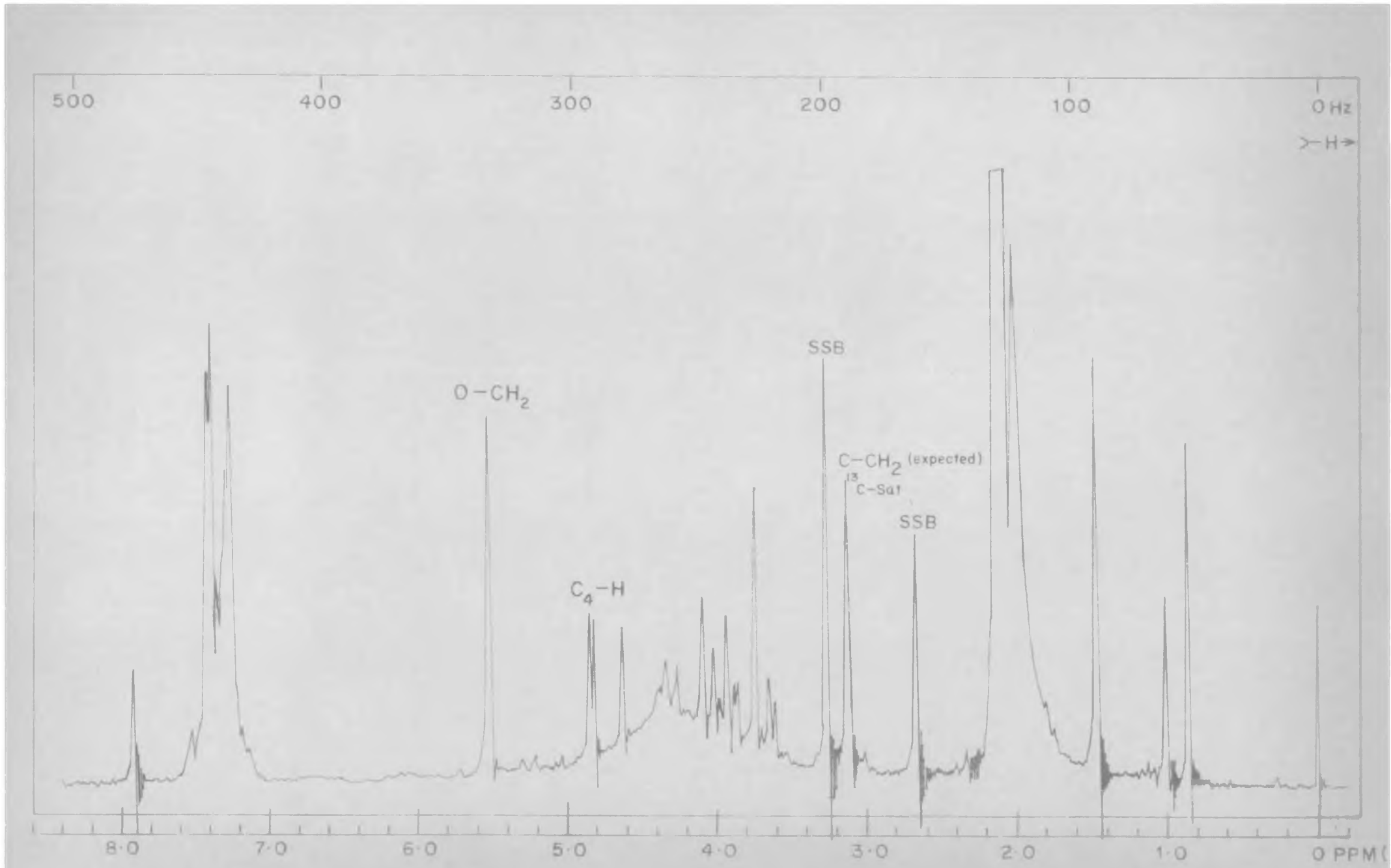



Fig.3 IR Spectrum of 2-C- Benzyl-AA




Fig·4 60 MHz PMR Spectrum in acetone of the residual oil of AA benzylation after all the *C*-benzyl derivative crystallised out

in the 2-position. The above experiment was repeated several times and the results were found to be reproducible. The total benzylation yields varied between 30 and 40%.

Prolongation of reaction times beyond 70 hrs did not give much effect on the benzylation yield. Neutralisation of ascorbic acid only to the extent of about 90% gave a pH of 5.8 to the solution. Reactions done at this pH gave slightly increased amounts of side products (products other than the C-benzyl and O-benzyl derivatives). Neutralisation of ascorbic acid almost completely gave a pH of 8.5 to the reaction medium. Benzylations at this pH reduced the amount of side products; however, there was no effect on the total benzylation yield. Since the reaction at a pH of 8.5 gave a cleaner product mixture, further repetitions were done only at this pH.

Benzylation of ascorbic acid using different solvents did confirm the conclusions of Kornblum[11] on the variation of O/C-alkylation ratio with change in solvents. DMSO which is a solvent with high dielectric constant, gave more of O-benzylation. The aqueous acetone reaction being heterogeneous and also having polar solvent like water gave more of C-benzylation. Reactions in pure MeOH and water gave much less total benzylation as compared to mixture of solvents.

The ratios of O and C-benzylation obtained in the various products were determined from the intensities of O- and C-benzylic methylene signals in their PMR spectra

and these are presented in Table I.

Table I

| | Solvent used | % C-benzylation | % O-benzylation |
|---|---|---|---|
| 1 | 50% aqueous acetone | 50 | 50 |
| 2 | DMSO * | 25 | 75 |
| 3 | MeOH/$H_2O$ * 25:75 | 40 | 60 |
| 4 | Water * | 60 | 40 |
| 5 | MeOH * | 40 | 60 |

In the work up procedure, the acetone was removed before extracting the benzylated products into hot ethyl acetate. The removal of acetone had to be done at about 25-30°C only. Increase in temperature at this stage could lead to the hydrolysis of the unreacted benzyl chloride generating hydrochloric acid. This acid might catalyse the formation of an acetonide of the benzyl derivatives formed. In fact, the increased temperatures did give rise to the formation of acetonides of the benzylated products. Even these reaction products were analysed chromatographically and it was found that only one C-benzyl derivative was formed in the reaction.

* Please see experimental section.

Table II shows the results of various benzylation experiments carried out. In each case, the C-benzyl derivative, which crystallised out from the oil was washed with chloroform to make it free of other products. The purity was checked each time by m.p., PMR spectrum and other physical and chemical properties. The aqueous layer was also examined in each experiments. The PMR spectrum (Fig.2) of the crude reaction product indicated a ratio of 1:1 for the O- and C-benzylations.

Table II

| No. of Expt. | Time of reaction in hrs. | % of neutra-lisation | pH of the solution | Total yield of benzyla-tion products (%) | Yield of C-benzyl derivative (%) |
|---|---|---|---|---|---|
| 1 | 60 | 90 | 5.8 | 38 | 17 |
| 2 | 100 | 90 | 5.8 | 36 | 16.5 |
| 3 | 70 | 90 | 5.8 | 28 | 12 |
| 4 | 70 | 90 | 5.8 | 39 | 17 |
| 5 | 60 | 100 | 8.5 | 40 | 17.5 |
| 6 | 70 | 100 | 8.5 | 35 | 15 |
| 7 | 80 | 100 | 8.5 | 42 | 18 |
| 8 | 100 | 100 | 8.5 | 42 | 18 |
| 9 | 100 | 100 | 8.5 | 39 | 17 |

The chromatographic analysis of the residual oil(Oil B) was done in five experiments and in all cases it was shown that only one C-benzyl derivative was formed.

Benzylation of ascorbic acid acetonide was also done under identical conditions. Since the acetonide is susceptible to hydrolysis under its own acidity when dissolved in water, care had to be taken to add it to an alkaline solution containing required amount of alkali. The net yield of benzylation in this case was in the range of 50 to 55 percent. The PMR spectrum (Fig.5) of the crude reaction product did not indicate any C-benzylation. It was very well resolved and showed sharp signals indicating the purity of the product. It showed signals due to O-$CH_2$ and acetonide group at 324 Hz and 78 Hz respectively. Their intensities were in the ratio of 1:3 so that they could together be taken to mean that it was mostly 3-O-benzyl-ascorbic acid acetonide. The crude oil eventually crystallised. On recrystallisation from 5 percent acetone in petroleum ether, it gave pure 3-O-benzyl-ascorbic acid 5,6-acetonide. All the physical constants and spectral data confirmed the purity of the sample. Only 5 to 6 per cent of the oil was left in the mother liquor. This was presumably a mixture of decomposition products.

The chromatographic analysis of this reaction product was also done on cellulose although, this was not really necessary. The TLC of the crude oil showed 3 spots, the middle spot being the major one. Column chromatography on

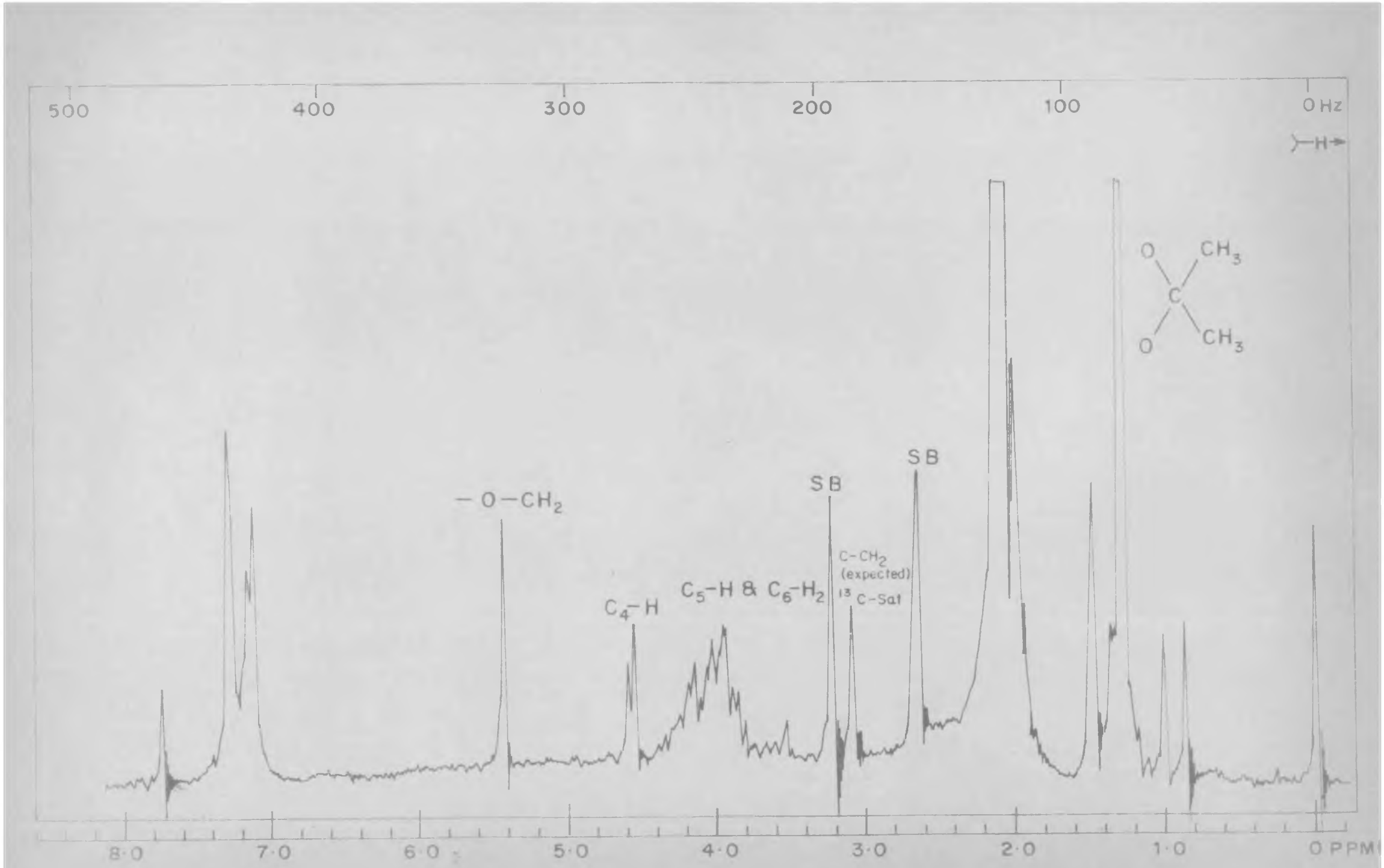



Fig. 5 60 MHz PMR Spectrum of the crude product of benzylation of 5,6 isopropylidene AA in acetone

cellulose showed that the major component constituted 90 per cent of the product mixture. The slow moving one was identified as the unreacted acetonide and the fast moving one was present in almost negligible quantities only. The latter was not identified. There was about 2-3 per cent residual material on the column which came out only on elution with methanol. This was found to be insoluble in most other organic solvents.

The aqueous layer was also examined by PMR spectroscopy, after concentration at low temperature ($30^{o}C$). It did not indicate the presence of any benzyl derivative. The only material it had was the unreacted acetonide.

The PMR spectrum of the crude oil had no signal that could be attributed to one coming from a benzylic $-C-CH_2$ group. The oil which crystallised after some time on recrystallisation from 5% acetone in petroleum ether gave the pure 3-O-benzyl-5,6-acetonide. The mother liquor after filtering off the crystals was concentrated to get a light brown oil which corresponded to only about 5 to 6% of the total oil. The PMR spectrum of this oil indicated that it was a mixture of some decomposition products. In this case, the chromatography was really not necessary because the oil eventually crystallised to give the pure 3-O-benzyl-acetonide.

Table III shows the results of the various acetonide benzylations carried out.

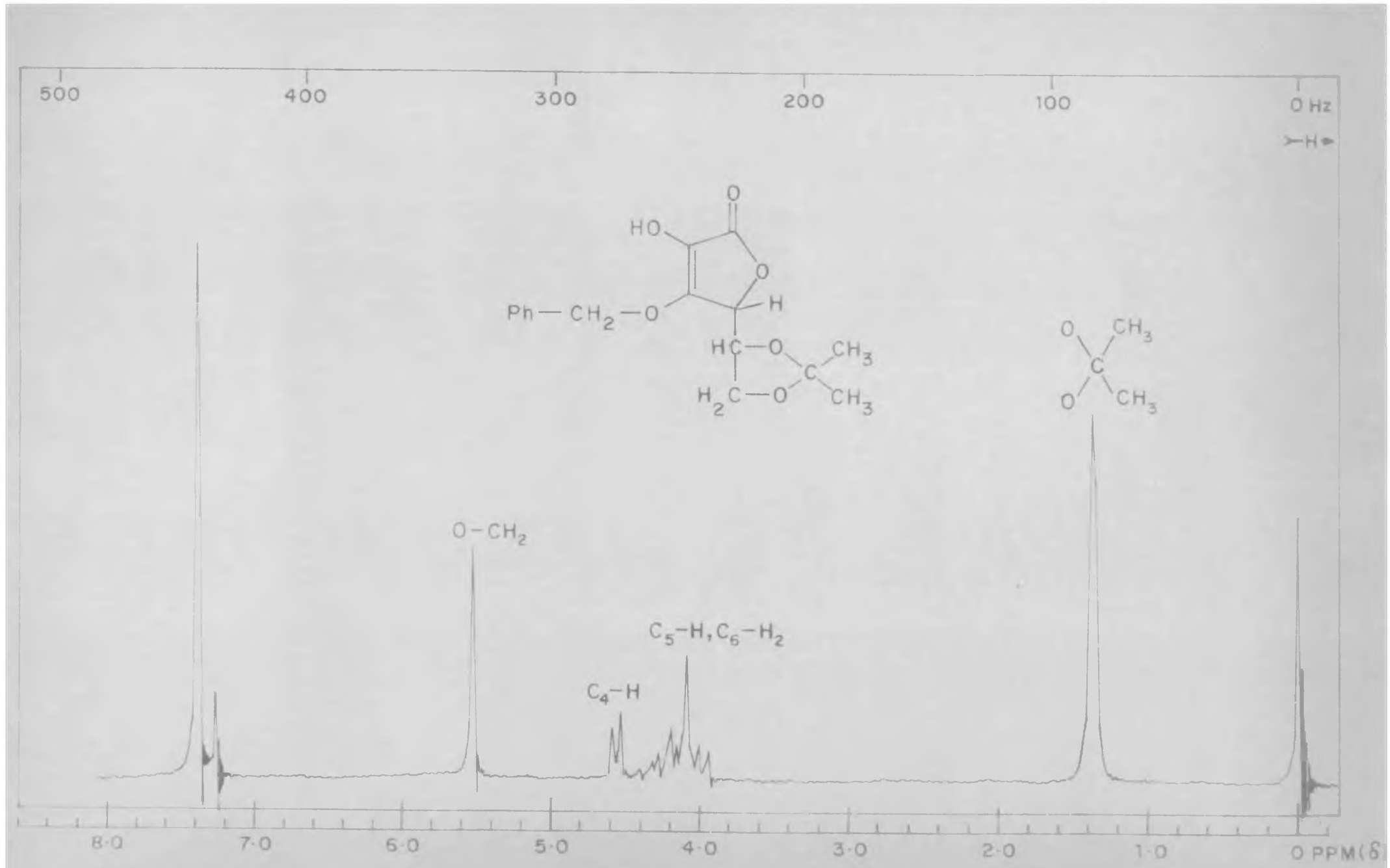



Fig 11 60 MHz PMR Spectrum of 3-O-Benzyl-5,6-isopropylidene ascorbic acid in $CDCl_3$

Table III

| No. | Time of reaction (hrs) | % of neutralisation | pH of the solution | Total yield (%) | Yield of the recrystallised product (%) |
|---|---|---|---|---|---|
| 1 | 70 | 100 | 8.5 | 53 | 50 |
| 2 | 80 | 100 | 8.5 | 50 | 47 |
| 3 | 90 | 100 | 8.5 | 55 | 51 |
| 4 | 100 | 100 | 8.5 | 58 | 54 |
| 5 | 70 | 100 | 8.5 | 50 | 46 |

Benzylation of D-iso-AA was also done under identical conditions. The total benzylation yields here were better as compared to those for AA benzylation. The average yield was about 50%. The oil obtained after concentration of the ethyl acetate layer showed 4 spots on TLC. The PMR spectrum (Fig.6) showed a signal at 3.7 $\delta$ which could be assigned to a $C-CH_2$ group. But its intensity was too low. The major spot corresponded to almost 80-85% of the total product. The other small components together were about 10-15%. This mixture was subjected to chromatography on a silica gel column. The major component was identified as 3-O-benzyl D-iso-AA. Another small fraction was 2,3-di-O-benzyl-D-iso-AA. The third component was small in amount and was 3-O-benzyl-

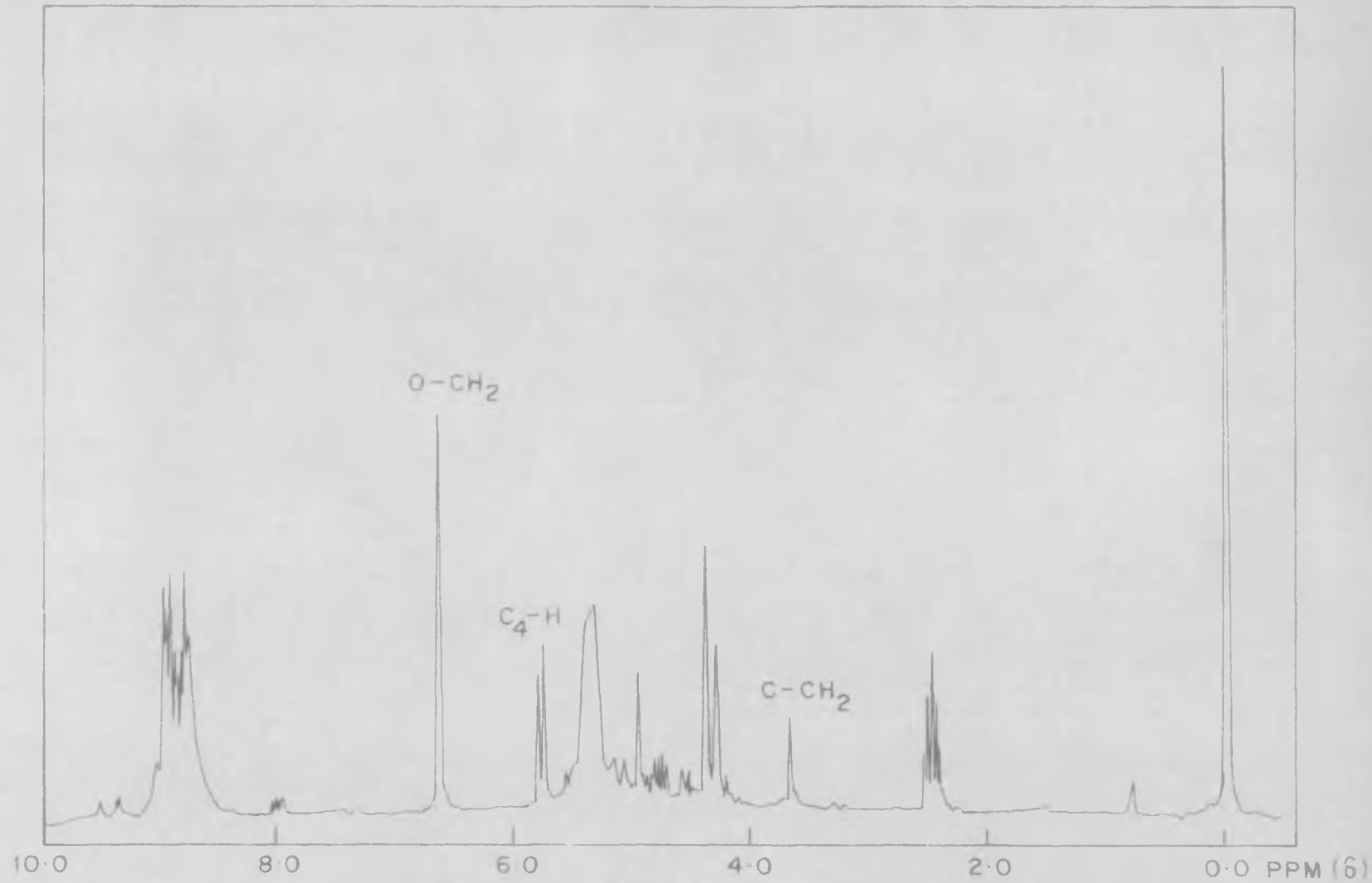



Fig 6 90 MHz PMR Spectrum of the crude product of D(-)-iso-AA benzylation in acetone-$d_6$

5,6-acetonide of D-iso-AA. The fourth component could not be identified and was likely to be a decomposition product. The PMR spectra were used to examine all the column fractions. About 2-3% of the total oil which remained on the column could be eluted out only with methanol which gave a brownish solid which was insoluble in all organic solvents. The chromatography was repeated on cellulose also and the results obtained were the same.

The aqueous layer was also examined by PMR spectra after concentrating it at room temperature. It had only unreacted potassium salt and no benzylated products. Table IV shows the results of different experiments.

Table IV

| No. | Reaction Time (%) | % of neutralisation | pH of the solution | Total yield of the benzylation (%) |
|---|---|---|---|---|
| 1 | 70 | 100 | 8.5 | 53 |
| 2 | 90 | 100 | 8.5 | 52 |
| 3 | 100 | 100 | 8.5 | 55 |
| 4 | 70 | 100 | 8.5 | 50 |

The results can be summarised as follows:-

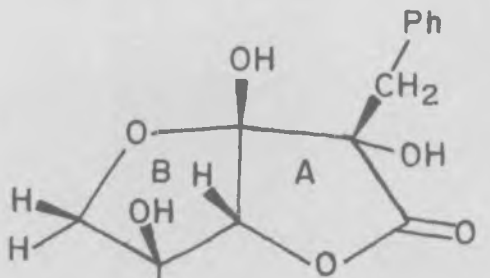
i) Ascorbic acid in 50% aqueous acetone gave a mixture of C-benzyl and O-benzyl derivatives in the ratio of 1:1. There is only one C-benzyl isomer formed and it had the benzyl in 2-position. The average yield of the total benzylation was about 35%.

ii) Ascorbic acid acetonide gave only an O-benzyl ether with an average yield of 50%.

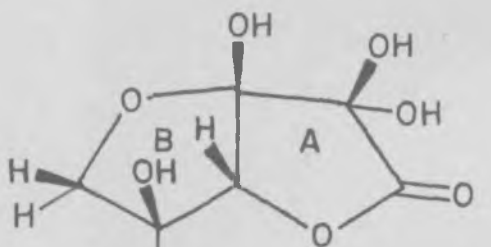
iii) D-iso-ascorbic acid gave O-benzyl derivative as a single large product (about 85%) with very small amounts of C-benzyl derivative which could not be isolated.

Discussion

The benzylation of AA described earlier[2] was repeated several times and the products carefully examined for the formation of a possible stereo-isomer of the reported product. The results were negative. But the stereochemistry of the product obtained appeared to be opposite to that which has been assigned to it. The PMR spectrum of 2-C-benzyl AA is in complete accord with the bicyclic structure (II).




II b




XV

| | AA | 2-C-Benzyl AA | DHA |
|---|---|---|---|
| $\delta_{C_{(4)}-H}$ | 4·98 δppm | 4·27 δppm | 4·75 δppm |
| $\delta_{C_{(5)}-H}$ | 4·09 | 4·46 | 4·59 |
| $\delta_{C_{(6)}-H}$ | 3·75 | 4·14 | 4·24 |

The spectrum of the compound in $D_2O$ (Fig. 7) which is displayed along with that of AA itself, shows a doublet at 4.27 δ which can readily be assigned to $C_{(4)}$-H, a multiplet centered at 4.46 δ due to $C_{(5)}$-H and another multiplet centered at 4.14 δ due to $C_{(6)}$-protons. The singlet at 3.13 δ may be assigned to the methylene protons of the benzyl group. The five aromatic protons of benzyl group

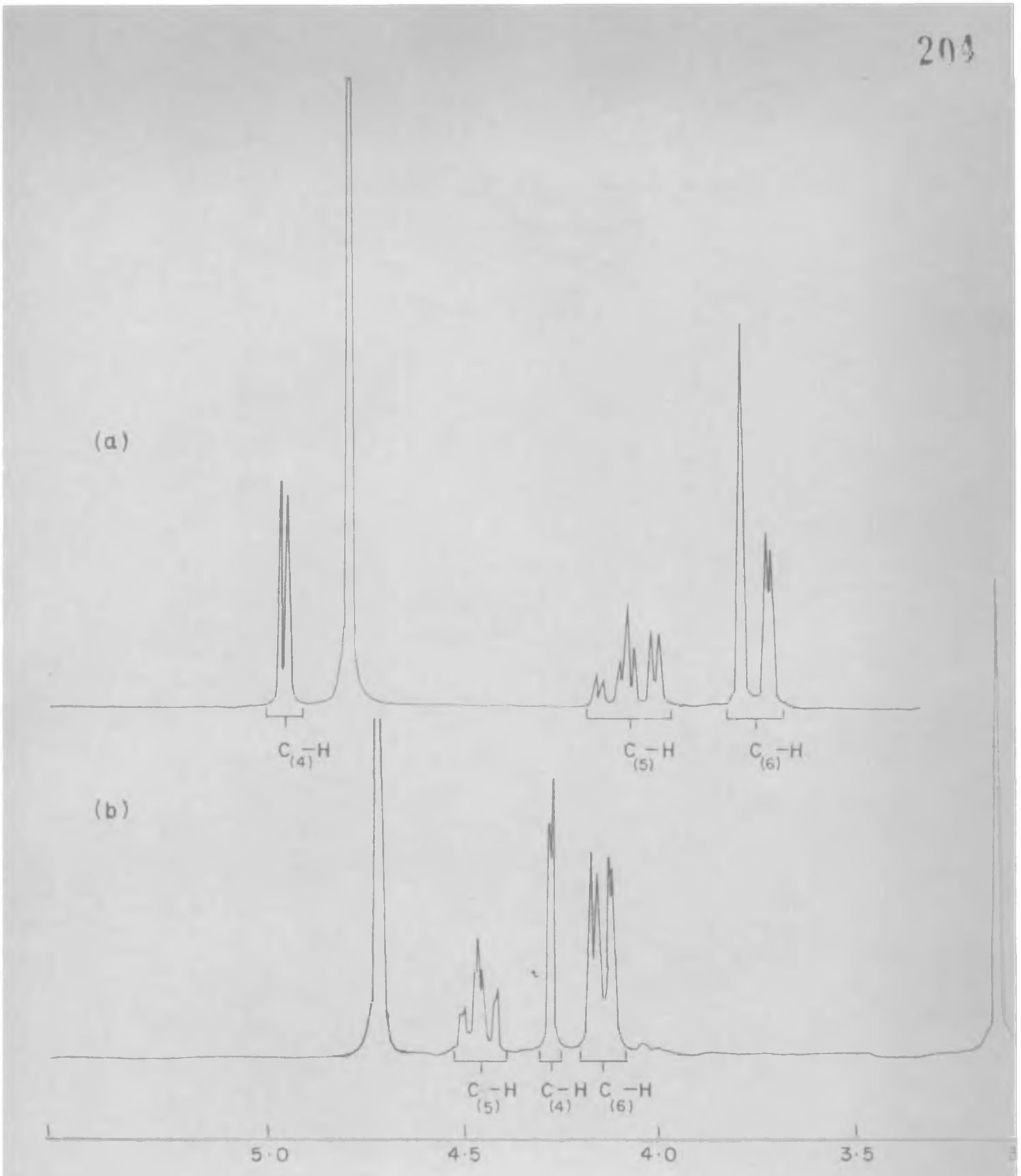



Fig. 7 90 MHz PMR Spectra in $D_2O$

(a) Ascorbic acid

(b) 2-C-Benzyl ascorbic acid

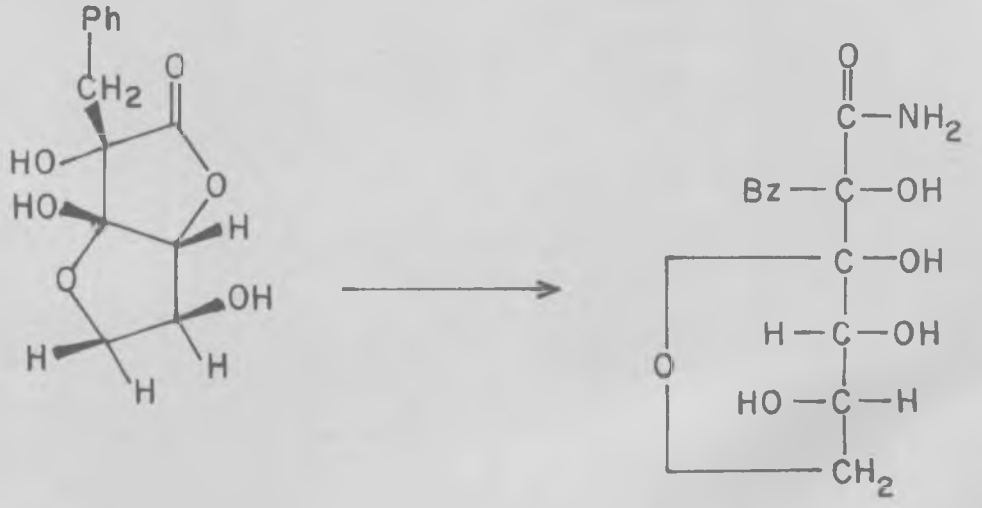
give an unresolved narrow band. The changes in shifts observed for the $C_{(4)}$-, $C_{(5)}$- and $C_{(6)}$-protons in the transition from AA to the benzyl derivative are quite striking. The $C_{(5)}$- and $C_{(6)}$-proton signals move downfield while that of $C_{(4)}$-H moves upfield. The downfield shifts (0.39 and 0.38 ppm respectively) are consistent with the formation of ring B. Similar downfield shifts* are seen for the $C_{(5)}$- and $C_{(6)}$-protons in the conversion of AA to DHA (XV). The $C_{(4)}$-proton of DHA is only slightly more shielded than the similar proton of AA. It would be reasonable from this to conclude that the anticipated effect of formation of ring B on the $C_{(4)}$-H absorption should be slightly shielding for II. What is actually found is a very pronounced upfield shift for $C_{(4)}$-H by about 0.68 ppm. This is undoubtedly an effect of the introduction of the C-benzyl group. The marked upfield shift* may be readily rationalised if the benzyl group at $C_{(2)}$ is exo. The $C_{(4)}$-H is likely to come under a large shielding effect of the phenyl ring of the benzyl group only when the benzyl is exo. If the benzyl group had an endo stereochemistry as assigned by the earlier workers[2], one would not expect an appreciable upfield shift for $C_{(4)}$-H as the benzyl group, being on the wrong side, would be kept far away from $C_{(4)}$-H by ring B. If it were possible for the phenyl ring to assume an orientation where it can provide a small shielding effect on $C_{(4)}$-H, it is found from an examination of a Dreiding model that such a phenyl

---

* For DHA, the downfield shifts for $C_{(5)}$-H and $C_{(6)}$-$H_2$ are about 0.5 and 0.49 ppm respectively, and the upfield shift for $C_{(4)}$-H is 0.22 ppm, the $C_{(4)}$-H signal appearing below that of $C_{(5)}$-H. In the spectrum of C-benzyl-AA the $C_{(4)}$-H signal is upfield from that of $C_{(5)}$-H.

conformation should result in very strong upfield shifts for the protons on $C_{(5)}$ and $C_{(6)}$ particularly those which are endo. Such effects are not observed for these protons. In fact, only the effect of formation of ring B is seen. Thus, the stereochemistry is undoubtedly exo for the benzyl group. The spectra of the compound in pyridine and DMSO are also shown in Figs. 8 and 9. The large upfield shift for the $C_{(4)}$-H is observed in all the spectra.

The earlier assignment of stereochemistry was made on the basis of the dextro rotation shown by the amide (XVI) obtained from (II). It was contended that Hudson's[62] lactone rule




II b XVI

would imply that the hydroxyl at the 2-position would be to the right in the Fischer-Rosanov projection if the amide is dexterorotatory. However, Hudson's rule assumes that the amide group is attached to a CH-OH group. In other words, the second group at $C_{(2)}$ must be a hydrogen in order for

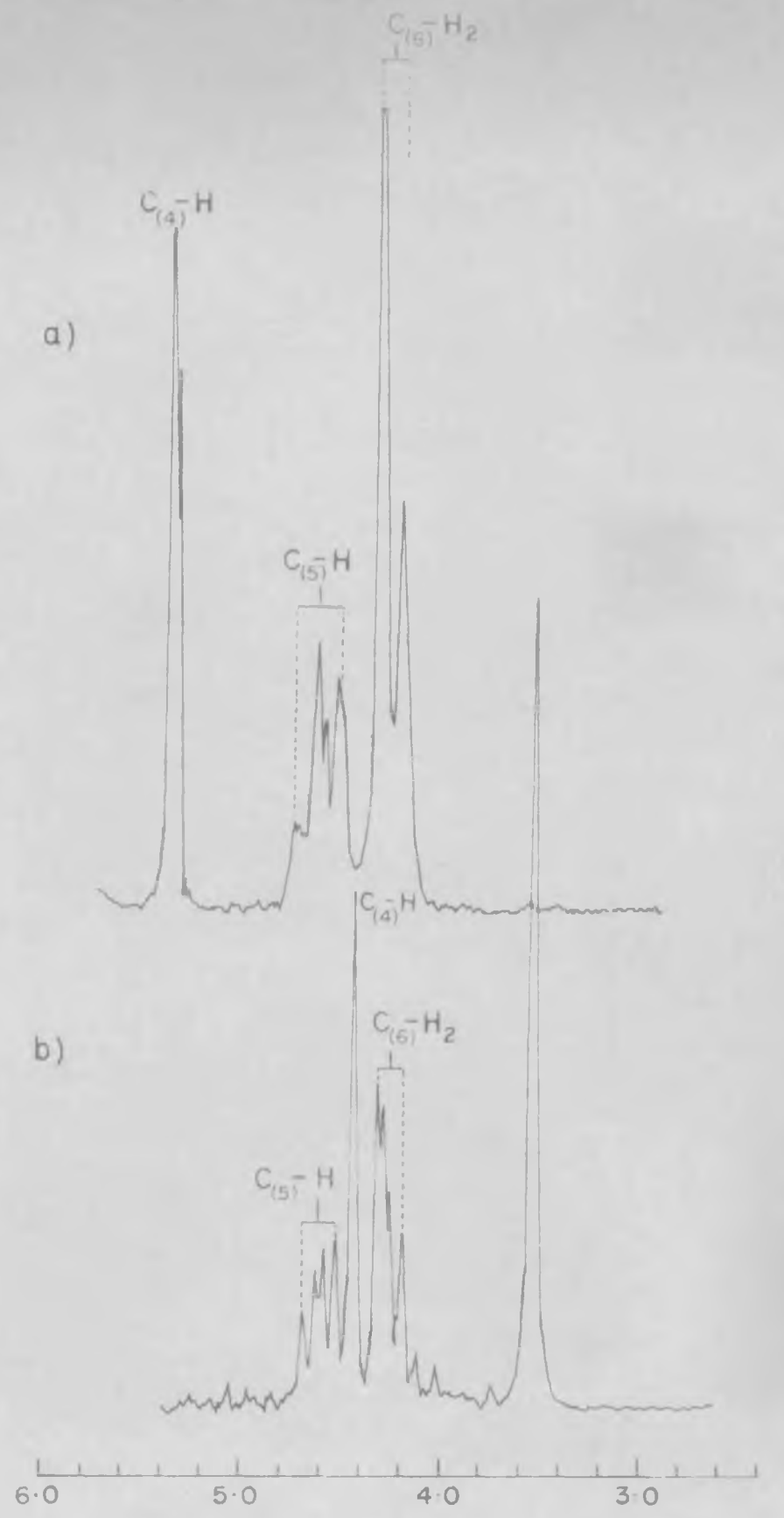



Fig. 8 60 MHz PMR Spectrum in pyridine

a AA

b) 2-C-benzyl AA

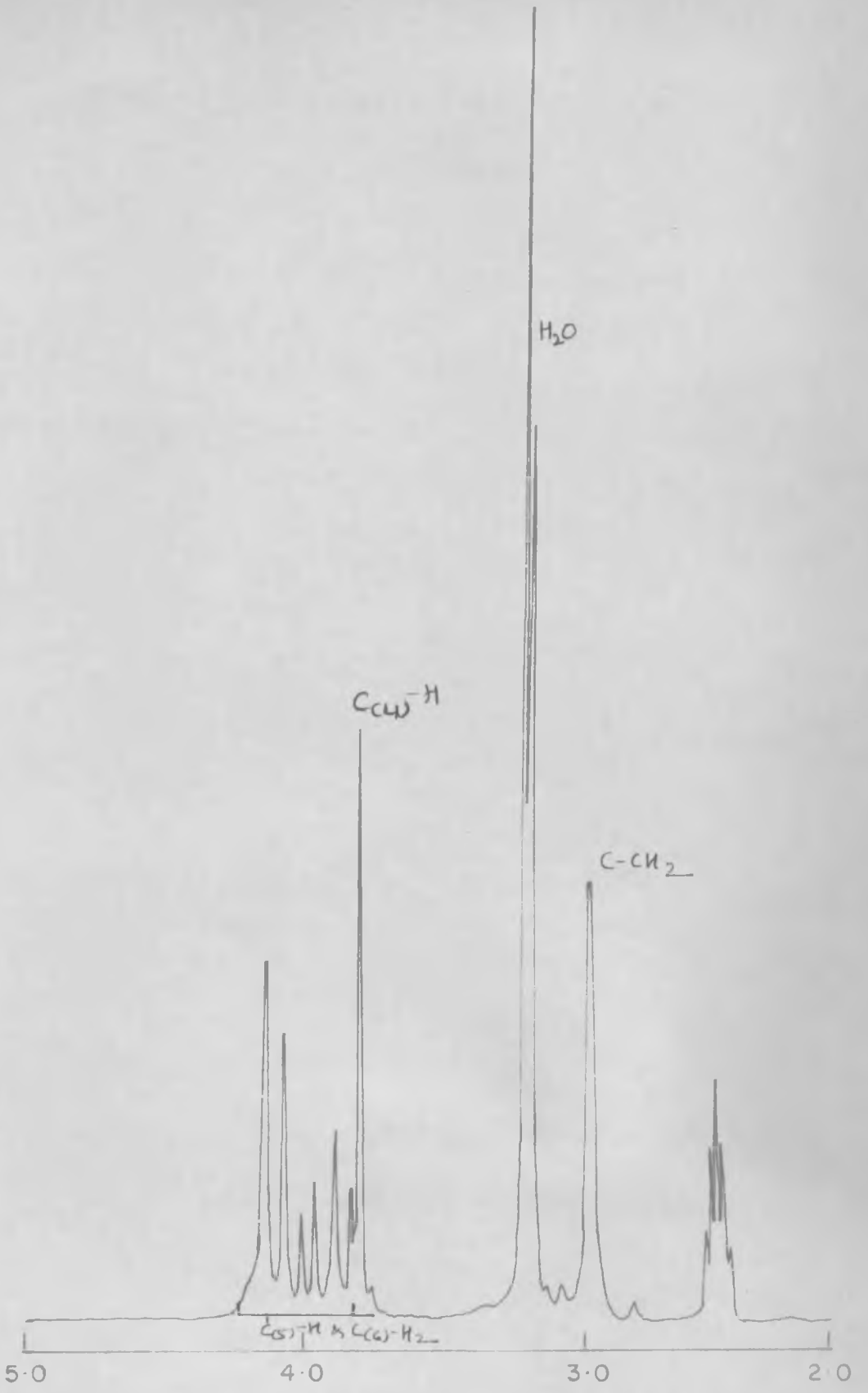
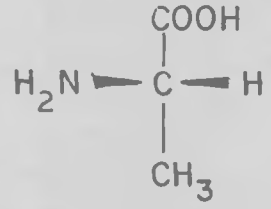



Fig.9. 90 MHz PMR Spectrum of 2-C-benzyl-AA in DMSO-$d_6$

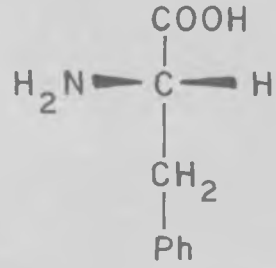
the rule to hold good. In the case under consideration, this is not so. Instead of a hydrogen, we have the highly polarisable benzylic group. The very large effects that a phenyl group can have in determining the rotation of an optically active compound even when it is on a carbon adjacent to the optically active centre may be illustrated with the change in rotation from L-alanine (XVII) to L-phenyl-alanine (XVIII). The molar rotation changes from +2 to -58 under comparable conditions on the introduction of $\alpha$ phenyl group one carbon away from the optically active carbon.




XVII

α-alanine

$M_D = +2$




XVIII

α-phenyl alanine

$M_D = -58$

The error in the earlier assignment has thus been caused by ignoring the benzyl substitution effect. The significance of Hudson's rules is presumably that the more polarizable group would be to the right in the Fischer projection. As between OH and $CH_2$-Ph the latter is apparently the one that is more polarizable. Such an interpretation would make

the results consistent with the stereochemistry implied by the PMR chemical shift data.

Now that the stereochemistry at $C_{(2)}$ is established unambigiously. the next question to be answered is why only the exo epimer is formed. The possibility of the side chain at $C_{(4)}$ exerting a large enough hindering effect on the attack of the ambident anion at the $C_{(2)}$ position by the benzyl group is very unlikely. Because of the planarity of the ring, any steric effect of the type mentioned has to be small. The nature of the results obtained in the benzylation of the 5,6-acetonide of AA and D-iso-AA show that steric effect has hardly any role to play. With the acetonide, no C-benzyl derivative was at all formed while 3-O-benzylation was quite appreciable. The acetonide function merely increases the size of the side chain of the molecule and can hinder the approach of benzyl chloride to $C_{(2)}$ only when it is from the same side. There is nothing at all to hinder the approach from the opposite side of the ring, and this derivative should have therefore given a C-benzyl derivative just as AA itself. But none was formed. With D-iso-ascorbic acid, the benzylation was mostly at the 3-oxygen. The reaction mixture seemed to show a signal in the PMR spectrum (Fig.6) assignable to a benzylic methylene, indicating the likelyhood of the formation of a small amount (about 1/6th of the 3-O-benzylation product) of the C-benzyl derivative. But, none could actually be isolated by chromatography of the

product. D-Iso-AA differs from AA only in the configuration at $C_{(5)}$. The bulk and nature of the side chain at $C_{(4)}$ is the same in both cases. However, there was considerable difference in its behaviour in benzylation as compared to that of AA. As in the case of the acetonide, the side of the $\gamma$-lactone ring opposite to the one having the side chain is completely free in D-iso-AA as it is in AA itself. However, the experiments done showed that formation of the C-benzyl derivative was not as facile as in the case of AA.

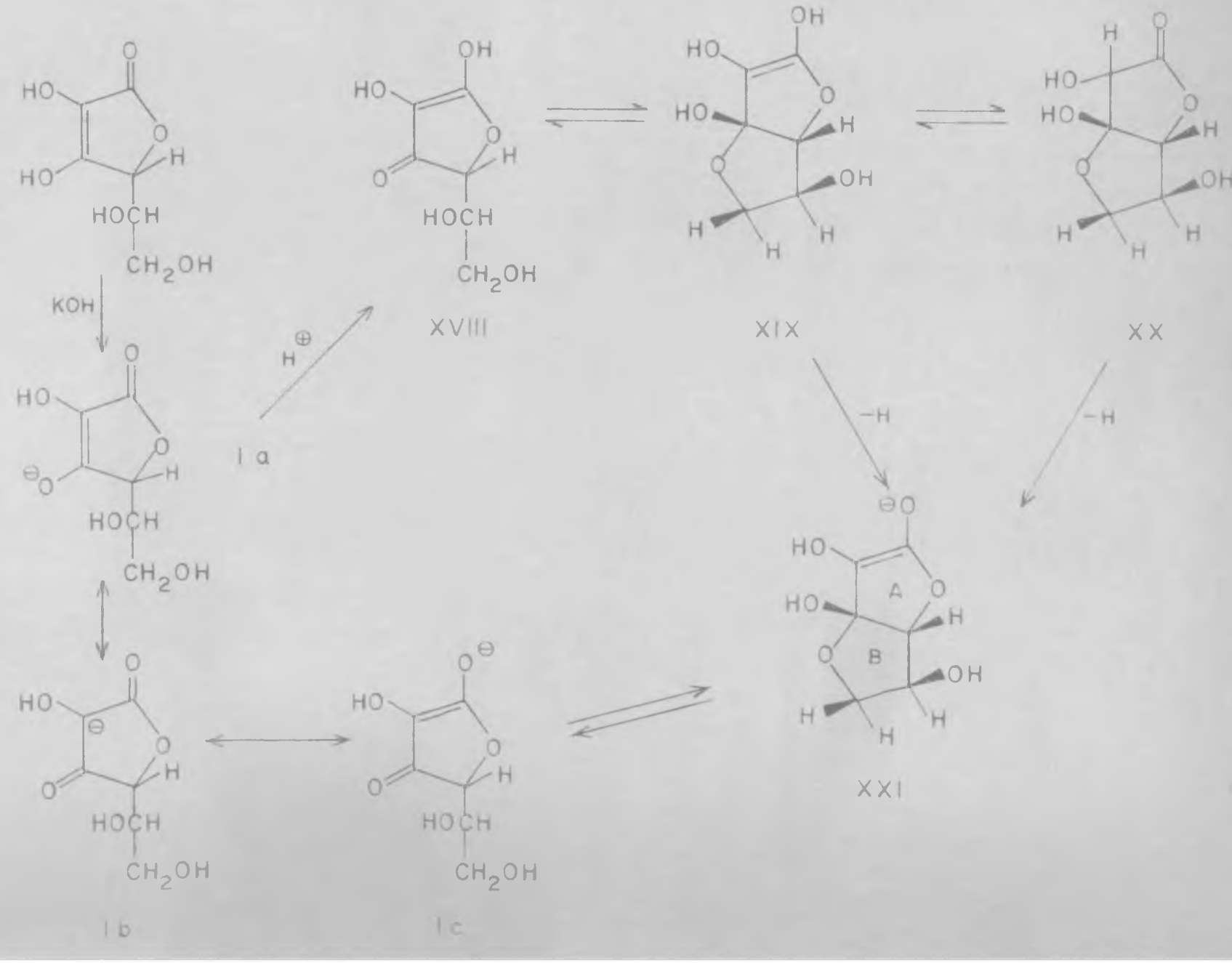
The differences in behaviour that have been observed cannot thus be rationalized if the reactive species is (I). It is, therefore, necessary to consider the other possible forms we had discussed in the last two chapters. We have already seen that the different isomeric forms of AA that have been postulated are not present in high enough concentrations to be directly observable. However, as noted earlier, some of these, though present in only very small concentrations, can be more reactive than the normal form and can have great significance for the reactivity of the vitamin. The isolation of the 1-O-methyl hetero AA[63], the likelyhood of AA existing in the hetero form (XVIII) and the possibility that some of this will go over to the bicyclic species (XIX) or (XX) have all been mentioned. These can give rise to the ambident anion (XXI) which can be the reactive species giving exclusively the

exo product in the C-benzylation. All these possible species are shown in Chart I. Potassium ascorbate is the salt of a weak acid and a strong base, and there will always be a hydrolytic equilibrium under the reaction conditions mentioned. This means that some free ascorbic acid and hetero ascorbic acid will always be available from which species (XXI) can be formed.

We may now consider the reactivity of species (XXI) in which the negative charge is delocalised in the $C_{(2)}$-$C_{(1)}$-O chain. The charge density here is distributed between $C_{(2)}$ and the lactone carbonyl oxygen. As compared with this, the negative charge of the ion I is more highly delocalised and the available charge density on $C_{(2)}$ should be expected to be lower. Further more, the energy for localisation of the charge at $C_{(2)}$ will be much higher for the ion I than it will be for (XXI). The activation energies for the alkylation reaction in the two cases will therefore be very different. The latter reaction will be more facile than the former.

As far as stereochemistry is concerned, it is eminently reasonable to consider that attack from the exo side would be preferred since it would be the less hindered side. The alternative mode which is attack from the endo side will be highly hindered. Thus, if it is the bicyclic anion that is the actual reactive species, it will furnish a ready explanation not only for the exclusive formation of

Chart I



only one product, but also the stereochemistry that is obtained for it.

Further, the behaviour of M. acetonide in the benzylation reaction lends strong support to the proposition that it is the bicyclic anion that is the reactive species. For the formation of the bicyclic intermediates, the free OH group at the $C_{(6)}$-position is a must. When this is blocked, the formation of the bicyclic form is also blocked. This is presumably what happens in the case of M.-acetonide where the OH at $C_{(6)}$ is blocked by the acetonide formation. Only the open chain ambident anion is available in this case, and this, apparently, gives only O-benzylation. Since the bicyclic anion which would have the higher reactivity required for C-benzylation cannot be formed at all, no C-benzylation can take place in the case of M.-acetonide.

We now come to the problem of the benzylation of D-iso-M. under identical conditions. The reaction was predominantly at the oxygen. The spectrum (Fig. 6) of the reaction mixture shows a low intensity signal that could be interpreted as a benzylic methylene on carbon. However, no C-benzyl derivative was isolated in the chromatography of the reaction mixture. It could be safely concluded that even if some benzylation had taken place, the extent of reaction was much smaller than in the case of M.( Fig. 10 shows the PMR spectrum of the pure 3-benzyl ether of D-iso-M.).

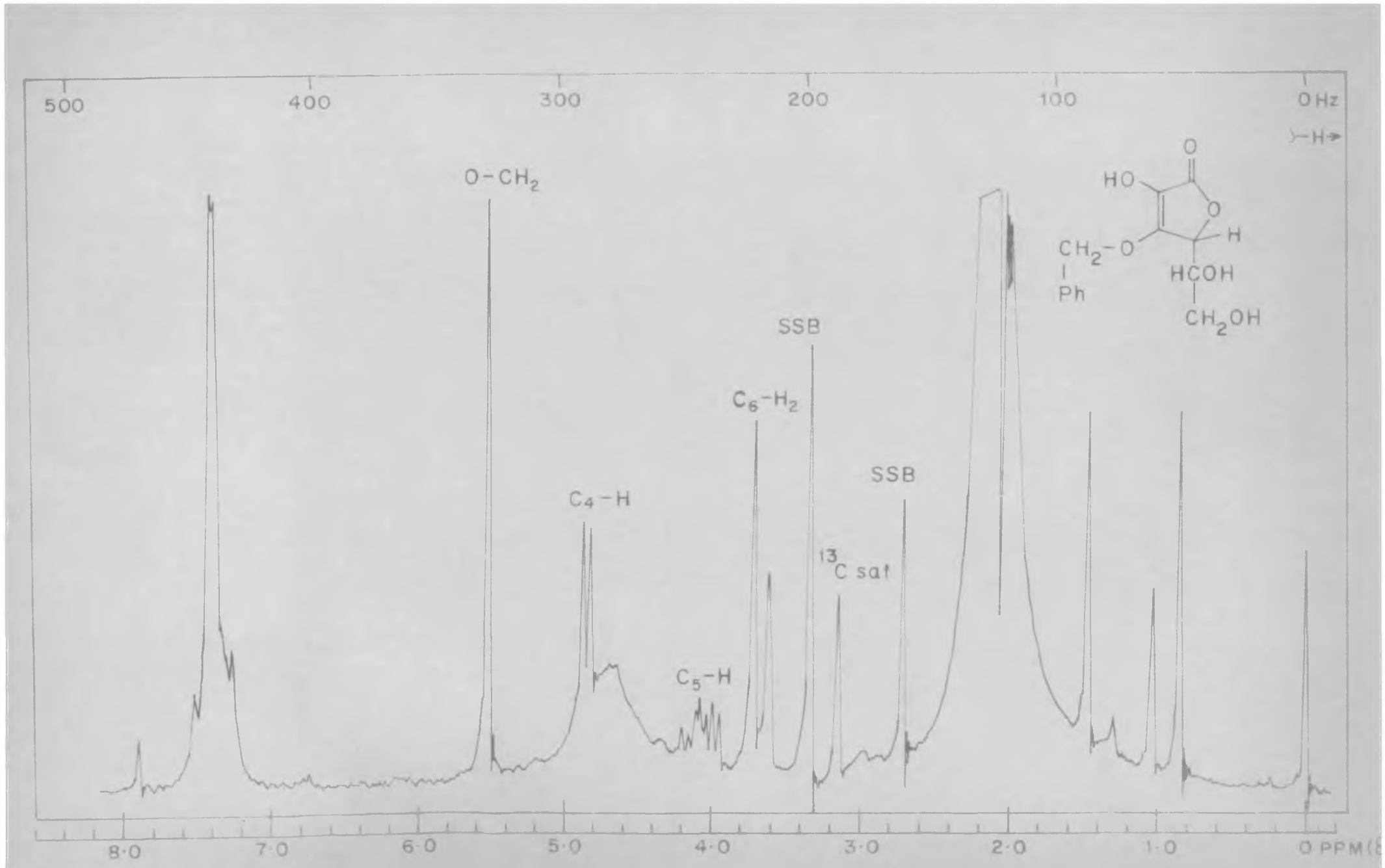



Fig. 10 60 MHz PMR Spectrum of the pure 3-O-benzyl-D(−)iso-AA in acetone

A plausible explanation for this difference may be furnished on the basis of the need for the formation of a bicyclic form for the reaction. When one considers the open-chain structure, it is difficult to see why this compound does not behave like AA in the benzylation reaction.

A careful examination of the molecular models of the bicyclic forms of AA and D-iso-AA reveals an interesting situation. The pictures of these two bicyclic models i.e. of AA (XX) and D-iso-AA (XXII) are shown in Fig. 11. The lactone oxygen attached to $C_{(4)}$ eclipses with the oxygen on $C_{(5)}$ in the latter case. This will raise the energy of the bicyclic form and increase the activation energy for the cyclisation reaction. This situation would tend to decrease the equilibrium concentration of the bicyclic form and reduce its rate of formation. Another factor that can help rationalise the C-benzylation yield is probably the repulsive interaction between the $C_{(5)}$ and $C_{(2)}$ oxygens which would both be endo in the benzylated product. The rate of reaction of the anion with benzyl chloride is in all possibility lower in this case.

These considerations show that the major difference between AA and D-iso-AA is only in the two factors mentioned. When the configuration at $C_{(5)}$ is changed from D to L these interactions disappear. Since the equilibrium concentration of the bicyclic form is low in the case of

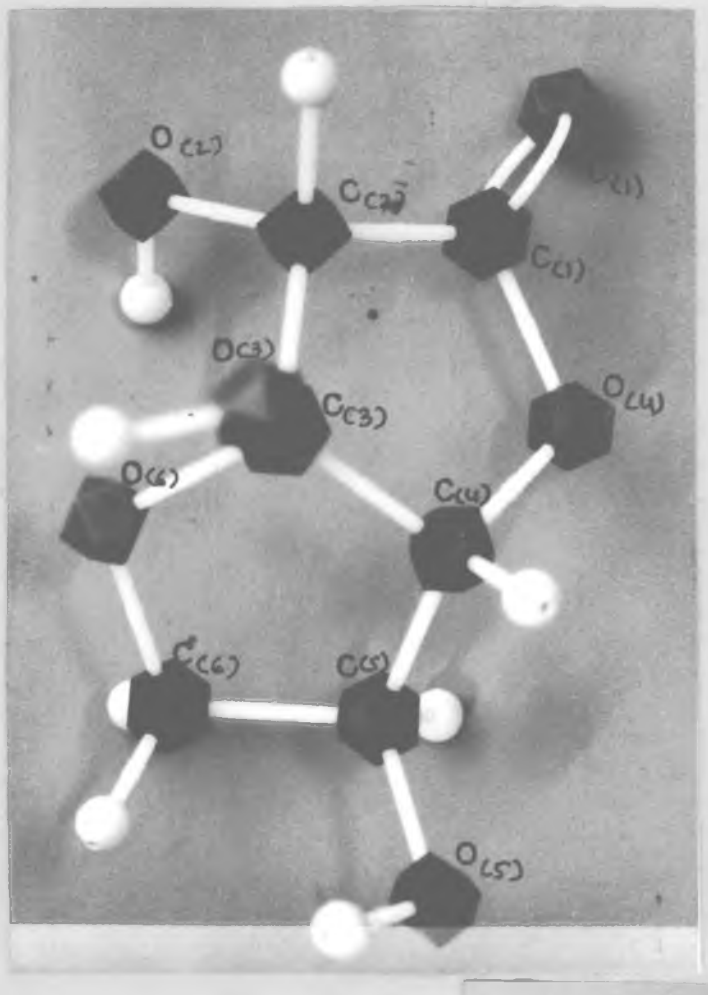



Fig. II (XX)

Bicyclic AA model

Distance between Oxygen

$O_{(5)}-O_{(4)} = 3{\cdot}5\ A^{\circ}$

$O_{(2)}-O_{(5)} = 5{\cdot}8\ A^{\circ}$

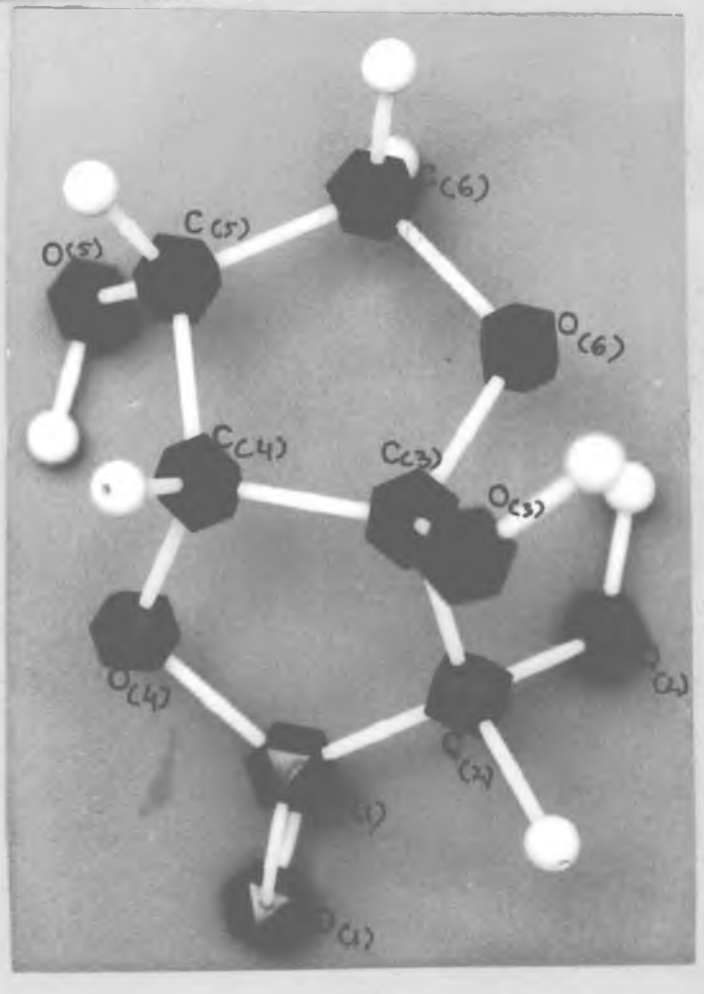



Fig. II (XXII)

Bicyclic D-iso-AA model

Distance between Oxygen

$O_{(4)}-O_{(5)} = 2{\cdot}5\ A^{\circ}$

$O_{(2)}-O_{(5)} = 4{\cdot}3\ A^{\circ}$

D-iso-AA and its rate of formation is also low, C-benzylation cannot compete effectively with O-benzylation, and only the O-benzyl derivative is formed. The energy raising interactions between the $C_{(4)}$ and $C_{(5)}$ oxygens in species (XXII) is a sum of Van der Walls and Coulombic repulsion terms. The hydrogen bonding interaction between the $C_{(4)}$ oxygen and $C_{(5)}$-hydroxyl, which is energy lowering, cannot compensate for the increase in energy arising from the repulsive terms mentioned. The repulsive interaction between the $C_{(2)}$ and $C_{(5)}$ oxygens will be mostly Coulombic in nature and it will get reduced with change in configuration from D to L at $C_{(5)}$.

The nature of the results obtained in benzylation of AA and its analogues are thus in very good agreement with the postulate that the reactive species involved is derived from a bicyclic form.

Conclusion

The 2-C benzylation product of AA has been shown to have its benzyl group in the exo configuration in its bicyclic structure on the basis of PMR data. This assignment is opposite to that made earlier. It has been pointed out that the error in the earlier assignment has apparently arisen from the lack of consideration of substituent effect of the benzyl group on the optical rotation of the derivative which was employed for the configurational assignment.

The fact that only one C-benzylation product is obtained in the reaction has been verified. The acetonide of AA was found to give only O-alkylation in the reaction, while, with D-iso-AA, O-benzylation predominated. The behaviours obtained in the benzylation of the three compounds have been rationalized in terms of reactions via bicyclic intermediates.

Experimental

1. Benzylation of AA[2]

AA (10.0 g) was dissolved in water (100 ml) to which KOH (3.4 g in 25.0 ml of water) was added. Benzyl chloride (7.2 g) dissolved in acetone (100 ml) was added to the above solution. This mixture was kept tightly closed on a mechanical shaker for the required period of time (normally about 80 hrs).

After completion of the reaction, acetone was removed at 25-30°C under vacuum on a rotary evaporater. The mixture was then washed with pet.ether (60-80° ; 3 x 150 ml portions) to remove the unreacted benzyl chloride. The aqueous solution was now extracted repeatedly with hot ethyl acetate (5 x 200 ml portions). The aqueous layer was concentrated and its PMR spectrum was checked to make sure that it did not contain any benzylated product.

The ethyl acetate layer was dried on anhydrous sodium sulfate. It was then concentrated under vacuum at 30-40°C using a rotary evaporater to get a yellow oil. The PMR of the oil obtained was checked each time to determine the proportion of O- and C-benzylation products. The thick yellow oil, on being allowed to stand, deposited crystals of C-benzyl-AA (seeding with C-benzyl-AA from a previous experiment hastened the crystallisation).

After the crystallisation was complete, the mass was extracted with $CHCl_3$. The crystalline C-benzyl derivative

being, insoluble in $CHCl_3$, was filtered off. The $CHCl_3$ layer, which contained mainly 3-O-benzyl-AA, was concentrated under vacuum at 30-40°C to get a yellow oil. The PMR spectrum of this oil was checked each time to make sure that no C-benzyl-AA was left in it.

This oil was now loaded on to a silica gel* column (250 g) of 1 inch diameter which was packed in benzene. The oil was dissolved in about 20 ml of acetone into which 10.0 g silica gel was added. The acetone was evaporated very slowly. The free flowing silica gel with the oil on it was loaded on to the column as a benzene slurry. The elution was started with benzene itself. The polarity was gradually increased with ethyl acetate. 150 Ml of the eluate was collected in each fraction. The elution was monitored with the help of silica gel TLC. The developing solvent system for TLC was a mixture of 15 parts MeOH, 15 parts of acetone and 70 parts benzene. 3-O-Benzyl-AA, the major component of the oil, came out of the column with 30-40% ethyl acetate in benzene. The column was washed with ethyl acetate, acetone and finally, with MeOH. All the fractions were analysed with the help of PMR spectra, and the other possible epimer of C-benzyl-AA was looked for. The entire experiment was repeated several times in order to make sure of the results. The second epimer was not found anywhere. The 2-C-benzyl-AA was recrystallised from 8:1 chloroform acetone mixture, m.p. 156°C.

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* The silica gel used here was prepared from the commercial grade material according to the procedure described in Chapter IV.

2. Benzylation of AA acetonide

KOH (3.4 g) was dissolved in 125 ml of water to which 12.0 g of the acetonide was slowly added. The addition of the acetonide to an alkali solution was necessary as the acetonide would readily hydrolyse to AA in pure water. Benzyl chloride (7.2 g) dissolved in acetone (100 ml) was added to this and the heterogeneous mixture was kept on a mechanical shaker for the required length of time. The work-up procedure for the isolation of the reaction product was identical to the one adopted in the case of AA. Here, the pet.ether washings contained the same benzylated product which crystallised out on concentrating the pet.ether layer. The m.p. (109-110°C) and spectral data for this crystalline compound showed that it was only pure 3-O-benzyl-AA 5,6-acetonide.

The ethyl acetate layer gave a yellow oil which crystallised readily. The PMR spectrum of this was checked each time. The solidified oil could be easily recrystallised from $CHCl_3$ and pet.ether (60-80°) mixture. The mother liquor after crystallisation was also examined each time by PMR spectrum.

However, in two experiments, the oil was subjected to chromatography on cellulose (250 g) (since the acetonide group was very susceptible to acids, this oil was separated on a cellulose column instead of silica gel). The column was packed in pet.ether in a one inch diameter column. The oil was loaded

on to the column after it was adsorbed on a small amount of cellulose. The 3-O-benzyl-AA acetonide got eluted out with 5 to 15% $CHCl_3$ in pet.ether. The recovery was almost 90 to 95%.

3. Benzylation of D-iso-AA

This was carried out exactly under the same conditions as described for the case of AA using the same quantities of materials.

The oil obtained from ethyl acetate layers refused to crystallise even after long standing. So, this was taken up for chromatography as such. The chromatography was also done under the same conditions. The behaviour of the oil on the column was similar to that of the AA benzylation product. Here also, all the fractions were checked by PMR spectra. Only 3-O-benzyl derivative could be obtained in the chromatography.

4. Benzylation of AA in solvent systems other than aqueous acetone

In case of purely aqueous system and aqueous-MeOH system the work-up procedure was almost the same. Only in case of aqueous-MeOH system, the ethyl acetate extraction was done after removing MeOH. In case of DMSO, the solvent was removed at 50°C in a rotary evaporator under vacuum and the residual oil was dissolved in ethyl acetate. The ethyl acetate layers dried and concentrated to get oils, which were analysed by PMR spectrum to get O/C benzylation ratio.

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# Chapter VI

## HYDROXYLATIONS MEDIATED BY ASCORBIC ACID

Introduction

Ascorbic acid (AA) is known to play a key role in many biochemical transformations[1]. An important type of reaction catalysed by AA in living beings is hydroxylation. AA has been shown to be essential for the conversion of proline to hydroxyproline in collagen synthesis[2], the biogenesis of corticosteroids[3,4] and the conversion of tryptophan to its 5-hydroxy derivative[5]. AA is also known to hydroxylate simple aromatic compounds for instance, acetanilide, in presence of NADH[6]. However, all these are enzymatic reactions.

In 1943, Warren demonstrated for the first time non-enzymatic oxidations catalysed by AA[7]. Hydrocarbons like anthracene, naphthalene and 3,4-benzpyrene were found to be hydroxylated in 80% aqueous acetone by air in presence of AA. The reactions were carried out in the absence of light to prevent photo-oxidation of AA. This was an important observation because the aromatic hydrocarbons are carcinogenic, and it is essential for the succeptible organisms to be able to eliminate or detoxicate them if they get into their bodies. It is of interest here to note that it has been shown that AA when fed to animals on naphthalene diet inhibits the development of cataract[8]. In the absence of AA, naphthalene added into their diets induced the development of cataract in the animals.

Udenfriend, Bordie for the first time, made a systematic study of aromatic hydroxylations mediated by AA[9-11].

They demonstrated that the products obtained in enzymatic as well as nonenzymatic systems are the same. They successfully carried out hydroxylations of various aromatic compounds like acetanilide to N-acetyl-p-aminophenol, aniline to p-aminophenol, salicylic acid to gentisic acid etc, with a system consisting of Aa, $FeSO_4.7H_2O$ and disodium salt of EDTA in a phosphate buffer in presence of molecular oxygen or $H_2O_2$[10,11]. This system has since been called the 'Udenfriend system' or a 'model system' for hydroxylation which mimicks the enzymatic reaction. Later, various workers have made use of the model system for different types of hydroxylations and suggested different mechanisms for the reactions involved. The transformations that have been claimed to be produced by the Udenfriend system include hydroxylations of amino acids[12-18], aromatic compounds including hydrocarbons[17,19-31], corticosteroids[32-34] and deoxychlolic acid[34], N-demethylation[35], oxidative decarboxylation of fatty acids[36-38], epoxide formation[39] etc., out of which aromatic hydroxylations is the one to which most of the studies have been devoted.

However, out of these, hydroxylations at $sp^3$ carbon atoms are of special interest since these are difficult functionalizations to bring about without the help of enzymes. Conversions of 11-deoxycorticosterone, 7-deoxycholic acid and proline into corticosterone[32-34], cholic acid [34] and hydroxyproline[17] respectively by the model system have

been reported. The products mentioned are the same as those obtained in the enzymatic reactions. These are highly regio and stereospecific transformations. In the conversion of deoxycorticosterone, for instance, the reported observation means that the model system produces a species that is able to selectively abstract a hydrogen from the 11-position and oxygenate the site selectively from the β-side. If the reactive part of [illegible] is the ene-diol-γ-lactone system as is generally assumed, and the reactive intermediate involved in the hydroxylation is considered to be one derived from it without any important structural change, it will be difficult to understand the reported specificity. The hydroxyl[9,19,21,23,27,30] and hydroperoxy[23,30,57] radicals, mono-dehydro-ascorbic acid[23,29] and the $OH^+$ ion[40] have been suggested as reactive intermediates in the reaction. But, it is inconceivable that these can give rise to the selectivity that has been claimed[17,32-34]. In this connection, it is natural to ask if the side chain has any role to play and whether the kind of bicyclic intermediates we have considered in the earlier chapters in connection with benzylation and autoxidation might not be involved. A reactive intermediate obtained from these would be more suitable for obtaining stereo-selective transformations.

It is also conceivable that the same question may be connected with another difficulty regarding the reactivity of [illegible]. Although it is a good hydroxylating agent in presence

of molecular oxygen, the substance and some of its derivatives are also known to be oxidation inhibitors[41]. For instance, the 6-esters of AA have been used for the stabilization of unsaturated fatty oils against autoxidation[42,43]. It is also generally assumed that, in non-enzymatic reactions, the behaviour of AA is governed by the properties of the ene-diol-$\gamma$-lactone system. The manner in which the same moiety can play these seemingly contradictory roles has never been explained. It is possible that it is again the involvement of the side chain that would help provide a satisfactory explanation for the difficulty. It was therefore thought that a comparison of the effectiveness of AA and some of its derivatives and analogues in Udenfriend hydroxylations might give useful information as in the case of the autoxidation experiments. But before we get to these experiments, it would be appropriate to give here a fuller account of the current status of knowledge on Udenfriend systems.

The substrates that have been studied and the transformations that have been achieved have been indicated. We shall restrict our attention here mostly to hydroxylations. The components of the Udenfriend system are AA or any other ene-diol, a metal ion, most often iron, a complexing agent like EDTA and molecular oxygen or $H_2O_2$. The medium of reaction is generally a phosphate buffer. Elimination of any one of these components produces a very remarkable reduction of the hydroxylation efficiency.

Several ene-diols including dihydroxymaleic acid and dihydroxyfumaric acid and catechol have been tried in place of AA with positive results[9,36]. Diketo compounds like DHA, which were initially claimed to be active, were later found to be inactive in Udenfriend hydroxylations[19]. In the case of oxidative decarboxylation of fatty acids by AA, it was shown that an ene-diol such as AA, where one proton is more acidic than the other (an unbalanced ene-diol), was necessary for activity as a catalyst[36]. The configuration at $C_5$ also seemed to have a significant effect. D-Iso-AA had half the activity of AA and DHA had none. DHMA and catechol were not very effective in producing oxidation[36].

Ions of copper or iron were essential for the Udenfriend system to work. Iron has been found to be more effective since copper destroys the ene-diols too rapidly by autoxidation[45]. There has been a great deal of controversy as to whether $Fe^{2+}$ or $Fe^{3+}$ is the effective catalyst and all conceivable possibilities have actually been suggested[9,19,20,21,24-27,30,31]. Those who favour monodehydroascorbic acid as the reactive intermediate assume $Fe^{3+}$ as the effective catalyst[22,29]. Replacement of iron by $Co^{2+}$, $Co^{3+}$, $Mg^{2+}$, $Mn^{2+}$, $Hg^{2+}$ gave little or no activity[37].

The use of a chelating agent, although not absolutely essential, enhances the rate and yield of hydroxylation by preventing wasteful oxidation of the ene-diol[9,15,27,28,36]. The disodium salt of EDTA is the most commonly used chelating

agent and an Fe-EDTA-$O_2$[21,44] complex has been proposed as an intermediate in the reaction. Although iron forms only a 1:1 complex with EDTA, the optimum metal to ligand ratio has been found to be 1:2[27,28].

As noted earlier, either molecular oxygen or $H_2O_2$ is required as the oxidant in the Udenfriend system[10]. Use of labelled oxygen demonstrated that the oxygen introduced into the substrate is mainly coming from the molecular oxygen[23]. Reaction conditions like temperature, pH and the concentrations of ene-diol and metal ion have also been studied[9,22,27,35,36]. The majority of hydroxylation have been done at 30 to 35°C. Hydroxylations have been carried out over the pH range 2 to 7.5, the optimal pH depending on the substrate. Many workers have preferred a pH of 6.8 which is close to physiological conditions. At higher pH, wasteful oxidation of AA or other ene-diol has been found to be greater[45]. The rate and amount of hydroxylation increased with increased concentration of the ene-diol upto a certain point beyond which no added advantage was obtained[9,35]. A similar effect was seen for the metal ion concentration also[9,27,35].

Although, the products in the enzymatic and non-enzymatic hydroxylations are the same, there is an appreciable difference between the two systems[21,25,26]. Hydrogen-peroxide can be used only in the nonenzymatic case[21,25]. The AA that is converted into DHA in the non-enzymatic reaction is irritrievably lost. But, in the

enzymatic case, the DHA formed is reduced back to AA. It must also be mentioned that though there are many reports claiming high specificity for the "model system", there are a few that seem to indicate formation of only mixtures of products[16,21-23]. There is at least one each of this type for proline[16] and some aromatics[21-23], including phenylalamine[14] and coumarin[21,47]. However, claims of specificity have been a predominant feature of the available literature.

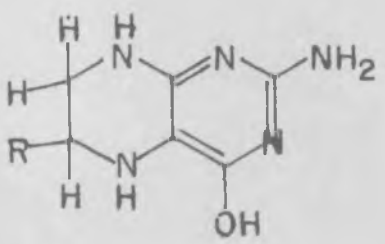
Various mechanisms have been suggested for these hydroxylations. All the proposals that have so far been made, relate to aromatic systems. In the earlier studies, no distinction was made between reactions using molecular oxygen and $H_2O_2$. The two cases were deemed identical and a common mechanism was assumed. In the initial phase of the extensive studies on the Udenfriend system, most workers were of the opinion that AA is oxidised in presence of molecular oxygen and metal ions, to give hydrogen peroxide, which in presence of $Fe^{2+}$ will give rise to OH radicals. This reaction mixture was thus assumed to act like Fenton's reagent[48]. Grinstead[23] showed that the enzyme catalase, which destroys $H_2O_2$, inhibited hydroxylation and assumed that this favoured the Fenton type of mechanism in case of aromatic substrates. He also suggested the possibility that $HO_2$ radicals might also be partly involved in the reaction. The OH or $HO_2$ radical would generate aryl free radicals which would

continue the chain. Similar free radical mechanisms were favoured by Douzue[31] and Buhler[21].

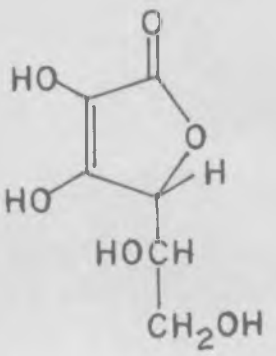
Many others, however, could not furnish proof for existence of $H_2O_2$ any time in the model system. Also it was shown that $H_2O_2$ could not replace the ene-diol in the system[25,36]. Udenfriend et al.[10] believed that the hydroxylating species is some unknown product formed by the reaction of AA and $H_2O_2$, which is itself obtained by the reduction of $O_2$ by AA. Dalgliesh[29] and Acheson[22], who supported this, considered a free radical electron acceptor like monodehydroascorbic acid (MDHA) as the active intermediate and ruled out the possibility of attack by $OH^+$ as suggested by Williams[40]. The fact that hydroxylations are at electron rich sites is consistent with a free radical mechanism[22]. The presence of free radical in the model system was proved by Acheson by initiation of polymerisation by the 'model system'[22]. The existence of MDHA during oxidation of AA in presence of metal ions has been proved by several workers using ESR spectroscopy[49].

Some more recent studies have suggested that the two types of model systems i.e. model system with $O_2$ and with $H_2O_2$ as the oxidants are different and act by different mechanisms[25,26]. Where $H_2O_2$ is oxidant, OH radicals are involved, and, when molecular oxygen is the oxidant, the reactive species is presumably different.

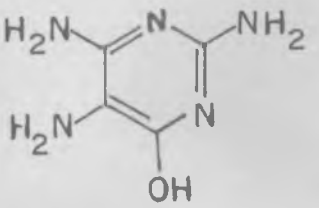
An interesting and attractive mechanism has been suggested by Hamilton for the Udenfriend type of oxidation[26]. This mechanism appears to be applicable for both $sp^2$ and $sp^3$ hydroxylations. It is similar to the one suggested earlier for hydroxylation of anisole by hydrogen peroxide requiring catalytic amounts of ferric ion and catechol[56]. The opinion has been expressed that such reactions are reminiscent of carbene reactions and the implication is that an oxygen species with six electrons and similar to a carbene or carbenoid species is responsible for oxidation. The evidence supporting the suggested mechanism (Chart 1) for the model system is the similarity of some of its features to those of the enzymatic system. The tetrahydropteridine cofactor (I) required by phenylalanine hydroxylase has structural features similar to [illegible] (II) and 2,4,5-triamino-6-hydroxypyrimidine (III) which can replace [illegible] in the model system.



I

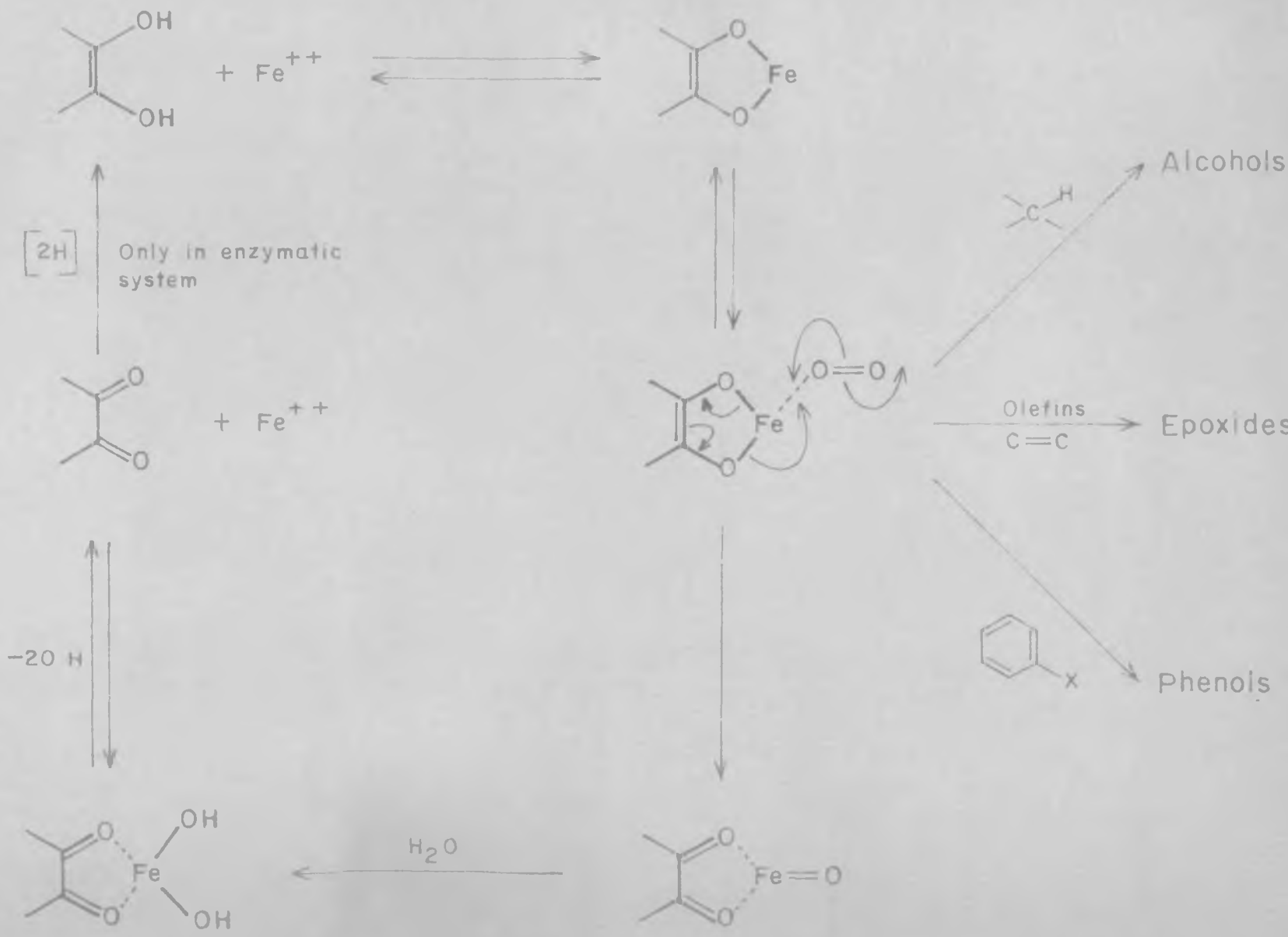


II



III

CHART I


OH
OH
+ Fe++
O
O
Fe
[2H]
Only in enzymatic system
O
O
+ Fe++
O
O
Fe
O=O
C
H
Alcohols
Olefins
C=C
Epoxides
X
Phenols
-20 H
OH
O
O
Fe
OH
H2O
O
O
Fe=O

Both model and enzymatic systems appear to require a metal ion usually $Fe^{2+}$. The stoichiometry of the reaction is as follows:

$$O_2 + S + AH_2 \rightarrow SO + A + H_2O,$$

where S is the substrate being oxidised and SO the product.

Most of the mechanisms suggested have been in connection with hydroxylations at $\underline{sp}^2$ carbon sites where the question of stereoselectivity is not involved. The reported hydroxylations at $\underline{sp}^3$ carbons are substantially with the same reagent and under the same conditions. It would therefore appear that the same reactive species would be involved. However none of the species suggested seem to be suitable for a stereoselective attack on the substrate. Further for hydroxylation to take place at an $\underline{sp}^3$ carbon, removal of a hydrogen atom and introduction of hydroxyl are necessary steps. If the intermediate is reactive enough for this purpose, it is not clear why the reported regioselectivities were obtained. As indicated earlier, it was therefore considered that it would be of special interest to see if one or more of the reported hydroxylations at $\underline{sp}^3$ carbons could be reproduced. This has been one of the important things done in this study described here. The problem of the dual role of AA, one as an efficient oxidation mediator and the other as an inhibitor of autoxidation, has been already mentioned and this needs clarification.

It is thus seen that there are many features in the chemical behaviour of ascorbic acid still which remain to be accounted for satisfactorily. The possibility that the involvement of the side chain may be one of the factors that complicates the behaviour of AA is the proposition we have studied in earlier chapters in this thesis. In the background of the review that has been made, it therefore appeared useful to compare the efficiencies of AA with that of some of its derivatives and analogues in the Udenfriend hydroxylation of a suitable substrate. The compounds studied include the 6-benzoate and the 6-palimate of AA, 5,6-isopropylidene AA, phenyl tetronic acid, phenyl tetronimide, dihydroxymaleic acid, dihydroxyfumaric acid and tetrahydroxyquinone.

Materials, procedures and methods

The choice of a suitable hydroxylation experiments for bringing out conceivable differences in the reactivities of various ene-diols was a difficult problem. A number of questions had to be answered before this choice could be made. This involved a great deal of experimentation. A suitable substrate had to be selected and the convenient concentration ranges for the reactants, methods for measurements of the progress of the reaction and the conditions best suited for ready recognition of differences had to be determined. We may begin with the preparation of the required materials.

6:A Materials

a) The following substances were used without further purifications. (1) $FeSO_4.7H_2O$ (BDH Analar sample), (2) Disodium salt of EDTA (Analar Merck sample), (3) Dihydroxy fumaric acid (Fluka sample), (4) Dihydroxymaleic acid (Aldrich sample 98% pure), (5) D(-) isoascorbic acid (Fluka sample 99% pure), (6) Gentisic acid (BDH sample).

b) Ascorbic acid: GR grade AA (Sarabhai Merck) was recrystallised twice from double distilled water before use.

c) Salicylic acid: Analar grade sample was recrystallised twice from double distilled water.

d) 5,6-Acetonide of AA: This was prepared according to the procedure of Jackson[50] which is better than others described earlier.

AA (10 g) was dispersed in acetone (40.0 ml) to which acetylchloride (1.0 ml) was added. The solution was stirred well at room temperature for about 4 hrs. The mass was then cooled to 0°C to crystallise out most of the acetonide formed. It was then filtered under suction. The white crystalline mass of acetonide was washed twice with cold dry acetone. This was then recrystallised thrice from dry acetone.to give needle shaped crystals ( m.p.: 222°C and showed $[\alpha]_D^{\cdot}$ + 28° in MeOH). Since, this derivative was very sensitive to moisture, it was stored in a desiccator. On standing it developed light brown colour so that it had to be recrystallised each time before use.

e) 6-Benzoate of AA[51]: AA (17.6 g) was dissolved in Analar (98-99%) $H_2SO_4$ (250 ml) to which benzoic acid (18.32 g) was added. The mass was stirred for 3.5 hrs at room temperature. The reaction mixture was then poured on 500 g of crushed ice, and then extracted three times with ether (300 ml). The ether layer was then washed twice with 100 ml of brine, dried on anhydrous sodium sulfate and distilled to remove the solvent. The dry residual solid was extracted several times with dry petroleum ether (60-80°C) to remove unreacted benzoic acid. The residue which was the 6-benzoate of AA was recrystallised thrice from ethyl acetate-Acetone-pet-ether mixture (m.p. 182°).

f) 6-Palmitate of AA[52]: This was prepared according to the latest procedure given by Cousins et al. AA (14 g) was

dissolved in Analar sulfuric acid (98-99%, 250 ml), to which palmitic acid (26.0 g, 90% pure) was added. The mass was stirred for about 2.5 hrs at room temperature, the clear solution was allowed to stand for 3.5 hrs more at room temperature. The mass was then poured on 500 g of crushed ice and then extracted thrice with 300 ml portions of ether. Ether layer was washed with brine (2x100 ml). The ether layer was dried on anhydrous sodium sulfate. The ether was then evaporated to get a dry solid. This solid was then extracted twice with 300 ml portions of pet-ether (60-80°C) to remove unreacted palmitic acid. The residue was recrystallised thrice from chloroform to get the product (m.p. 110-115°C).



g) Phenyltetronimide: KCN (35.0 g) in 2 N aqueous soda solution (1 litre) was treated with stirring and under a current of oxygen free nitrogen with glyoxal hydrogen sulfite dihydrate (87.5 g) and a solution of freshly distilled benzaldehyde (26.0 g) in dioxane (50 cc). The olive green solution gave a colourless precipitate after 30 minutes. The pH of the solution at this stage was adjusted to 6 with glacial acetic acid. The solution turned yellow and was stirred for 3 more hours. The precipitate was filtered and washed with lot of distilled water, methanol and ether. The precipitate was dried in a vacuum desiccator and then recrystallised several times from ethanol to get a m.p. of 173-177°C (decomposition).

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h) Phenyltetronic acid: Phenyltetronimide (1.91 g), prepared as above was dissolved in a mixture of 2 N $H_2SO_4$ (25.0 ml) and acetone (10.0 ml). The solution was cooled to 0°C and 10% $NaNO_2$ solution (15 ml) was added over a period of 15 minutes. The solution was stirred for 1 hr and allowed to stay overnight. The precipitate was filtered under suction and washed with water. On concentrating the filtrate some more of the product was precipitated.

1.0 g of this compound was dissolved in t-amyl alcohol (60 ml) and absolute methanol (20 ml). This solution was saturated with $H_2S$ gas at room temperature and allowed to stay for 14 hrs. Excess $H_2S$ was then flushed out with a current of $CO_2$. The solution was concentrated under vacuum to get a residue. The residue was redissolved in t-amyl alcohol and sulfur was removed by filtration. The filtrate was concentrated under vacuum. The residue obtained was recrystallised thrice from acetone pet-ether (60-80°C) to get the pure product (m.p. 154-155°C).

55

i) Tetrahydroxyquinone: A solution of anhydrous sodium sulfite (40.0 g) and anhydrous sodium bicarbonate (15.0 g) in 300 ml of water was heated to 40-45°C. To this was added 50% of glyoxal solution (48.0 ml) and a brisk stream of air was drawn through the solution for 1 hr without application of heat. Within a few minutes, greenish black crystals of the sodium salt of tetrahydroxyquinone began to separate. The flask was then warmed to 80-90°C over a period of one hr.

The air current was then stopped and the mixture was heated to incipient boiling and set aside for 30 minutes. On cooling this solution, the sodium salt of tetrahydroxyquinone separated out, which was filtered and washed successively with cold 15% NaCl solution and finally 50.0 ml of cold water.

This salt was now added to 50.0 ml of 2N HCl and the mixture was heated to incipient boiling. The resultant solution was cooled in an ice bath when the glistening black crystals of tetrahydroxyquinone precipitated out. The crystals were filtered under suction, washed with ice cold water and dried. The quinone failed to melt even at 320°C.

6:B Procedures

All the glass vessels and burettes used were thoroughly washed with chromic acid, then rinsed several times with double distilled water and then dried without using any organic solvent.

Both air and oxygen were used as oxidants. The procedure using air as oxidant was used only in the initial experiments and it was later abandoned.

Preparation of buffers: Solutions of 1M strength of $K_2HPO_4$ and $KH_2PO_4$ were prepared in double distilled water and were stored at 0°C. For each experiment, different amounts of these solutions were mixed to get the required pH and then diluted with double distilled water to make 0.1M buffer. The pH of the buffer was measured on a Elico pH meter. The

stock solutions standing for more than fifteen days were discarded.

6:B.1 Chromatographic procedures

Chromatograms were developed on strips of Whatman No.1 filter paper (14 cms x 46 cms). A maximum of four samples were spotted on each strip. For comparative studies, equal amounts of reaction mixtures were spotted with a graduated capillary to ensure comparable conditions. Each time the capillary was thoroughly washed before use. A hot air blower was used to dry the spots, thus preventing the over spreading of the spots. The chromatogram was developed with the solvent system described below by the descending technique. The chromatographic chamber was saturated with vapours of the same solvent system. Each run took about 14 hrs. The papers were then removed from the chamber and then dried in air. The dry paper was then sprayed uniformly with the desired spraying reagent for identification of products. Only when ninhydrin was sprayed the paper was to be slightly heated with a hot air blower. For the other two reagents (A and B) the colour development was immediate. But the chromatograms were not very stable. Photographic records were therefore made immediately after spraying the reagent.

The developing solvent system was prepared in the following manner. n-Butanol, acetic acid and water were mixed in the ratio of 4:1:5 (by volume) in a separating funnel. The mixture was shaken well and allowed to settle

for 1/2 hr. The lower aqueous layer was discarded. The upper organic layer was used for developing the chromatograms.

Spraying agents were prepared in the following manner.

(a) Ninhydrin solution: One percent (w/v) ninhydrin solution was prepared in acetone.

(b) Reagent A: A solution of one percent (w/v) analar $FeCl_3$ in distilled water was prepared.

(c) Reagent B: 3 volumes of the above solution was mixed with one volume of a one percent solution of potassium ferricyanide in distilled water. This reagent was not stable and was freshly prepared each time.

6:B.2 Procedures for hydroxylation

a) The Model system consisted of the following: 15.0 mg of $FeSO_4.7H_2O$, 75.0 mg of disodium salt of EDTA and the chosen amount of ene-diol (AA, its derivative or analogue).

b) Substrate: Proline, salicylic acid or gentisic acid was used in quantities indicated below. Proline used was 10.0 mg for all the experiments done on its hydroxylation. The SA amount was 20.0 mg for most of the experiments and was then decreased to 10.0 mg for experiments described in 6:C.13.

Though the substrate to ene-diol ratios varied in several experiments, the amounts of $FeSO_4.7H_2O$ and disodium salt of EDTA were the same. The buffer used was 0.1M of pH 6.8 for most of the experiments, though 0.3M strength and

different pH's were also tried in a few experiments.

Procedure I: Using air as the oxidant: The buffer components and the substrate to be hydroxylated were dissolved in 25.0 ml of buffer and this solution was placed in a reactor as shown in Fig. 1.

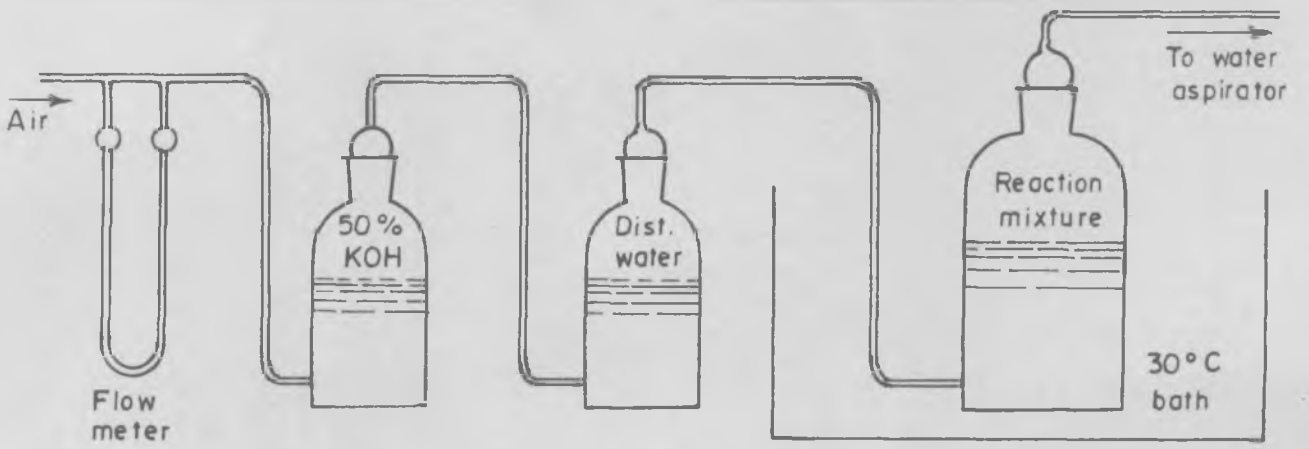



FIG. 1.

Air was drawn through the solution at a controlled rate (90-100 cc/min) using a flow meter connected to the system. At different time intervals, samples were drawn using the same graduated capillary and were spotted on filter paper strips.

Procedure II: Using oxygen as the oxidant - This was the procedure used for most of the experiments described here. For all the comparative studies, only this procedure was employed.

The components of the model system were accurately

weighed into a clear 250 ml Erlenmeyer flask fitted with a head having a dropping funnel and a set of inlet and outlet tubes as shown in Fig.2.

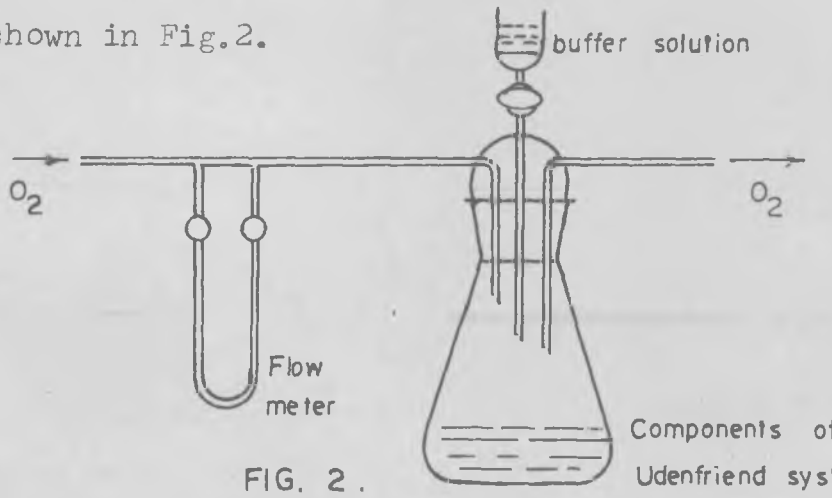



FIG. 2.

20.0 ml of the phosphate buffer was placed in the funnel. Oxygen from a cylinder was now passed through the inlet at a constant pressure for 30 seconds. The buffer was then dropped into the flask in a period of 30 seconds while maintaining the $O_2$ flow. The $O_2$ was passed for a further period of 30 seconds. The assembly piece was then removed and the flask was immediately stoppered with a ground glass stopper. This flask was then kept on a mechanical shaker for the desired length of time. Samples were then drawn with a graduated capillary. For each reaction time the progress of hydroxylation was measured in a separate run so that no ambiguity arose from the opening of the reaction vessel for sampling purposes. This procedure was specially suited for making accurate comparisons of hydroxylation.

6:C Methods

As mentioned in the proceding survey, our first aim

was to check the reproducibility of the reported highly specific hydroxylations at $sp^3$ carbon atoms. For this purpose, we chose proline as substrate which has been studied quite a few times with the Udenfriend system and more frequently under enzymatic conditions.

6:C.1 Hydroxylations of proline by model system

Even after several repetitions of the reported procedures of hydroxylation of this compound, we were unable to detect any hydroxylation. The reported methods claim very poor yields in these reactions. The procedures employed used very high proportions of AA in relation to the substrate taken.[17,33,34] As a consequence, the buffer used was unable to maintain the pH mentioned. In this study, several other reaction conditions including variations of the concentrations of AA, metal ion and EDTA, buffer strengths and pH were tried. Continuous bubbling of air or oxygen as well as filling of the closed vessels containing the Udenfriend system with oxygen was also tried. However, in no case could we get a detectable hydroxylation. All the reactions were monitored by paper chromatography. The reaction mixtures were checked periodically from the beginning of the mixing of the reagents for about 5 hrs. A reference sample of a mixture of proline and hydroxyproline dissolved in the buffer was spotted on the same paper for comparison.

In many of the hydroxylations reported at $\underline{sp}^3$ carbon atoms, the identification of the products has been done by paper chromatography alone. In our experiments, we also got a new spot after a few hours of reaction which had the same $R_f$ value as that of the reference hydroxyproline. However, a careful examination showed that this spot gave a light brown colour with ninhydrin, while the reference hydroxyproline gave an intense lemon yellow spot. To avoid any confusion, a small amount of the reference compound (hydroxyproline) was added to the reaction mixture after 5 hrs of reaction (which was giving a brown spot) and the mixture was rechromatographed. It was found that the light brown diffuse spot was clearly superposed on an intense lemon yellow one in the centre. This therefore meant that the brown spot having the same $R_f$ value as that of hydroxyproline was not really due to hydroxyproline. It appeared very likely that this represented a product of AA decomposition. This suspicion was proved to be correct experimentally. A blank reaction with the model system was repeated for a period of 5 hrs, which had no substrate, viz. proline. As in other cases, the AA present decomposed during this period. The chromatogram of the reaction mixture gave a brown spot at the same place as before, confirming that it was only an AA decomposition product. Another study had reported that hydroxylation of proline was observed only within the first three minutes.[15] In order to confirm this, the experiment was repeated with reaction

times varying from 1 to 60 minutes. The chromatograms of none of these reactions showed even traces of hydroxyproline.

6:C.2 Hydroxylation of aromatic substrates

For comparing the efficiencies of AA, its derivatives and analogues, choice of a proper substrate and a procedure for determining the extent of hydroxylation was necessary. In the light of the results just mentioned, it was clear that an aromatic substrate had to be employed. Some of the reported procedures, involving tedious and lengthy solvent extractions, did not appear convenient for our purpose.[11] The more the number of operations, the more was the loss of the product formed. For the comparative studies of our interest here, methods not involving any processing were more suitable. Also, in the first place, one had to see if any qualitative difference between these various ene-diols could be recognised before going into elaborate quantitative procedures for their measurement. In this regard, paper chromatography was more attractive. This technique has been used by several workers both for hydroxylations of saturated and aromatic substrates. The difficulties in using this method were the choice of a suitable developing solvent system and a spraying reagent. The spraying reagent should preferably give different coloured spots for substrate and product. Also, the recognition of changes in the concentrations of the product and substrate should be possible by visual comparison of the colour intensities produced.

6:C.3 Choice of oxidising agent

Out of the two oxidising agents, $H_2O_2$ and molecular oxygen generally used with the model system, we selected the latter in this study. This was closer to the physiological conditions. The $H_2O_2$ system has been reported to act in a manner similar to that of Fenton's reagent, which has been shown to be different from that obtained with $O_2$.[25,26]

6:C.4 Choice of substrate

Various aromatic compounds were successfully hydroxylated by the AA model system under varying conditions of buffer strength and pH. The following aromatic compounds were successfully hydroxylated; benzene, toluene, nitrobenzene, aniline, acetanilide and salicylic acid. The corresponding phenolic derivatives were the products. In the case of benzene and toluene, the substrates were very poorly soluble in the model system and also were volatile and would not show up in chromatography. With nitrobenzene, aniline and acetanilide, the spraying reagents were not very satisfactory. Out of all these, we found the hydroxylation of salicylic acid (SA) to gentisic acid (GA) the most convenient one. The spraying reagent A gave a pink spot for SA, the grey blue colour for GA was not deep and stable. It was therefore not convenient for visual comparison of intensities. However, reagent B was very sensitive to GA, giving a stable dark blue spot. The colour with SA was again pink. The solvent system used for developing the chromatograms gave

different $R_f$ values for SA, GA and the decomposition products of AA. Thus, there was no room for confusion. However, it was found later that the derivatives of AA employed as mediating agents in this study had the same $R_f$ value as GA. The overlap problem was solved by methods described elsewhere.

Though the majority of our experiments were done with SA as substrate, it was found later that hydroxylation of GA could be employed with greater advantage. With this substrate, the differences in the reactivity of AA and its derivatives could be brought out more clearly. Also, the spot for GA with reagent B was more stable and intense as compared to the weak and unstable one for SA. Stable spots were obviously more suitable for obtaining photographic records.

6:C.5 Choice of reaction conditions

To avoid the decomposition of the phenolic products of hydroxylation we did all our hydroxylations at room temperature (25°C). The most commonly used phosphate buffer was chosen for the purpose. Though different molar strengths were successfully tried, it was decided to employ a 0.1M phosphate buffer. SA hydroxylation with AA model system was carried out in the pH range of 4 to 7. A pH of 6.8 was very suitable for us for this study since the 5,6-isopropylidene and the 6-benzoate derivatives of AA involved in our studies here were relatively more stable at this pH. Further, this pH was closer to physiological conditions.

Initially, only air was employed as the oxidant in the model system. But, as we have seen in the kinetic studies on autoxidation of AA, the composition of laboratory air was not the same all the time and this did affect the AA oxidation. So, it was decided to use only oxygen from a cylinder which ensured uniformity. In the initial experiments, $O_2$ was bubbled continuously throughout the run. However, this was not really necessary because the oxygen in the reaction vessel itself was more than sufficient for the oxidation. Closed vessels of uniform capacity and having ground glass stoppers were preferred, so that the comparison would be unexceptionable. However, there was not much difference between the chromatograms of hydroxylations when air or $O_2$ was used.

Experiments were also tried replacing $O_2$ by $N_2$ in the model system. No detectable hydroxylation was observed when $N_2$ was used.

6:C.6 Hydroxylation of SA mediated by AA under different conditions.

In the initial sets of experiments, we used 20.0 mgs of SA, 15 mg of $FeSO_4 7H_2O$, 75.0 mg of AA and 75 mg of disodium salt of EDTA in 20.0 ml of 0.1 M phosphate buffer of pH 6.8. Samples of reaction mixtures were analysed after 5,10,15 .... 60 min, 2 hrs and 3 hrs. The hydroxylation appeared to be very fast and was substantial within the first 10 minutes itself. Detectable hydroxylation was

observed just after one minute of the mixing of all the compounds and filling of the oxygen to initiate the reaction. However, after 15 minutes, the spot intensity for GA fell with time. This was probably due to further hydroxylation of GA, which was confirmed in a separate experiment. The hydroxylation was carried out exactly under the conditions mentioned above using GA as a substrate. The chromatograms of the reaction mixture after 5,10 ... 30,60 min. showed the gradual decrease of intensity for the GA spot confirming its hydroxylation. With SA as substrate, its concentration would show a monotonous decrease while that of product GA would first increase and then decrease if the first hydroxylation has the higher rate. It was also seen from the chromatograms of SA hydroxylation that, even after 3 hrs, the SA spot intensity appeared to decrease indicating that the model system was still active. In one case, we continued the reaction for 24 hrs, with refilling of oxygen every 6 hrs. Although the SA spot intensity decreased to a considerable extent, it did not completely disappear. There was not much difference between the 5 hr. and 24 hr. chromatograms.

6.C.7 Effect of varying AA concentration on hydroxylation

An attempt was also made to find out the minimum amount of AA needed in the model system to bring about detectable hydroxylation. A series of experiments were done with no AA and with increasing amounts of AA (5.0 mg, 10 mg, 20.0 mg, etc). The experiment with no AA called the blank run, also gave a small amount of hydroxylation, as indicated

by the tiny faint blue spot for GA. The amount of hydroxylation increased with increasing amount of AA. Increase in the amount of GA i.e. amount of hydroxylation with increase in AA was detectable by the naked eye upto the limit of 10.0 mg. The differences in colour intensities obtained for product GA with larger amount of AA (for instance, 15 mg and 20 mg) in the model system could not be visually ascertained since they were too deep. They appeared to give the same intensity. Also, increasing concentration of AA in the system increased the hydroxylation of the product GA. This would add to the difficulties in comparison. These experiments implied that smaller amounts of AA than customarily employed would be adequate and convenient for the purpose of the present study.

6:C.8 Limitations of the spraying reagents selected

In the initial experiments, we were spraying the chromatograms only with reagent A. This reagent was quite sensitive to SA, giving a pink spot which was not stable for a long time. It also gave a grey blue spot for GA, the apparent colour of which did not seem to be a quantitative index and could not be used for visual comparison. Reagent B was very sensitive for GA. For SA, its colour was same as that produced by reagent A. The chromatograms had to be photographed immediately after spraying the reagents. In the case of reagent A the spots are not stable and in the case of reagent B the entire paper turned blue within a few minutes. For following only the disappearance of SA,

reagent A was more suitable. It did not affect the colourless background of the paper, making photography easier. For following the changes in the amounts of SA and GA with time, reagent B was more suitable. The intensity of the blue spot for GA obtained was so high that, beyond certain concentration, visual comparison became too difficult. For this reagent, the concentration had to be modified such that the visual comparison of colour produced would be meaningful. Different amounts of GA (2,5 .. 25 mg) were dissolved in 20.0 ml of buffer. Equal amounts of these solutions are spotted with a graduated capillary. The chromatograms were developed in the usual manner. They indicated that the intensities obtained with more than 8 to 10 mg were too deep for visual comparison.

Experiments on the hydroxylating efficiency of MA system with that of its 6-substituted derivatives were undertaken under the optimum conditions thus established.

6:C.9 Some difficulties encountered in the comparative studies.

In the comparison of the relative activities of MA and the acetonide of MA in the Udenfriend hydroxylations, the amount of acetonide employed was the molar equivalent of MA and the proportions of other reagents of the model system remained the same including the substrate SA. Substantial amount of hydroxylation was obtained even with acetonide. The chromatograms of the reaction mixture for a

5 min. reaction showed that the GA spot was less than that for the one mediated by AA. For a 15 minute reaction, the chromatogram showed almost equal intensities for both the systems. For the 25 minute reaction chromatograms, however, the order was reversed, the GA obtained with the acetonide being more than that obtained with AA. This was so apparently because, the GA formed with AA was further hydroxylated more rapidly. This fact was later confirmed when GA was used as the substrate. It appeared to us that the apparent reactivity of acetonide would not be the correct figure if the material was contaminated with small amounts of AA. Our acetonide sample was twice recrystallised from acetone giving correct melting point and rotation. However, the chromatograms of the material showed a trace amount of residual AA in it. So, the hydroxylation observed with it could also be attributed, at least in part, to the trace amount of AA. Such small amounts would be adequate to produce appreciable hydroxylation as seen in 6:C.7.

6:C.10 Pre-incubation experiments

For a comparison of the stabilities of AA and acetonide a qualitative experiment was done in which these ene-diols were incubated with $O_2$ in the model system for an hour. The chromatograms of the reaction mixture showed a spot for acetonide, but none for AA. It appeared that acetonide could withstand the pre-incubation, i.e. acetonide appeared to be more stable than AA. This was, of course a qualitative

result. It was not clear as to how much acetonide was remaining. However, it suggested pre-incubation of the reaction mixture with acetonide before the addition of substrate would destroy the trace amount of AA, perhaps without doing much damage to the acetonide and would solve the problem. These experiments were actually done before the autoxidation work described earlier was carried out. The comparative hydroxylation experiments employing pre-incubation for the reaction with acetonide were actually carried out, the pre-incubation period being 1 hr. The substrate SA was then added to the reaction mixtures and the flasks refilled with oxygen. Hydroxylation was done for different lengths of time. The chromatogram even after 15 min. of reaction showed only a trace amount of GA, with the pre-incubated acetonide, which was comparable to that obtained in the blank run where no ene-diol was added. The chromatograms for acetonide reaction with pre-incubation were checked at different time intervals also. In comparison, a strong spot for GA was seen in the experiment with AA. On the surface, it looked as if there was a dramatic difference between AA and its acetonide. However, experiments with specially purified samples of the acetonide having no trace of AA showed that the acetonide was capable of giving quite substantial hydroxylation. Similar experiments were tried with other 6-substituted derivatives, viz. AA 6-benzoate. This also gave a very substantial hydroxylation, although

it appeared to be less as compared to with that obtained with AA. The amount of hydroxylation in the case of benzoate was even less than that obtained for the acetonide.

This meant that pre-incubation before reaction in the case of the acetonide had destroyed not only the AA in it, but also most of the acetonide. Not only acetonide, but also AA and its benzoate gave similar results, i.e. there was no hydroxylation of SA with model system when the pre-incubation with $O_2$ was adopted before additions of the substrate. In any case, the pre-incubation procedure became irrelevant when a satisfactory procedure of making the pure acetonide, absolutely free of AA, became available. Although the experiments with the pre-incubation were thus not of much use from the present point of view, it brought out clearly that earlier claims to the effect that hydroxylations were not affected by pre-incubation were definitely wrong.

Another 6-derivative we choose for the comparative study was AA 6-palmitate. This is a very well known antioxidant.[41-43] This however had a limited solubility in the buffer employed. The use of ethyl alcohol for solubilising the difficultly soluble steroidal substrates had been already reported by several workers. So, it was decided to use alcohol for solubilising the palmitate. Hydroxylation experiments were done for various reaction times, 10,15 ... and 60 minutes. But, to our surprise, none of these chromatograms showed even a trace of GA. It should be

emphasized here that the experiments referred to were not merely with the palmitate. The experiments were of a comparative nature and the other ene-diols were examined under parallel conditions i.e. with addition of the same amount of alcohol as for the palmitate case. Alcohol inhibited hydroxylation in all cases including the one with AA. Use of methanol in place of ethyl alcohol gave the same result, the amount of alcohol used being 2 ml for each reaction.

6:C.11 Disadvantages of following GA spot intensity for comparative studies.

It was observed that the $R_f$ values of GA, the acetonide and the benzoate were the same with the solvent system that was employed here for development of chromatograms. Also, they gave the same colour with reagent B. So, the corresponding spot intensities in the chromatograms of the reaction mixtures obtained with the benzoate and the acetonide could not be used as measures of GA produced. In an experiment for determining relative efficiencies of various ene-diols including these, comparisons of GA intensities were not therefore meaningful. Of course, as can be seen from the decay kinetics dealt with elsewhere, there was little ene-diol left unoxidised after 2-3 hrs. If one compared GA intensities after 3 hour reaction periods, than the spot intensities would probably be truly representative. However, by this time (i.e. after a lapse of 2 to 3 hrs) the intensities were themselves not true indices of SA hydroxylation, since GA

itself was getting further hydroxylated to different extents with the different ene-diols employed.

One way of solving the difficulty here was solvent extraction of the aromatic acids, both SA and GA. This was in fact tried. After a 10 minute reaction, 2 ml of concentrated HCl and 5 g of NaCl were added to all the systems. Then, they were all extracted thrice with 30 ml portions of ether. The ether layer was then dried on anhydrous $Na_2SO_4$ and the solvent removed by distillation. The residue was dissolved in 20 ml of ethanol. Now, equal amounts of these solutions were spotted.

In another set of experiments, after 10 minute reactions, 5.0 ml of 0.1N $I_2$ solution was added to each of the reaction mixtures to destroy any ene-diol present and then they were directly spotted. The excess iodine in the system did not pose a problem since it got out of the system very readily during chromatography.

Experiments under above mentioned conditions were repeated using different analogues of AA viz. phenyltetronic acid, phenyl-tetronimide, dihydroxymaleic acid, dihydroxy-fumaric acid and D(-)-iso-AA and tetrahydroxyquinone.

6:C.12 Better way of comparison

Following the disappearance of SA: In all the above experiments, it was observed that while the GA spot intensity showed first an increase and then a decrease with time, the SA spot intensity showed a monotonous decrease. This meant

that the fall in SA spot intensity would be a more meaningful criterion for comparative studies. This was confirmed when all the above mentioned experiments were repeated and reagent A was employed for detection of SA. This reagent did not give much colour with decomposition products from the ene-diols and did not change the colour of the paper adversely as did reagent B. Spraying with reagent A gave pink spots for SA in a lightly yellow background making the comparison facile. As compared with this, the dark blue spots of GA in a background which was steadily getting more and more blue, was much more inconvenient for purposes of comparison.

The efficiencies of AA, its 6-derivatives and analogues in hydroxylations were now compared in fresh set of experiments using changes in SA spot intensities in the chromatograms.

6:C.13 Optimum conditions for comparison of hydroxylation efficiencies.

As has been declared earlier, it would be desirable to obtain a qualitative demonstration of differences in hydroxylation efficiencies in the Udenfriend system between AA on the one hand and its derivatives and analogues on the other, if such differences exist and are of suitable magnitudes. From the point of view of visual comparison of the extents of hydroxylation, we have already seen that it is desirable to limit the amounts of substrate used (or rather their concentrations) to modest levels so that the

problem of judging relative colour intensities was made easier. From the experience gathered in the experiments described above, it became clear that it was best to compare the reaction mixtures when most of the substrate was consumed in the hydroxylation reaction, at least in one case. The conditions of the following experiment with AA were found satisfactory for our purpose.

AA (50.0 mg), $FeSO_4 7H_2O$ (15.0 mg) and disodium salt of EDTA (75 mg) were dissolved in 20.0 ml of buffer (0.1M, 6.8 pH) to which 10.0 mg of SA was added. The experiments were repeated with varying reaction times. Comparisons after 4 hrs were found to be most useful. When the ene-diol used was AA, the SA taken was completely hydroxylated while residual amounts were left in the other cases.

The use of GA as substrate instead of SA under these conditions made these comparison much easier. The slower reaction and the stronger colour obtained with this substrate were helpful to bring out the differences under the reaction conditions employed.

Results and Discussion

The best of our attempts to reproduce the reported hydroxylation of proline to hydroxy proline were not successful. Many of the earlier positive claims in this regard were based on paper chromatographic evidence. In our experiments on the Udenfriend hydroxylation of proline, chromatography of the reaction mixture did give a spot with the same $R_f$ value as hydroxy proline. But the colour with ninhydrin was light brown instead of the lemon yellow expected for hydroxyproline. Addition of authentic hydroxyproline to the reaction mixture and re-chromatography brought out the difference clearly. The material that gave the light brown colour with ninhydrin tended to give a diffuse spot while in the case of hydroxyproline the spot was more localized. The spots were superposed on each other. Since AA was known to give rise to a number of decomposition products it was suspected that the compound giving the brown spot in question actually represented one of the decomposition products. This suspicion was shown to be correct by an experiment in which no substrate proline was used. The chromatograms of the reaction mixtures obtained in 3 minute and 30 minute reactions employing 10 mg of proline and 500 mg AA are shown in Figs.1a and 1b along with the references required. The reference spots have been heavy in these figures.

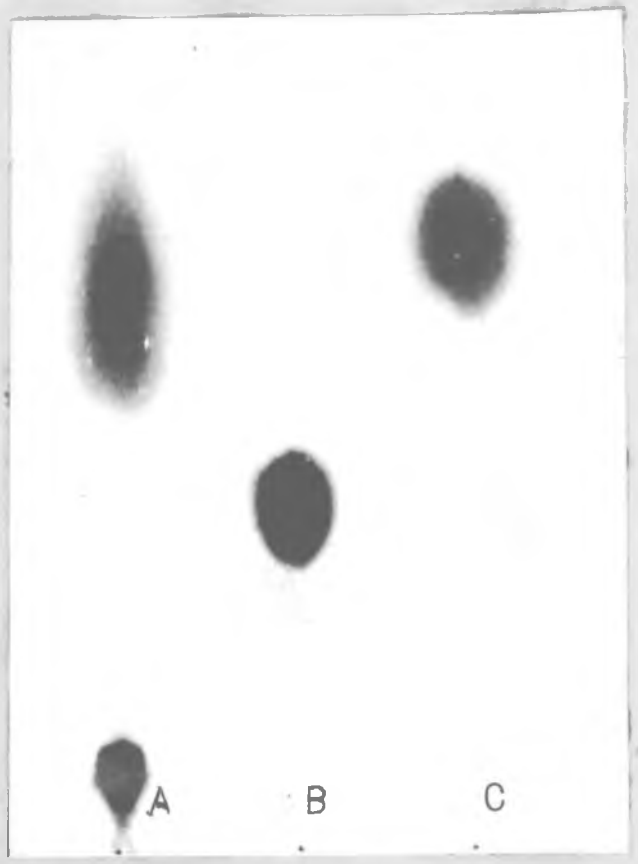
It appears from these results that the identification of products in the experiments reported in the literature

Fig. 1 · Hydroxylation of Proline to Hydroxy proline

System : 15 mg $FeSO_4.7H_2O$ + 75 mg disodium salt of EDTA + 10 mg proline + 500 mg AA dissolved in 25 ml of 0.1 M phosphate buffer of 6.8 pH.

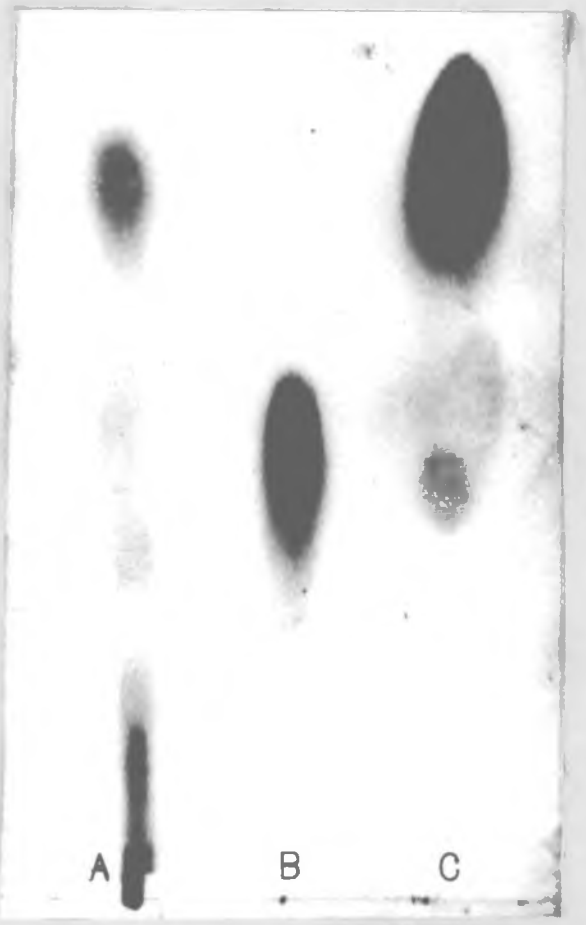
(a) 3 minute reaction chromatogram sprayed with 1% ninhydrin solution.
[A - Reaction mixture, B - standard hydroxy proline C - standard proline].

(b) 30 Minute reaction chromatogram sprayed with 1% ninhydrin solution.
[A - Reaction mixture, B - standard hydroxy proline C - standard mixture of proline and hydroxy proline].




← Fig. 1a

Fig. 1b →



has been defective and unreliable[17,33,34]. Although, inordinate quantities of [illegible] were used, there is no mention of any difficulty arising out of the presence of [illegible] decomposition products in the reaction mixtures. Most of the reported hydroxylations of this type, i.e. at $sp^3$ carbon atoms[17,25,26,32-34], the yields reported are very poor and the method of identification of products has been paper chromatography. In the context of the findings here, it is felt that reports of the highly regio- and stereo-specific hydroxylations at unactivated $sp^3$ carbon atoms, that seem to have been obtained in Udenfriend reactions must be viewed with considerable skepticsm. This does not however affect the question that has been posed about the reactivity of ascorbic acid, its derivatives and analogues.

In contrast to our experience with proline, all the aromatic substrates we tried (toluene, benzene, acetanilide, nitrobenzene, aniline, salicylic acid, etc.) underwent hydroxylation under the Udenfriend conditions. From the point of view of convenience in following the extent of hydroxylations, it was found that salicylic acid (SA) would be a convenient substrate. This gives a pink colour with $FeCl_3$ (Reagent A) and the product of hydroxylation gentisic acid (GA) gives a blue-grey colour. With a mixture of $FeCl_3$ and potassium ferricyanide (Reagent B) the colour with SA was the same, but, with GA it was dark blue. The suitability of the substrate and the spraying reagents for the paper

chromatograms for recognition of quantitative differences was brought out by experiments using varying amounts (0.0, 5.0, 10.0 and 15.0 mg) of AA with a fixed quantity (20 mg) of SA. Fig. 2a shows the chromatogram of four reaction mixtures obtained in 30 minute reactions using the quantities of AA specified above with spraying reagent A. The increase in yeild of GA (lower spots) with increasing concentration of AA is clearly seen. Fig.2b shows the parallel chromatogram obtained for the same reactions with reagent B. The decrease in SA and the corresponding increase in GA with the increase in the concentration of AA are clearly seen. Fig. 2c shows the chromatogram after it was washed with water when only the spots for GA remained on the paper.

In the initial attempts to compare hydroxylation efficiencies, attention was concentrated on AA and its acetonide. The course of this effort has been sketched in the experimental section. Since the initial samples of the acetonide had traces of AA in it, and it was known that AA was more susceptible to autoxidation than the acetonide, a selective pre-incubation of the reaction mixture for the acetonide case was tried. The results of these experiments are shown in Fig. 3a and 3b. The former is the chromatogram of the reaction mixtures after one hour of reaction, the spraying agent being reagent A. Fig. 3b shows the result of spraying a chromatogram of the same reaction mixtures with reagent B. All comparison studies included parallel blanks. One would have been tempted to conclude from these

Fig. 2 Hydroxylation of SA to GA

Sytem : 15 mg $FeSO_4.7H_2O$ + 75 mg disodium salt of EDTA + 20 mg SA + varying amounts of AA (0, 5, 10, 15 mg) dissolved in 20,0 ml 0.1 M phosphate buffer of 6.8 pH.

(a) 10 minutes reactions chromatogram sprayed with reagent A.

(b) 30 minutes reactions chromatogram sprayed with reagent B.

(c) The above chromatogram was washed with water when SA spots were washed away leaving only the GA spots. [A - 0.0 mg, B - 5.0 mg, C - 10.0 mg, D - 15.0 mg of AA].


Fig. 2a

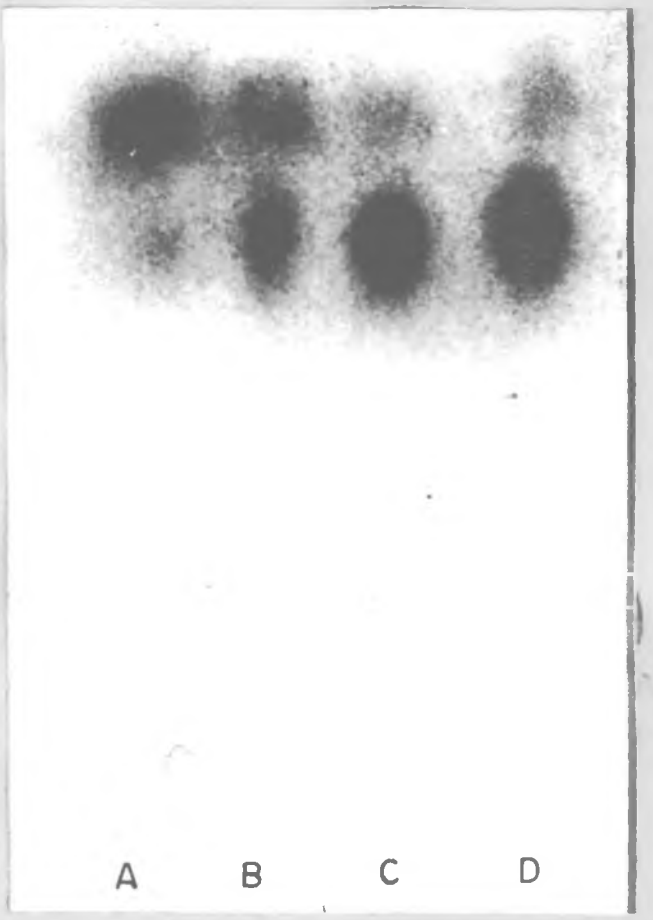
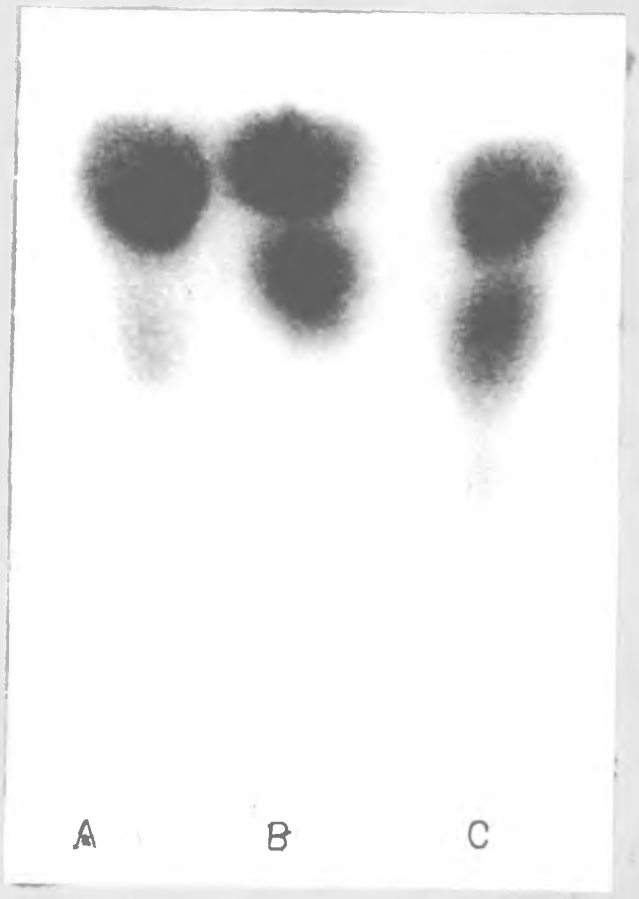



Fig. 2b


Fig. 2c


← Fig. 3 a

Fig. 3 b →



not high and that of the palmitate was quite poor. It was thought that we might use a little methanol or ethanol to solubilize these things in the buffer as has been done in the reported work on steroidal hydroxylations[33,34]. However, it was found that the presence of methanol or ethanol inhibited the hydroxylation of SA completely. A similar observation was made by Acheson and Hazelwood[22] earlier who commented on the use of alcohol for solubilization in the earlier studies. Fig. 6 shows the chromatogram of the reaction mixtures for experiments using AA, its 6-benzoate and the 5,6-acetonide along with a blank. It is seen that the SA used remained unaffeted. It is of interest here to recall that it has been shown that methanol and ethanol are oxidised to the corresponding aldehydes in the Udenfriend reaction[57,58]. Ethanol has also been shown to stabilize AA against metal catalyzed oxidation[59]. As mentioned already, ethanol has been used for purposes of solubilization of substrates in severals reported hydroxylation of saturated compounds[17,33,34]. (i.e. at $sp^3$ sites). This has apparently created considerable confusion since no transformation of the substrate or the decomposition of AA itself is possible until after all the alcohol employed is oxidised.

The comparison was done in the absence of alcohol in a set of experiments using 5 mg of AA and corresponding amounts of the other ene-diols i.e. the acetonide and the benzoate. The amount of substrate (SA) used was 20 mg.

Fig. 5 - Hydroxylation of SA to GA

System : 15 mg $FeSO_4.7H_2O$ + 75 mg disodium salt of EDTA + 20 mg SA + x mg ene-diol dissolved in 20.0 ml of 0.1 M phosphate buffer of 6.8 pH. The chromatogram was sprayed with reagent B.
[x = 50.0 mg AA, 62 mg acetonide].
(A - acetonide system, B - standard mixture of SA + GA, C - AA system).

Fig. 6 Hydroxylation of SA to GA

System : 15 mg $FeSO_4.7H_2O$ + 75 mg disodium salt of EDTA + 20 mg SA + x mg ene-diol + 2.0 ml of ethanol dissolved in 18.0 ml of 0.1 M phosphate buffer of 6.8 pH.
( x = 50 mg AA, 62 mg acetonide, 80 mg benzoate).
[A - AA system, B - acetonide system, C - benzoate system, D - blank run].

Fig. 7 Hydroxylation of SA to GA

System : 15 mg $FeSO_4.7H_2O$ + 75 mg disodium salt of EDTA + x mg of ene-diol + 20.0 mg SA dissolved in 20 ml of 0.1 M phosphate buffer of 6.8 pH.
(x = 10 mg AA, 16 mg benzoate, 13 mg of acetonide).
[A - AA system, B - acetonide system, C - benzoate system, D - blank run].


Fig. 5


Fig. 6


Fig. 7

The chromatogram of the reaction mixtures is shown in Fig. 7, the spraying reagent being $FeCl_3$. This picture made it clear that the reactivity order was AA > acetonide > benzoate.

As mentioned in the experimental section, it was desirable to obtain reaction conditions where the differences would be even clearer. It was decided to see if 20 mg of SA could be totally hydroxylated by allowing enough reaction time using only 10 mg of AA. The progress of the reaction with a set of four ene-diols was followed and the results are illustrated in the chromatograms of the reaction mixture (Fig. 8) at various time intervals. It was seen that even after 48 hours, there was residual SA left in the reaction mixtures. Reagent B was used for spraying the chromatograms in these cases. The GA spot intensities here are not accurate indices of the extents of hydroxylation since GA itself is further hydroxylated under the reaction conditions to varying extents.

It was eventually found that if the substrate (SA) amount was reduced to 10 mg and the ene-diols used were equivalent to 50 mg of AA, one could obtain a better comparison of their hydroxylation efficiencies in 4 hour reactions. The results of a comparative study involving AA, its acetonide and 6-benzoate are presented in Fig. 9. The first picture (a) is that of the product chromatogram after 5 minute of reaction, the second (b) was obtained after 4 hours. The picture 9(a) clearly shows that the spot intensity for SA in the case of

Fig. 8 - Hydroxylation of SA to GA

System : 15 mg $FeSO_4.7H_2O$ + 75 mg disodium salt of EDTA + 20 mg SA + x mg ene-diol dissolved in 20 ml of 0.1 M phosphate buffer of 6.8 pH.

( x = 10 mg AA, 16 mg benzoate, 13 mg acetonide, 8 mg dihydroxy-maleic acid). All the chromatograms were sprayed with reagent B.

(a) 10 minutes reactions chromatogram

(b) 30 minutes reactions chromatogram

(c) 12 hours reactions chromatogram

(d) 48 hours reactions chromatogram

[A - AA system, B - acetonide system, C - benzoate system, D - dihydroxy-maleic acid system).

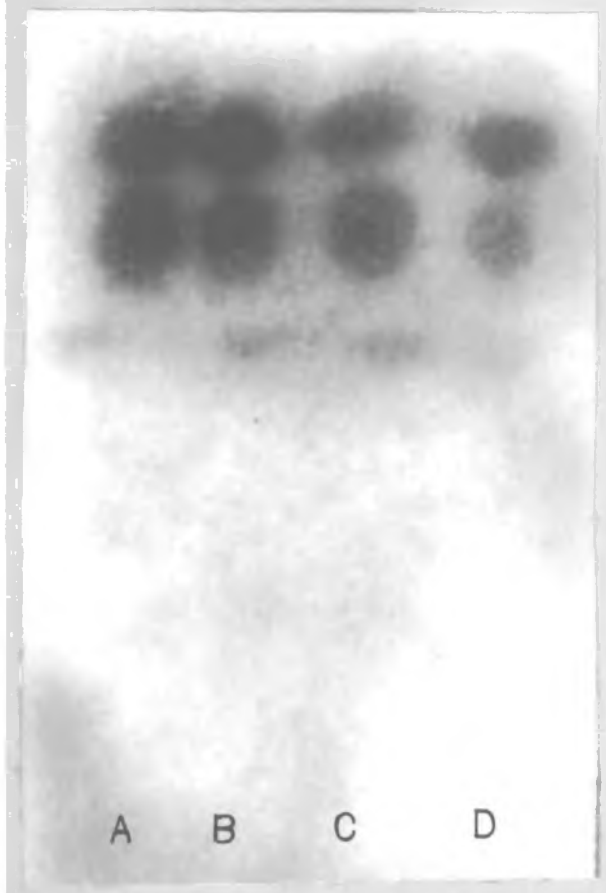



Fig. 8 a


Fig. 8 b

Fig. 8 c

Fig. 8 d


AA has already got reduced even with 5 minute of reaction as compared to the acetonide and benzoate systems. The spots here were obtained with reagent A. When 9(b) was further sprayed with reagent B the result shown in 9(c) was obtained. The relative reactivity order suggested earlier, i.e. AA > acetonide > benzoate, was thus confirmed. The results of a completely similar set of experiments with phenyltetronic acid, phenyltetronimide, tetrahydroxyquinone and dihdroxymaleic acid are shown in Fig. 10. This group of compounds can be placed between ascorbic acid and its two derivatives we have dealt with here as far as hydroxylation efficiency is concerned.

Since it was found that the hydroxylation of GA was slower than that of SA, it was thought that this would be capable of giving greater selectivity. Sets of experiments were therefore done with GA as substrate, the result of one of which is illustrated in Fig. 11. The figure shows that AA and D-iso AA are nearly equally efficient in the reaction, while AA benzoate ranks much lower.

To sum up, one may say that although only one compound (proline) has been examined in connection with the behaviour of the saturated type of substrate in Udenfriend hydroxylations, the negative results obtained here cast serious doubts about the authenticity of earlier claims in this regard. It appears that the efficacy of ascorbic acid as a mediating agent in the Udenfriend hydroxylation of aromatic substrates is significantly reduced in its derivatives where the 6-hydroxyl

Fig. 9 - Hydroxylation of SA to GA

System : 15 mg $FeSO_4.7H_2O$ + 75 mg disodium salt of EDTA + 10 mg SA + x mg of ene-diol dissolved in 20 ml of 0.1 M phosphate buffer of 6.8 pH.
(x = 50 mg AA, 62 mg acetonide, 80 mg benzoate).

(a) 5 minute reactions chromatogram sprayed with reagent A.

(b) 4 hour reactions chromatogram sprayed with reagent A.

(c) 4 hour reactions chromatogram sprayed with reagent B.

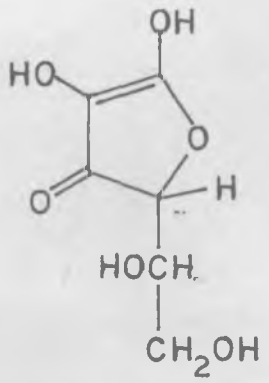
[A - AA system, B - benzoate system, C - acetonide system, D - blank run].


Fig. 9a

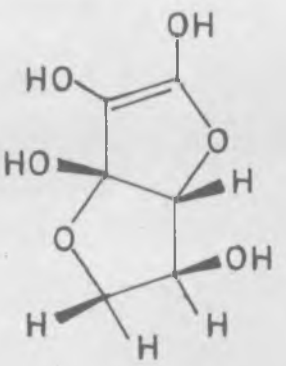

Fig. 9 b


Fig. 9 c

is no longer free. Thus, both the 5,6-acetonide and the 6-benzoate are less effective hydroxylating agents. The significance of this is presumably that, in the presence of the 6-hydroxyl group, a more effective ene-diol can be generated from ascorbic acid and that this new intermediate is probably the active material involved. The results obtained here are thus consistent with those obtained in the autoxidation and benzylation studies. As we have seen earlier, it is possible for AA in its hetero-form (IV) to go over to a more active ene-diol (V) when the 6-OH adds to the 3-carbonyl.




IV




V

We had also noted earlier that such a possibility could account for several things, e.g. the disappearance of the UV absorption of AA in dilute solution even in conductivity water and under nitrogen atmosphere, the existence of a very large negative entropy of activation for the uncatalyzed autoxidation of AA which is in other respects[46], i.e. enthalpy-wise, a more facile reaction and the formation of only one C-benzylation product instead of two in the benzylation of AA.

Fig. 10 -- Hydroxylations of SA to GA

System : 15 mg $FeSO_4.7H_2O$ + 75 mg disodium salt of EDTA + 10 mg SA + x mg ene-diol dissolved in 20 ml of 0.1 M phosphate buffer of 6.8 pH.

(x = 55 mg phenyltetronic acid, 55 mg phenyltetronimide 25 mg tetrahydroxyquinone, 42 mg dihydroxy maleic acid).

(a) 5.0 minutes reaction chromatogram sprayed with reagent A.

(b) 4 hours reaction chromatogram sprayed with reagent B.

[A - Phenyltetronic acid, B - phenyltetronimide, C - Tetrahydroxyquinone, D - dihydroxymaleic acid].

Fig. 11 Hydroxylation of GA to Trihydroxy-benzoic acid

System : 15 mg $FeSO_4.7H_2O$ + 75 mg disodium salt of EDTA + 10 mg of GA + x mg ene-diol dissolved in 20 ml of 0.1 M phosphate buffer of 6.8 pH.

(x = 50 mg AA, 50 mg D-iso-AA, 80 mg benzoate).

A 4 hours reaction chromatogram was sprayed with reagent B.

[A - AA system, B - D-iso-AA system, C - benzoate system, D - blank run].


Fig. 10 a

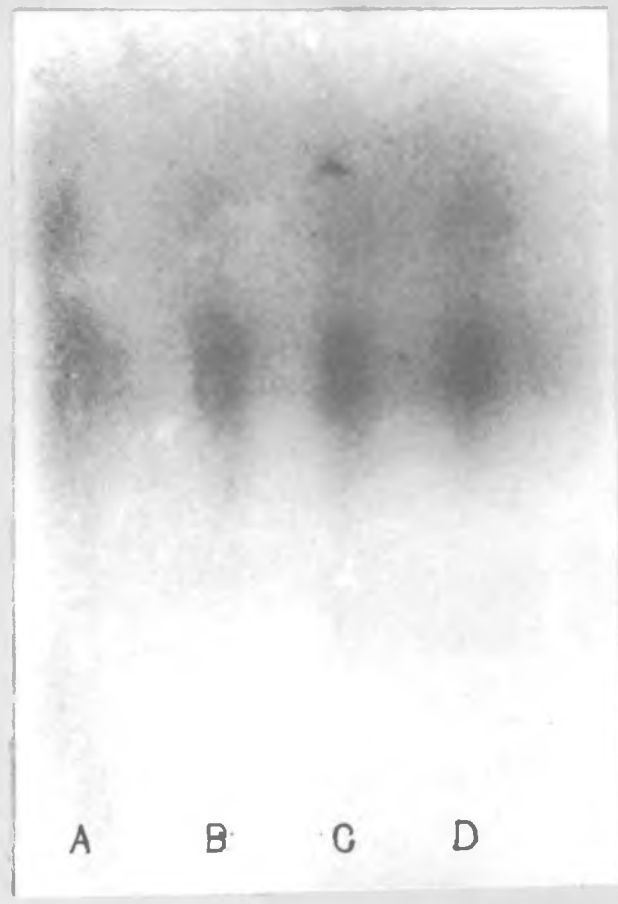
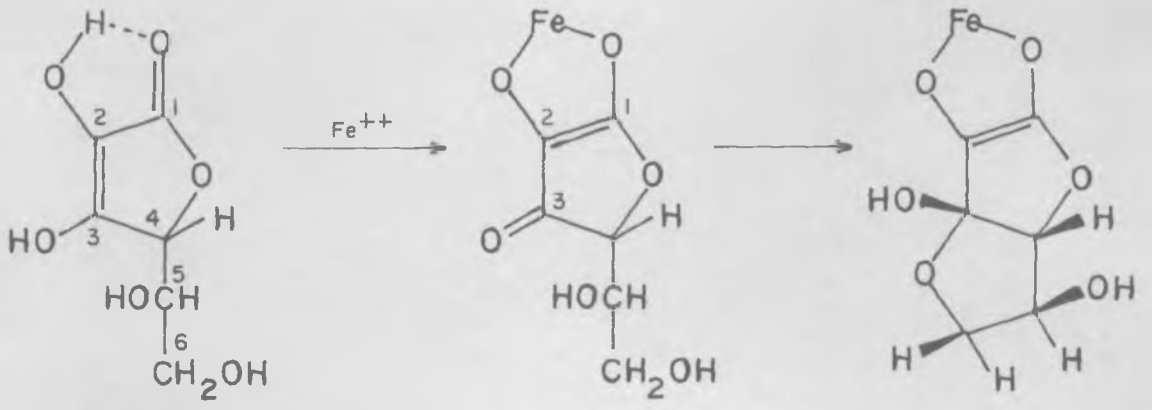



Fig. 10 b


Fig. 11

It would, of course, have been preferable if the role of the side chain could result in even more dramatic effects on reactivity. The Udenfriend hydroxylation reaction, being a metal catalyzed one, is apparently not the most suited for the purpose. Apparently, the reactivity of the system is high and the selectivity low. However, the differences found have been consistent and genuine. It appears to be quite clear that the side chain of ascorbic acid has a very definite influence on its reactivity even in non-enzymatic reactions. In the oxidation of AA to DHA, which has a bicyclic form[60], the cyclization step need not necessarily be subsequent to oxidation under all conditions.

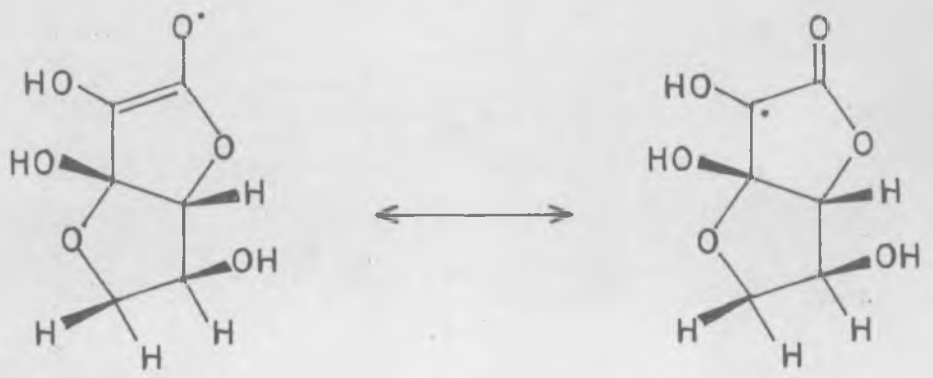
As far as the mechanism of the Udenfriend hydroxylation itself is concerned, our findings here may be deemed to have the following significance. There has actually been only one mechanistic proposal which ties up most of the available




VI VII VIII

information on this system[25,26]. This is the one made by Hamilton which might be recast in a completely parallel way involving the iron complexes of the hetero-ascorbic acid and the bicyclic ene-diol. In ascorbic acid, the 2-OH is chelated to the lactone carbonyl (VI), and, when $Fe^{++}$ replaces proton in the chelated ring, the product obtained can directly be the covalently bound ferrous complex of hetero-ascorbic acid (VII). In other words, formation of the iron complex of hetero-ascorbic acid need not at all be <u>via</u> the free hetero acid. When the 6-OH adds on to the 3-carbonyl, this gets highly activated (VIII) for reaction with $O_2$. The parallel mechanism may be put down as shown in Chart II.

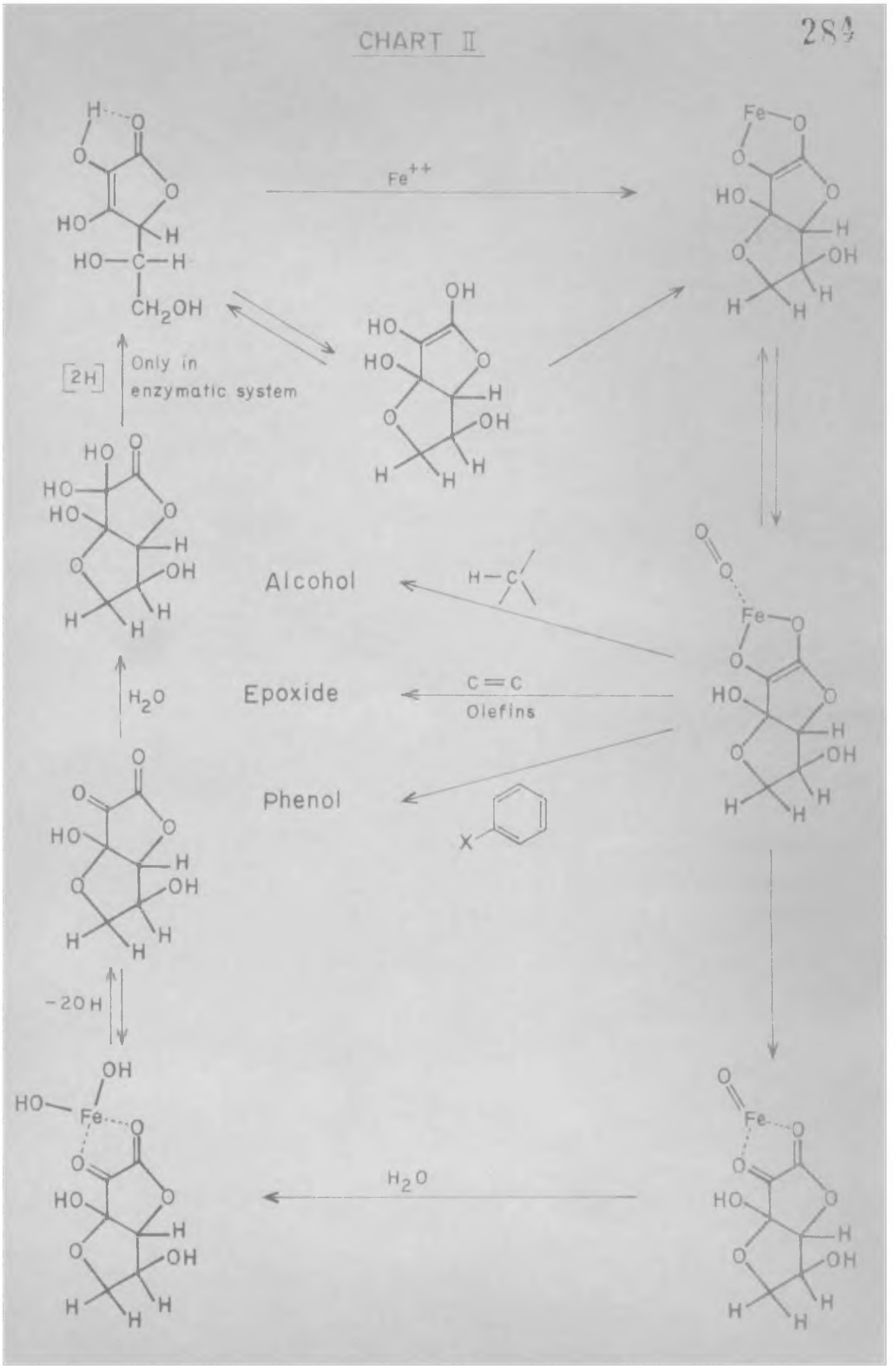
It is of course, not quite clear what the actual mechanism is. If mono-dehydro-ascorbic acid is a reactive intermediate, our findings here would imply that its structure is (IX). This would be consistent with the observation




IX

that the 6-protons of mono-DHA are non-equivalent.

CHART II


Fe++
[2H]
Only in
enzymatic system
Alcohol
H—C
H2O
Epoxide
C=C
Olefins
Phenol
X
-2OH
H2O

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# Chapter VII

## MASS SPECTROMETRIC AND NMR SPECTROSCOPIC STUDIES OF ASCORBIC ACID

Introduction

In a number of the studies discussed so far, we have made use of chemical shift data for several derivatives, particularly $^{13}C$ spectral data, for structural and stereochemical assignments, often without any detailed or explicit discussion, but assuming the validity of some statements which were to be discussed later. The difficulty that arose in the context of the confusing reports on DHA may be recalled. The availability of a number of derivatives prepared in connection with the various studies here have been particularly helpful in chemical shift assignments in some instances. It is of considerable interest therefore to assemble all the spectral data obtained and see to what extent their variation can be correlated with structural changes.

The mass spectra of a large number of these derivatives have also been obtained with the hope that some useful information may be gleaned from them. No attempt has been made to interpret all the main features of the fragmentation patterns. What has been done is to see if one or two of the more important features of each spectrum can be accounted for. A matter that is of special interest to us here is to see if any feature of the mass spectrum of AA or its derivatives can be correlated with the status of the side chain. In this respect, the results obtained have been useful. Carbohydrate mass spectrometry has normally employed methyl or silyl ethers or acetates. In the study here, no special derivatization has been made. AA itself gave

a spectrum without difficulty. The presence of ether functions in many of the derivatives concerned mitigated the problem of volatility.

Results and Discussion

The $^{13}C$ NMR shift data for AA and a number of its derivatives are presented in Tables I and II. We may first take up for consideration the problem of assignments of secondary carbons that arose in connection with the iso derivatives of 2,3-dialkyl ethers of AA and the clarification required on the nature of newly formed DHA in aqueous solution, in the context of the divergent views expressed in literature. The data required in this connection are presented in Table I*. The primary, secondary or tertiary character of the carbons was ascertained from off-resonance spectra.

Berger had reported shifts of 8 .2 and 76.7 for $C_{(5)}$ and $C_{(6)}$ respectively, for an aqueous solution of an authentic sample of dimeric DHA. The value for $C_{(5)}$ was suggestively close to 8 .4 which we had assigned to the $C_{(4)}$ of a freshly prepared aqueous solution of DHA. All three of the iso derivatives we had made from 2,3-dimethyl, 2-benzyl-3-methyl and 3-benzyl-2-methyl ethers of AA had three secondary carbons each in the regions of 74-77, 77-80 and 86-89. Out of the

* The $^{13}C$ shifts are all expressed in ppm downfield from TMS i.e. in units. The spectra were recorded at 22.63 MHz using dioxane as an intermediate external standard with a value of 67.4.

Table - I

| | Compound | Solvent | $^{13}C$ chemical shifts | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | $C_{(1)}$ | $C_{(2)}$ | $C_{(3)}$ | $C_{(4)}$ | $C_{(5)}$ | $C_{(6)}$ |
| 1. | AA | $D_2O$ | 174.0 | 118.9 | 156.1 | 77.1 | 69.9 | 63.0 |
| 2. | DHA ($I_2$ oxidation) | $D_2O$ | 174.3 | 92.0 | 106.3 | 88.3 | 73.6 | 76.9 |
| 3. | DHA (Hg (II) acetate) | $D_2O$ | 173.3 | 92.0 | 106.3 | 88.3 | 73.6 | 76.8 |
| 4. | DHA (Hg (II) acetate) | MeOH | 175.2 | 94.1 | 106.4 | 88.3 | 73.9 | 76.4 |
| 5. | Iso-dimethyl AA | $Me_2CO$ | 171.7 | 79.9 | 109.8 | 88.3 | 74.5 | 75.4 |
| 6. | Iso-dimethyl p-nitrobenzoate of AA | $Me_2CO$ | 163.9 | 79.8 | 121.4 | 85.1 | 77.0 | 72.8 |
| 7. | Iso-3-O-benzyl-; 2-O-methyl AA | $Me_2CO$ | 171.3 | 80.0 | 109.6 | 88.3 | 74.4 | 75.3 |
| 8. | Iso-3-O-Methyl-2-O-benzyl AA | $Me_2CO$ | 171.6 | 77.8 | 109.9 | 88.2 | 74.5 | 75.3 |
| 9. | 2-C-benzyl AA | $Me_2CO$ | 174.7 | 79.7 | 107.6 | 86.2 | 74.7 | 75.0 |

three carbons concerned, only $C_{(4)}$ is having an oxygen involved in an ester linkage. Since the $^{13}C$-H coupling is sensitive to the electronegativity of substituents, it may be anticipated that $^{13}C$-H coupling for $C_{(4)}$-H would be somewhat larger than for the others. The difference in the magnitude of this coupling from the others could be made out even from the off-resonance spectra. The assignment for $C_{(4)}$ was thus unambiguous (88-89 ). In the spectrum of the p-nitrobenzoate of iso-dimethyl AA, an additional secondary carbon showed the enhanced $^{13}C$-H coupling characteristics of esters. This absorbed in the range 74-75 . The assignment for $C_{(5)}$ was made on this consideration. Further, the spectrum of 2-C-benzyl-AA, which is also a bicyclic 3,6-anhydro derivative like the iso ethers, had a third tertiary carbon, while the iso derivative had only two each. The additional one in the C benzyl derivative was therefore readily marked, and the shift concerned was very close to that found for $C_{(2)}$ of iso-dimethyl AA and its p-nitrobenzoate. The assignments for the other ethers were made on grounds of analogy and the whole set of assignments were mutually consistent.

In the context of the assignments mentioned, there were no doubts at all as regards the assignments shown in Table I for DHA. It is clear that there is only one carbonyl in the molecule. The 3,6-anhydro form is confirmed by the $C_{(4)}$, $C_{(5)}$ and $C_{(6)}$ shifts. The carbonyl at $C_{(2)}$ is apparently hydrated. It was thus clear that Berger's assignment for $C_{(5)}$

was clearly erroneous, and, although the solution concerned was made from dimeric DHA, the species in solution had apparently the 3,6-anhydro monomeric structure and was in its hydrated form. In the change from the open chain to the bicyclic form, it is apparently the $C_{(6)}$ and $C_{(4)}$, among the proton bearing carbons, that suffer the larger shifts downfield. In comparison, the shift for $C_{(5)}$ is smaller. For $C_{(2)}$ and $C_{(3)}$ the shifts involved are larger and in the opposite direction, i.e. upfield. This is to be expected from the change in character from vinylic to tetrahedral. The shift for the hydrated carbonyl group at $C_{(2)}$ is naturally at lower field from those of the others. The $C_{(3)}$ of iso-dimethyl-A suffers a pronounced downfield shift in its conversion to the p-nitrobenzoate. This is presumably a proximity effect.

The question of dimeric structure for the DHA samples obtained here is apparently something that need not be seriously considered in the context of the present measurements. According to indications from literature, it appears impossible to obtain DHA in this form when it is generated in aqueous solution, as has been done here by the use of mercuric acetate or iodine as the oxidising agent. In the case of iodine oxidation, as the reaction was reversible, bicarbonate had to be used to neutralise the HI formed. Berger's $^{13}C$ shift measurements for 'dimeric' DHA would seem to indicate that this form is rapidly converted into the monomeric form in aqueous solution. Also, as mentioned elsewhere it is not clear to us by what

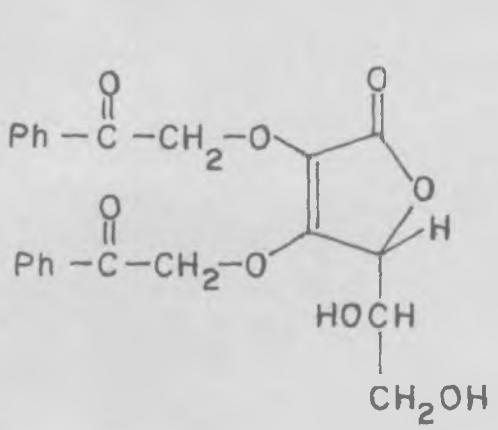
procedure he managed to get DHA in the open chain form in aqueous solution in the iodine oxidation of AA.

Table II gives the $^{13}C$ chemical shifts for various open chain derivatives of AA. One thing that is very clear from these shifts as compared with those of Table I is that it is easy to distinguish between an open chain and a bicyclic 3,6-anhydro structure by means of a $^{13}C$ NMR spectrum. The pattern of shifts observed is the same for various 2,3,5 and 6-O-derivatives of ascorbic acids in which the side chain is open. The effect of acetonide formation on $C_{(5)}$ and $C_{(6)}$ is deshielding and is more pronounced for $C_{(5)}$ and $C_{(6)}$. The downfield shift for the α-carbon atom and upfield shift for the β-carbon atom on esterification is well illustrated in the case of the 6-benzoate of AA.

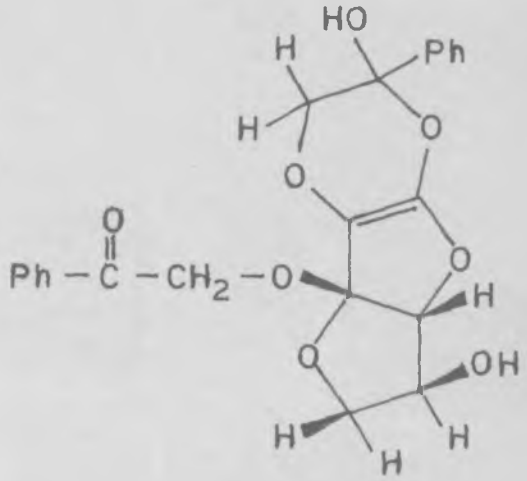
The proton chemical shifts have been discussed in greater detail in other chapters of this thesis. The data for a few more derivatives of AA, which were prepared for some other purposes, have also been obtained since they are likely to be of help in connection with studies on AA derivatives. These are presented in Table III.

Earlier, in this laboratory the PMR spectrum of the 2,3-diphenacyl ether of AA was examined. The spectrum was recorded at 60 MHz on a T-60 spectrometer which gives a S/N ratio of only 20 to 22 for 1% ethyl benzene. The compound had a good solubility in DMSO and the PMR spectrum in DMSO

was as expected for the normal structure (I). Both the methylenes of the phenacyl ether groups had the same gem coupling constant. When the PMR spectrum of this compound was recorded in $CDCl_3$, in which it had poor solubility, the noise level was inconveniently high. Some of the noise signals were mistaken for compound signals. The non-equivalence of the protons of one of the phenacyl methylenes appeared to have become very large while the gem coupling for the other appeared decreased. In an effort to rationalise these changes the possibility that the compound may exist in form (II) was considered.




I




II

Structure (II) could very well account for the observed differences provided they were genuine. The phenacyl ether at the 3-position will be on an asymmetric carbon atom and would thus account for a larger chemical shift between the methylene protons of the group. Since the CO group of the

Table - II

| Compound | Solvent | $^{13}C$ chemical shifts | | | | | |
|---|---|---|---|---|---|---|---|
| | | $C_{(1)}$ | $C_{(2)}$ | $C_{(3)}$ | $C_{(4)}$ | $C_{(5)}$ | $C_{(6)}$ |
| 1. AA | $D_2O$ | 174.0 | 118.9 | 156.1 | 77.1 | 69.9 | 63.0 |
| 2. AA | MeOH | 172.4 | 118.1 | 153.5 | 75.1 | 68.7 | 61.8 |
| 3. AA | DMSO | 171.7 | 119.3 | 153.9 | 75.8 | 69.3 | 63.3 |
| 4. D-iso-AA | $D_2O$ | 173.8 | 119.0 | 156.2 | 78.2 | 71.8 | 62.3 |
| 5. D-iso-AA | MeOH | 172.4 | 119.3 | 153.9 | 77.7 | 72.0 | 62.7 |
| 6. 3-O-Methyl AA | $Me_2CO$ | 170.6 | 120.0 | 150.8 | 75.4 | 69.8 | 62.8 |
| 7. 2,3-di-O-Methyl AA | $Me_2CO$ | 169.4 | 123.2 | 158.2 | 75.0 | 69.7 | 62.5 |
| 8. 3-O-Methyl 2-O-benzyl AA | $Me_2CO$ | 167.7 | 120.4 | 157.0 | 75.0 | 69.7 | 73.9 |
| 9. AA acetonide | DMSO | 171.3 | 119.5 | 153.3 | 75.4 | 74.7 | 66.0 |
| 10. 2,3-di-O-Methyl acetonide of AA | $Me_2CO$ | 168.8 | 121.2 | 157.4 | 74.5 | 74.2 | 65.4 |
| 11. 2-O-Methyl 3-O-benzyl 5,6-acetonide of AA | $Me_2CO$ | 168.3 | 123.4 | 156.2 | 74.7 | 74.2 | 73.4 |
| 12. 6-Benzoate of AA | $Me_2CO$ | 170.2 | 119.5 | 151.0 | 75.8 | 67.4 | 65.6 |
| 13. 2,3-di-O-phenacyl AA | $CDCl_3$ | 168.4 | 122.2 | 155.0 | 77.3 | 70.1 | 62.8 |

Table -III

| No. | Compound | Solvent | Proton chemical shifts in ppm from TMS | | | |
|---|---|---|---|---|---|---|
| | | | $C_{(4)}$-H | ($C_{(5)}$-H | $C_{(6)}$-$H_2$ | Other protons |
| 1. | 6-Benzoate of AA | $Me_2CO$ | 4.80 | ~ 4.40 | ~ 4.40 | aromatic protons at 7.90 and 7.33 |
| 2. | 6-Trityl ether of AA | $Me_2CO$ | 4.86 | ~ 4.01 | ~ 3.3 | aromatic protons ~ 7.27 |
| 3. | 5,6-Acetonide of AA | DMSO | 4.57 | ~ 4.01 | ~ 4.01 | -$C(CH_3)_2$ at 1.12 |
| 4. | 3-O-Methyl 5,6-acetonide of AA | $CHCl_3$ | 4.50 | ~ 4.05 | ~ 3.9 | -$OCH_3$ at 4.1, -$C(CH_3)_2$ at 1.33 |
| 5. | 2,3-di-O-methyl-5,6-acetonide of AA | $CHCl_3$ | 4.50 | ~ 4.13 | ~ 4.13 | -$OCH_3$ at 4.13 and 3.8-$C(CH_3)_2$ at 1.33 |
| 6. | 2,3,6-tri-O-methyl AA | $CDCl_3$ | 4.68 | 3.90 | 3.51 | -$OCH_3$ at 3.42, 3.86 and 4.18. |
| 7. | 2,3-di-O-methyl-6-trityl AA | $CDCl_3$ | 4.79 | 3.92 | 3.31 | -$OCH_3$ at 3.82 and 4.12 Trityl protons at ~ 7.37 |
| 8. | 2,6-di-benzoate of AA | $Me_2CO$ | 5.16 | ~ 4.53 | ~ 4.53 | Aromatic protons at 8.0 and 7.53 |
| 9. | 2-O-benzyl 3-O-methyl 5,6-acetonide of AA | $CCl_4$ | 4.4 | ~ 4.0 | ~ 4.0 | -O-$CH_3$ at 5.05 -$C(CH_3)_2$ at 1.36 and aromatic protons at 7.3. |
| 10. | AA tetra acetate | $CDCl_3$ | 5.38 | ~ 4.31 | ~ 5.44 | Acetate protons at 2.05, 2.08, 2.25 and 2.27 |

second phenacyl ether at the 2-positon is destroyed in the trans-formation, a reduction in the gem coupling between its non-equivalent protons could be expected.

However, the 90 MHz FT-PMR spectrum in $CDCl_3$ (Fig.1) made it very clear that both phenacyl methylenes have the same coupling constant, which is in agreement with structure (I). Apparently, the prying apart of the chemical shifts of the protons of one of the phenacyl methylenes has presumably to be rationalised in terms of a change in conformation of the side chain at $C_{(4)}$ involving hydrogen bonding interaction between one of the hydroxyls and one of the oxygens of the phenacyl ether moiety at $C_{(3)}$. There is a close similarity between the absorption patterns of the $C_{(6)}$-protons of DHA and the di-phenacyl ether of AA. One would be tempted to conclude from this that the di-phenacyl ether is also bicyclic. However,the open chain structure was also confirmed by the $^{13}C$ NMR spectrum of 2,3-di-phenacyl ether of AA in $CDCl_3$ which showed a typical pattern for an open chain structure. This is of interest in connection with the use of proton chemical shifts made by Pfeilsticker for assigning a bicyclic structure for DHA.

The mass spectral data for a series of derivatives are presented in Table IV. M/e values starting with the molecular ion down to 100 are indicated along with peak intensities shown in brackets. Perhaps the most important finding from

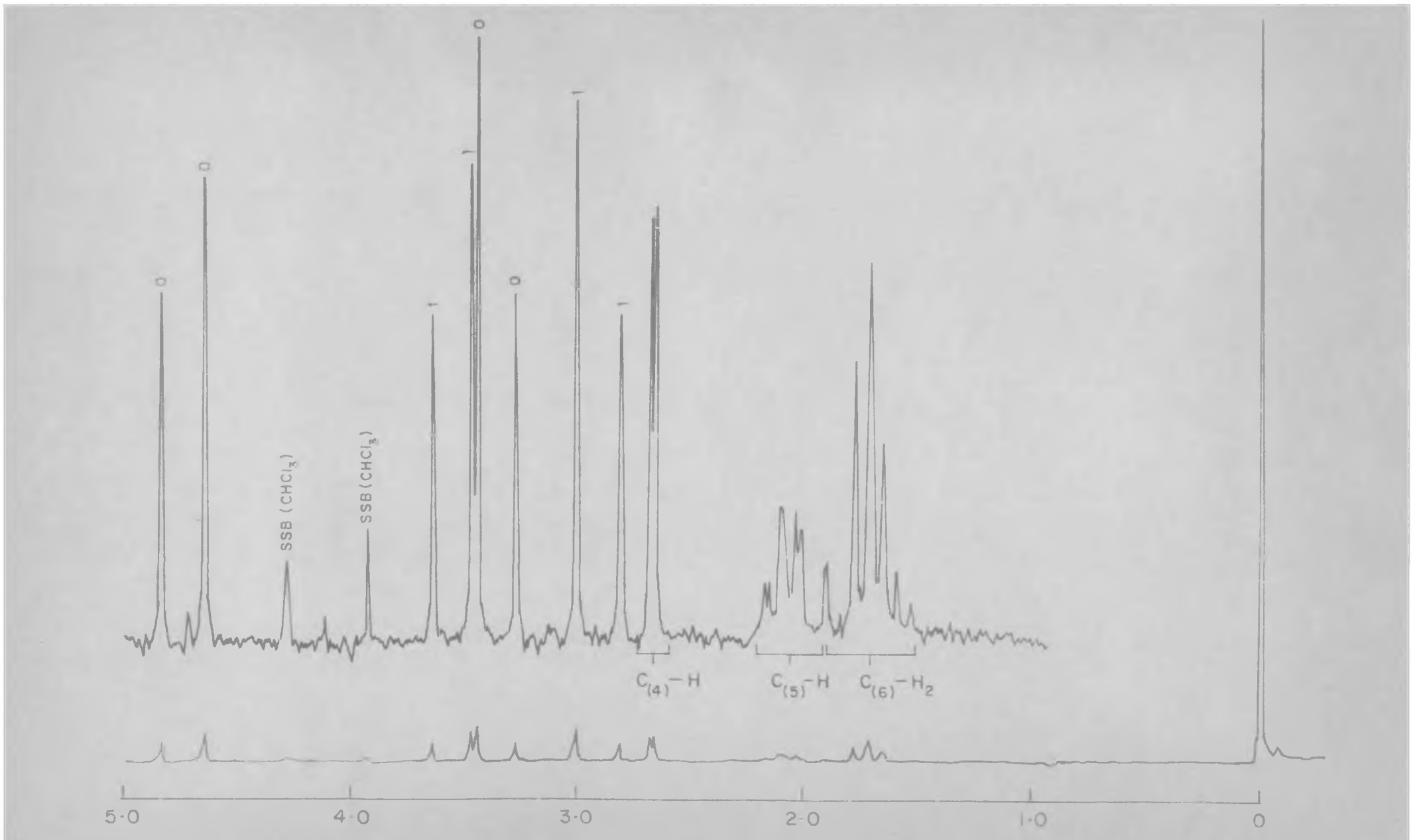



Fig.1 90 MHz PMR Spectrum of 2,3-di-O-phenacyl-AA in $CDCl_3$

Table - IV

1. AA
176(81), 145(21), 140(18), 130(10.5), 131(10.5), 119(86), 117(39.8), 116(100), 115(24.3), 102(65.2), 100(64.5), 101(69), 99(19.33), 98(15), 96(15), 95(65), 91(72), 89(50).

2. 3-O-Methyl-AA
190(99), 186(17), 159(26), 144(83), 141(12), 131(27), 130(100), 129(35), 127(28), 118(17), 115(26), 114(33), 101(30), 100(25), 99(19), 90(25).

3. 2,3-di-O-methyl-AA
204(51), 186(29), 173(34), 172(20), 157(8), 146(26), 145(66), 144(100), 143(67), 142(13), 130(47), 129(73), 127(19), 117(33), 116(51), 115(49), 114(42), 113(43), 102(22), 101(63), 100(42), 99(61).

4. 1-O-Methyl hetero-AA
190(75), 172(10), 159(53), 144(23), 141(23), 131(52), 130(100), 129(38), 127(39), 117(18), 116(18), 115(47), 114(51), 113(39), 103(26), 102(33), 101(66), 100(66), 99(60), 98(27).

5. 3-O-Methyl-2-O-benzyl-AA
280(91), 222(70), 220(24), 207(59), 204(17), 190(60), 189(37), 179(54), 177(55), 159(55), 144(67), 145(22), 131(55), 130(93), 129(69), 127(46), 117(28), 116(37), 115(56), 114(34).

6. 2,3-di-O-Methyl-6-O-trityl-AA
446(72), 369(40), 303(49), 259(27), 245(57), 244(99), 243(100), 242(58), 241(65), 240(28), 229(18), 228(46), 227(16), 226(25), 215(28), 183(74), 167(52), 166(52), 165(74).

7. AA-5,6-acetonide
216(77), 202(24), 201(100), 185(7), 178(10), 159(9), 141(62), 129(65), 116(22), 115(21), 113(31), 105(32), 102(22), 101(86), 92(18), 87(35).

8. 2,3-di-O-Methyl-5,6-acetonide of AA
244(68), 245(25), 231(28), 230(56), 229(90), 199(33), 189(100), 183(52), 136(25), 170(20), 169(72), 157(44), 155(12), 144(24), 143(52), 141(27), 129(17), 128(36), 127(17), 125(24), 115(39).

9. Iso-dimethyl-AA
204(69), 189(73), 187(57), 186(100), 172(26), 171(81), 157(10), 144(30), 143(48), 142(16), 131(24), 130(35), 129(53), 117(70), 116(66), 115(73), 114(61), 113(62), 103(26), 102(18), 101(46), 100(11), 99(88).

10. Iso-2-O-benzyl-3-O-methyl-AA
280(53), 262(65), 191(31), 190(58), 189(100), 175(20), 172(22), 171(58), 145(27), 144(36), 143(27), 127(9), 117(13), 116(37), 115(43), 114(37), 108(34), 107(36), 106(27), 105(39), 103(21), 101(22), 100(25).

11. 2-C-Benzyl-AA
266(100), 218(31), 205(11), 170(10), 148(42), 131(25), 129(19), 128(61), 127(19), 121(19), 120(53), 119(63), 118(26), 116(22), 115(29), 105(31), 104(26), 103(46), 102(65), 101(47), 94(71).

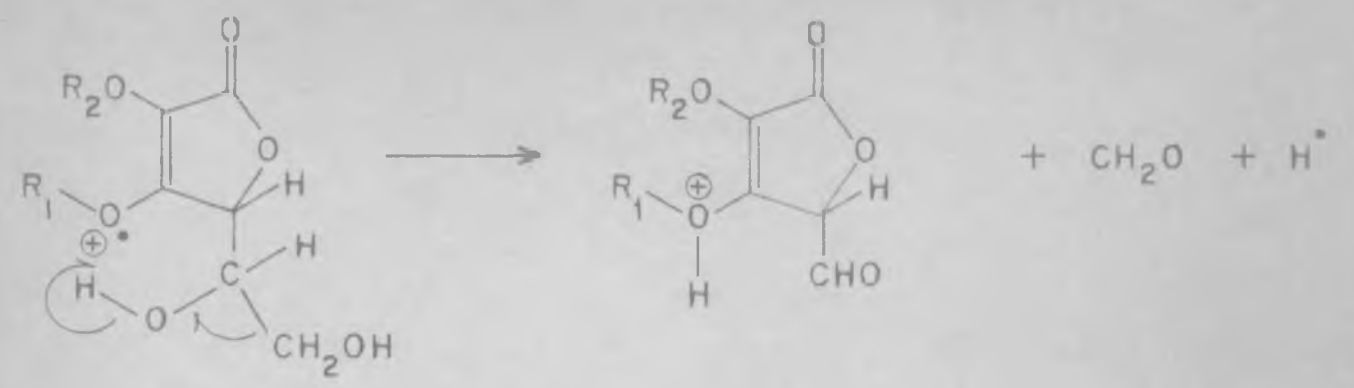
12. Phenyltetronic acid
193(77), 192(100), 191(31), 174(39), 164(21), 163(29), 148(60), 147(65), 146(58), 136(35), 134(22), 131(25), 130(44), 129(31), 121(31), 120(57), 119(81), 118(87), 105(59), 103(57), 104(50).

13. 6-Benzoate of AA
280(8), 262(2), 223(3), 165(6), 123(8), 122(21), 106(8), 105(100).

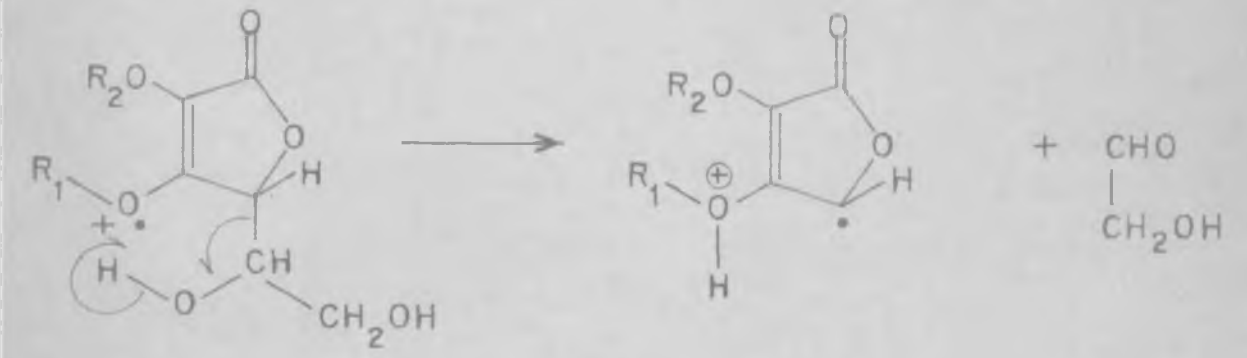
these spectra is the common presence of M-31 and M-60 peaks in the spectra of AA, 3-O-methyl AA, 1-O-methyl-hetero-AA and 2,3-di-O-methyl AA. The spectrum of 3-O-methyl-2-O-benzyl-AA shows an (M-31) peak, but instead of the anticipated (M-60) peak on grounds of analogy, what is obtained is an (M-58) peak. The formation of the ions with m/e values of (M-31) and (M-60) appears to be characteristic of the side chain at $C_{(4)}$. Actually, the (M-60) peak represents the predominant cleavage. The likely site of first ionization is the oxygen on $C_{(3)}$ or the oxygen on $C_{(4)}$ involved in lactone formation. Both are involved in conjugation with the lactone carbonyl. Perhaps ionization at the former site is somewhat easier. If we assume that this is so, a ready rationalization of the two fragmentation modes may be made as indicated in mode 1 and 2 (Chart I). Following Djerassi's notation, fish hooks have been used to indicate likely single electron transfers and curved arrows for two electron transfers. According to the first of the suggested mechanisms, both modes of cleavage have the same deriving force, namely, homolytic transfer of a hydrogen from the hydroxyl on $C_{(5)}$ to the oxygen of $C_{(3)}$ via a six membered transition state accompanied in concerted manner. The other similar possibilities that may be considered involve transfer of the hydrogen to the oxygen on $C_{(4)}$ either from the $C_{(5)}$-OH as in modes 3 and 4 (Chart I) or from $C_{(6)}$-OH as in modes 5 and 6 (Chart II). These two characteristic cleavages giving M-31 and M-60 fragments, are absent in the spectra of all the bicyclic derivatives as well as those in which the side chain hydroxyls are not free.

Chart I

Mode 1



Mode 2



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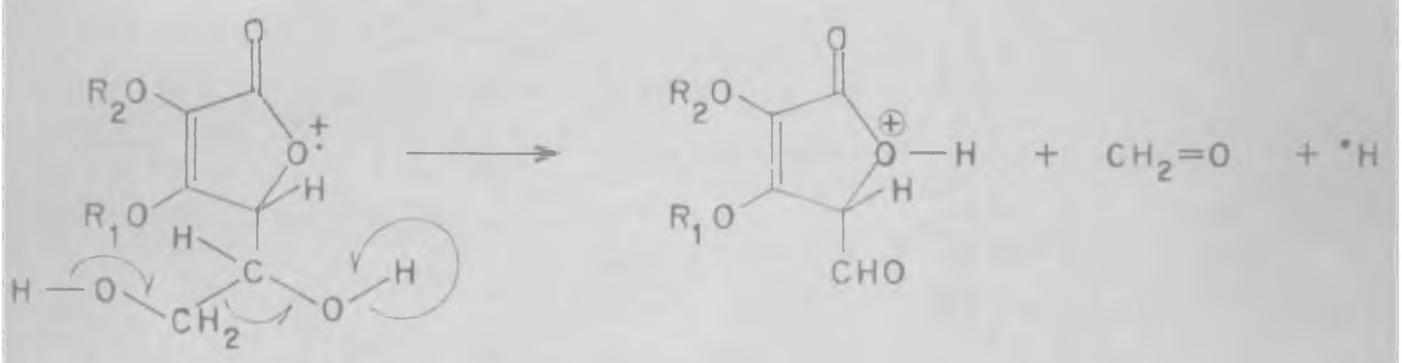


Mode 4

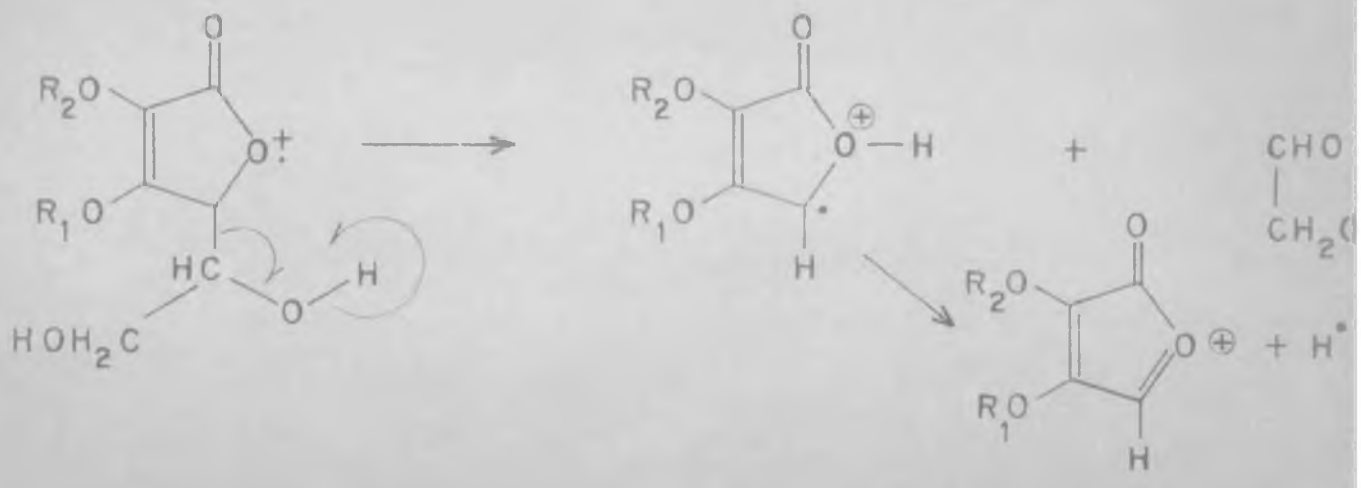
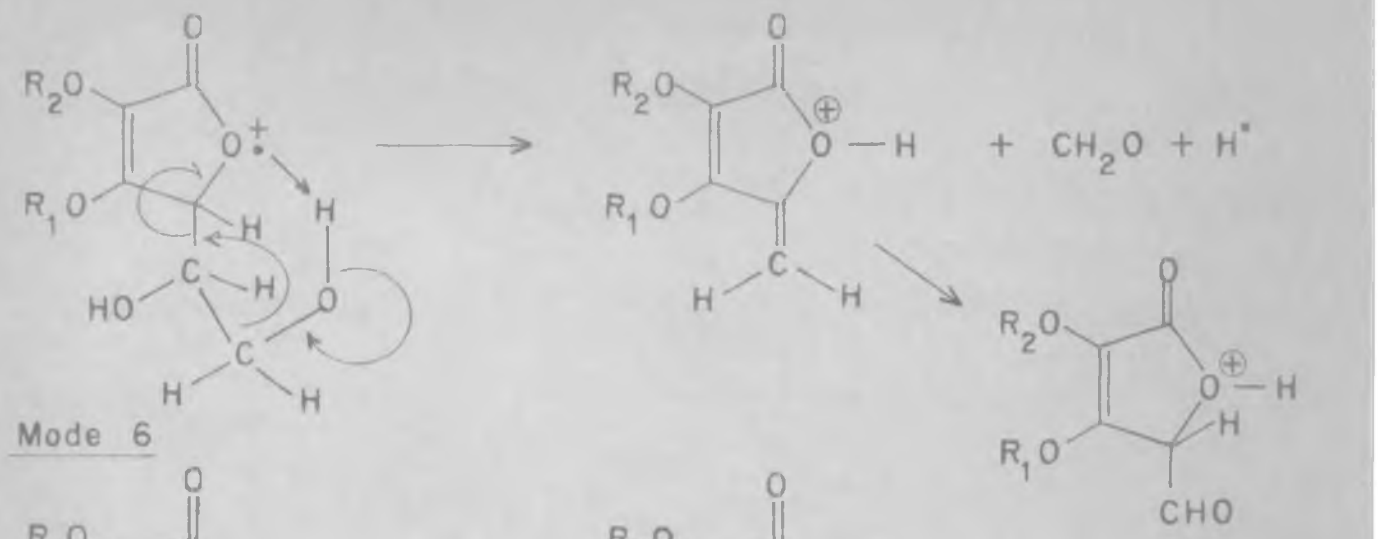
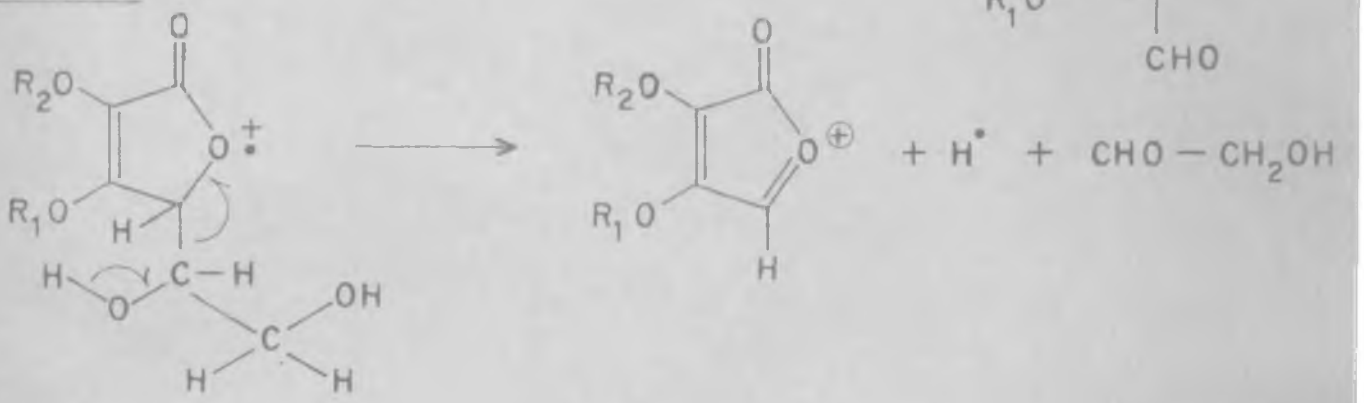


Chart II

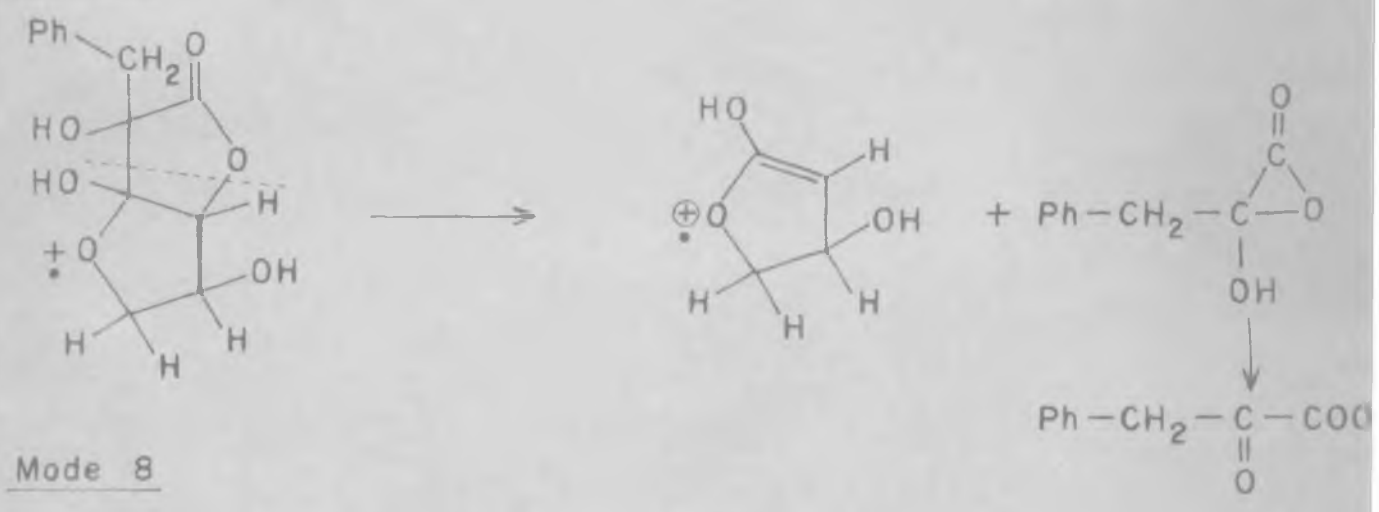
Mode 5



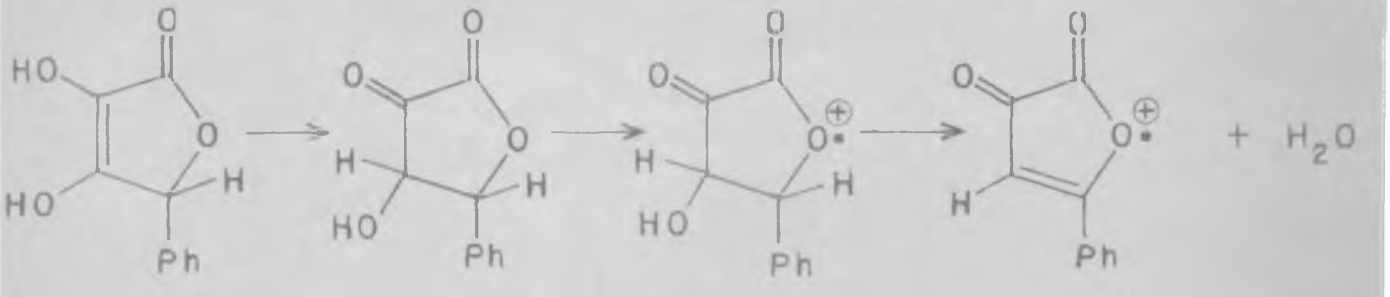
Mode 6



Mode 7



Mode 8



One of the characteristic features of the bicyclic derivatives in which the $C_{(3)}$ is present as a full ketal and not as a hemi-ketal is the loss of water from the molecular ion. Apparently, the $C_{(5)}$-hydroxyl and one of the $C_{(6)}$ hydrogens are involved in this elimination. The (M-18) peak is missing from the spectrum of 2-C-benzyl-ascorbic acid in which the $C_{(3)}$ is a hemi-ketal. The significance of this observation is not clear. A characteristic cleavage of the molecular ion in this case involves ring A as indicated in mode 7 (Chart II). Ring B gives the positive ion fragment (m/e 102). When one checks if this mode is seen in the other bicyclic derivatives as well, what is found is that in addition to the expected peak, one with a mass number smaller by one unit is found in larger abundance. The species concerned is probably a positive ion radical. The difference of one unit probably corresponds to a proton loss from the alkoxy group at $C_{(3)}$. In any case, cleavage of the A-ring in the manner suggested appears to be very likely. A derivative in which the main fragmentation path is diverted away from the ascorbic acid moiety is provided by the case the trityl ether. The main fragmentation mode here gives the trityl cation (m/e 243). Phenyl loss from the molecularmion also is one of the strong modes seen (M-77 peak). In the 5,6-acetonide of AA, the fragmentation starts from the acetonide moiety with an initial loss of a methyl group. The strongest peak is one which is characterised by a loss of 57 mass units from the molecular ion, representing the loss of a methyl group

and ketene from the molecule. This pattern is characteristic of the acetonide function. Phenyl tetronic acid shows an (M-18) peak apparently on account of loss of water, the molecular ion from an isomeric keto form as shown in mode 8 (Chart II).

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Earlier spectroscopic work on AA and DHA has been reviewed and studies that have a bearing on the question of the existence of isomeric forms have been specially mentioned. The PMR spectra of AA and D-iso-AA have been compared, and the conformation of the side chain of AA around the $C_{(4)}$-$C_{(5)}$ bond has been deduced from the low values of the $C_{(4)}$ H - $C_{(5)}$ H coupling constants. The conclusion obtained is in agreement with that arrived at from more laborious and sophisticated measurements involving deuterium labelling.

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The action of the Udenfriend reagent (ferrous-ascorbate-EDTA) on proline was re-examined under a variety of conditions. The reported formation of hydroxyproline could not be confirmed. All the earlier product identifications had been by paper chromatography. The chromatogram of the reaction products did indeed have a spot with the same $R_f$ value as hydroxy proline, but the colour with ninhydrin did not match, and the spot actually represented one of the decomposition products of AA.

In the comparative study of hydroxylation efficiency, there was need for a very great deal of preliminary experi-

mentation in order to find out appropriate reaction conditions which could bring out the genuine differences in reactivity. Salicyclic and gentisic acids were found to be suitable substrates. It was found that 10 mg of the former substrate was completely hydroxylated in 4 hrs when 50 mg of AA was employed. The use of equivalent amounts of other ene-diols in place of AA provided a method of comparison. It was found that the hydroxylation efficiencies of dihydroxy maleic acid, phenyltetronic acid and phenyltetron-imide were about as efficient as AA itself. Among the closer analogues including derivatives of AA, the reactivity order was AA ~ D-iso-AA > 5,6-acetonide > benzoate. The results suggested that the blocking of 6-OH very definitely leads to reduction of hydroxylation efficiency. It appeared likely that the iron complex of a bicyclic form of AA is involved in the reaction and a likely mechanistic scheme has been suggested. The demonstration that the side chain of AA has a role in determining its reactivity was consistent with the conclusions of quite a few other studies reported in this thesis.

Chapter VII - Mass spectrometric and NMR spectroscopic studies of AA and its derivatives

Some useful CMR, PMR and mass spectral data are presented and discussed. The use of $^{13}C$ spectra for assignment of the structure of freshly formed DHA in aqueous solution and for the recognition of other similar bicyclic structures of derivatives of AA has been discussed with the help of data for

a number of compounds. Residual C-H coupling in off-resonance spectra could be made use of in distinguishing between secondary carbons.

PMR spectra of the 2,3-diphenacyl ether of AA showed that the gem coupling in the phenacyl methylenes to be the same. Although the $C_{(6)}$-proton absorption pattern showed a close similarity to that of DHA, a $^{13}C$-spectrum of the derivative showed it to be an open chain compound.

The mass spectra of a number of open chain and bicyclic derivatives have been presented. It has been shown that the open chain compounds, where the side chain hydroxyls are free, give characteristic M-31 and M-60 peaks. Probable mechanisms for these fragmentations have been suggested. Also, a predominant cleavage mode of the bicyclic derivatives has been recognized.

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The Udenfriend hydroxylation is a non-enzymatic reaction in which mediation by AA gives results similar to

those obtained in enzymatic hydroxylations. There has been a great deal of interest in the reaction because of this similarity. The associated literature has been surveyed. Although a number of studies have been devoted to mechanistic aspects, a good mechanistic understanding of the reaction is still lacking. A number of highly regio- and stereoselective hydroxylations have been claimed to have been obtained at quite unactivated carbon atoms of various substrates. There has been no information at all about the nature of reactive intermediates which can give rise to such tremendous selectivity under non-enzymatic conditions. In this context, and in the context of the results obtained in the other studies described here, it appeared desirable to make a comparative study of the efficiency of AA and some of its derivatives and analogues as mediators of hydroxylation in the Udenfriend reaction. It was also thought that there was need to check the reports of the specificities obtained were true.

The action of the Udenfriend reagent (ferrous-ascorbate-EDTA) on proline was re-examined under a variety of conditions. The reported formation of hydroxyproline could not be confirmed. All the earlier product identifications had been by paper chromatography. The chromatogram of the reaction products did indeed have a spot with the same $R_f$ value as hydroxy proline, but the colour with ninhydrin did not match, and the spot actually represented one of the decomposition products of AA.

In the comparative study of hydroxylation efficiency, there was need for a very great deal of preliminary experi-

mentation in order to find out appropriate reaction conditions which could bring out the genuine differences in reactivity. Salicyclic and gentisic acids were found to be suitable substrates. It was found that 10 mg of the former substrate was completely hydroxylated in 4 hrs when 50 mg of AA was employed. The use of equivalent amounts of other ene-diols in place of AA provided a method of comparison. It was found that the hydroxylation efficiencies of dihydroxy maleic acid, phenyltetronic acid and phenyltetron-imide were about as efficient as AA itself. Among the closer analogues including derivatives of AA, the reactivity order was AA ~ D-iso-AA > 5,6-acetonide > benzoate. The results suggested that the blocking of 6-OH very definitely leads to reduction of hydroxylation efficiency. It appeared likely that the iron complex of a bicyclic form of AA is involved in the reaction and a likely mechanistic scheme has been suggested. The demonstration that the side chain of AA has a role in determining its reactivity was consistent with the conclusions of quite a few other studies reported in this thesis.

Chapter VII - Mass spectrometric and NMR spectroscopic studies of AA and its derivatives

Some useful CMR, PMR and mass spectral data are presented and discussed. The use of $^{13}C$ spectra for assignment of the structure of freshly formed DHA in aqueous solution and for the recognition of other similar bicyclic structures of derivatives of AA has been discussed with the help of data for

a number of compounds. Residual C-H coupling in off-resonance spectra could be made use of in distinguishing between secondary carbons.

PMR spectra of the 2,3-diphenacyl ether of AA showed that the gem coupling in the phenacyl methylenes to be the same. Although the $C_{(6)}$-proton absorption pattern showed a close similarity to that of DHA, a $^{13}C$-spectrum of the derivative showed it to be an open chain compound.

The mass spectra of a number of open chain and bicyclic derivatives have been presented. It has been shown that the open chain compounds, where the side chain hydroxyls are free, give characteristic M-31 and M-60 peaks. Probable mechanisms for these fragmentations have been suggested. Also, a predominant cleavage mode of the bicyclic derivatives has been recognized.

# APPENDIX

# Preparation & Properties of 3-Methyl & 2,3-Dimethyl Ethers of Ascorbic Acid*

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**In view of the divergent reports on the properties of 3-methyl ether of ascorbic acid, a procedure for the preparation of the authentic material and its chromatographic purification is described. A similar procedure has been developed for the 2,3-dimethyl ether as well.**

IN connection with some of our studies, we required relatively large amounts of 3-methyl and 2,3-dimethyl ethers of ascorbic acid. However, procedures available in the literature[1-4] did not appear to be suitable for obtaining the quantities that we were interested in. Further, there was need to sort out the reported differences in the properties of the former derivative. We have therefore investigated different procedures for making these derivatives and worked out suitable chromatographic procedures for obtaining them in high yields and purity.

In earlier work, methylations to the monomethyl ether with diazomethane have been carried out at temperatures in the range from −40° to −10° and solvent methanol stripped at around 15°. We have found that reactions at temperatures around 0°, not exceeding 5°, give oils which behave very well in chromatography. After the addition of the requisite quantity of diazomethane in ether,

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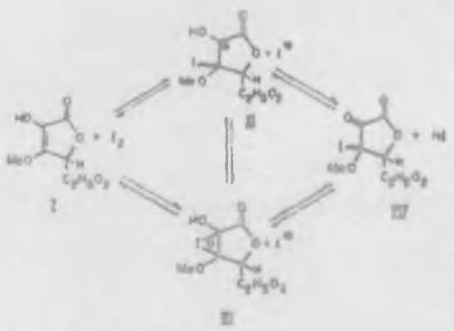

CHART 1

the reaction mixture need be allowed to stand only for two to three hours at 10° to 15°. The solvent may be stripped at 40° to 50° under vacuum without any deleterious effect.

For obtaining the pure compound, column chromatography of the oil on cellulose and different types of alumina and silica gel was tried. Grade-2 silica gel was found to be suitable, and it was prepared from commercial silica gel as follows: The commercial material was stirred with excess of saturated sodium bicarbonate solution for 3 to 4 hr and left overnight in the same medium. The material was filtered off, washed well with distilled water and activated at 700-800° for 4 to 5 hr. From the material thus obtained, grade-2 silica gel was made in the usual way by treatment with the requisite quantity of water, and the grade obtained was checked with the standard dyes[8].

The m.p., UV ($\lambda_{max}$) and $[\alpha]_D$ values of the chromatographed product were similar to those reported by Haworth *et al.*[1]. It was also acidic to litmus as reported by them. The $\epsilon_{max}$ value (13050) was somewhat higher than those reported earlier. The results obtained were consistent and in no case could we get a neutral product. The neutral product[2] has been reported to be non-reactive to iodine. The addition of iodine to the material obtained here was rapid to start with, but slowed down as the reaction proceeded and hydrogen iodide concentration built up in the reaction mixture. With acidified iodine in potassium iodide, the reaction is very slow as described by Haworth and others. The likely reason is that as in the case of ascorbic acid, this reaction is reversible as shown in Chart 1.

The positively charged intermediate (II or III) involved does not lose a proton readily to give the 3-iodo 3-methoxy-2-oxo derivative (IV) which is the expected product. This product appears to be quite stable. The reluctance of the intermediate to go over to IV would be more consistent with the iodonium structure (III) than with the 2-keto-hydroxy carbonium structure (II).

The reported enhancement in reactivity towards iodine on treatment with alkali and reacidification is most probably due to ring opening. It is not clear if any δ-lactone is formed in the transformation. It is relevant in this connection to recall that open chain or six-membered 1,2-diketones require very drastic conditions for reduction with hydrogen iodide[9]. The presence of a chelated hydroxyl signal in the NMR spectrum* of the methyl ether confirmed the presence of the enolic function at $C_{(2)}$ and the nature of the $C_{(5)}$—H absorption (δ 5·284 Hz, *d*, $J$ = 1·9 Hz) and the band at 1740 cm$^{-1}$ in the IR showed that the γ-lactone structure was intact and that the product was the authentic 3-methyl ether.

For the dimethyl ether the methylation procedure was similar to that for the monomethyl ether. The reaction mixture was allowed to stand overnight as usual and solvent stripped at 50° to 60° under suction. Only about 50% of the product could be recovered from the oil obtained by the crystallisation procedures that have been described earlier[1]. The simple chromatographic procedure described below gives about 85 per cent recovery. No inconsistencies in the properties of this material have been reported.

*3-O-Methylascorbic acid* — Ascorbic acid (3 g) was dissolved in dry methanol (50 ml) and the solution cooled to 0°. A calculated quantity (one molar equivalent, standardized against ascorbic acid) of a diazomethane solution in ether was similarly cooled and added slowly to the ascorbic acid solution so that the temperature did not rise above 5°. After the reaction mixture was allowed to stand for 2 to 3 hr at 10-15°, the solvent was stripped in a rotary vacuum evaporator at 40-50° to get a colourless oil. The average yield was 97-98% as judged from NMR spectra of the oils.

The crude oil from the reaction was loaded with a little acetone onto a one-inch silica gel column, prepared from 200 g of silica gel grade-2 in pet. ether (60-80°) slurry. It was first eluted with acetone-pet. ether (20:80). Some unidentified impurity was obtained in this fraction. The elution was continued until no further material came out. The pure 3-*O*-methyl derivative was then eluted out with 45% (v/v) acetone in pet. ether. This fraction was concentrated by removing solvent under suction. It was then allowed to stand overnight when 3-*O*-methylascorbic acid separated out as beautiful needles, m.p. 121-22°, yield 80-85% (UV $\lambda_{max}$ 245 nm, ε 13050; IR: $\nu_{max}$ 1740 and 1675 cm$^{-1}$; $[\alpha]_D$ = +28·9° in water; NMR in DMSO: single proton doublet with $J$ = 1·9 Hz at 284 Hz δ due to $C_{(5)}H$, three proton singlet at 242 Hz due to OMe, and multiplets at 217 and 204 Hz representing $C_{(6)}$—H and $C_{(6)}$—$H_2$ (Found: C, 43·9; H, 5·0. Calc. C, 44·2; H, 5·3%).

*2,3-Di-O-methylascorbic acid* — Ascorbic acid (3 g) was dissolved in MeOH (50 ml) and cooled to 0° and the diazomethane solution, similarly cooled, was added slowly, not allowing the temperature to rise above 5°, until a yellow colour persisted. The mixture was allowed to stand overnight at 10-15° after addition of a 5% excess of diazomethane. The solution was then stripped at 50-60° under

*NMR spectra were recorded at 60 MHz and chemical shifts are from internal TMS.

suction in a rotary evaporator yielding a light yellow oil.

Crystallization of the dimethyl ether straight from the oil is more troublesome than in the case of the monomethyl ether, and, when it succeeds, only about half the material comes out in crystalline form. The following simple procedure furnishes a neat solution for the difficulty and gives complete recovery of the product.

The oil was dissolved in a small quantity of acetone and loaded on to a one-inch column of cellulose (200 g) which was packed in ether slurry. The dimethyl ether was eluted out neatly with peroxide-free ether. Nearly all the impurities remained on the column. On concentration of the ether solution and allowing it to stand, the dimethyl ether separated out as sugar-like crystals, m.p. 59°. After drying at room temperature in a vacuum desiccator over $P_2O_5$ and recrystallization from dry chloroform, very pure material was obtained, m.p. 62-63°; yield 85%; UV: 232·5 nm ($\epsilon$ = 11,300); IR: 1740 and 1650 $cm^{-1}$; $[\alpha]_D = +30°$ in water; NMR (DMSO): single proton doublet with $J$ = 1·9 Hz at 282 Hz, three proton singlets at 242 and 220 Hz due to $C_{(2)}$—OMe and $C_{(3)}$—OMe and multiplets at 215 and 203 Hz representing $C_{(3)}$—H and $C_{(6)}H_2$ respectively.

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