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ENZYMIC METHYLATION OF L-ASCORBIC ACID BY CATECHOL *O*-METHYLTRANSFERASE*

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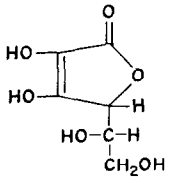
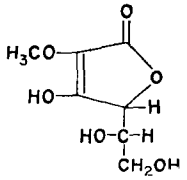
Abstract—L-ascorbic acid (L-AA) was found to be methylated *in vitro* by catechol-*O*-methyltransferase (COMT) isolated from the soluble fraction of rat liver, S-adenosylmethionine (SAME) serving as coenzyme. In the assays either a labelled methyl donor ($^{14}CH_3$-SAME) or, reciprocally, a labelled substrate (1-^{14}C-L-AA) were used. The *O*-methylated product was identified as 2-methyl-L-ascorbic acid (2-MeAA) by cochromatography with synthetic 2-MeAA. This metabolite was recovered also *in vivo* from the urine of rats given 1-^{14}C-L-AA. It accounted for less than 5 per cent of the total activity excreted in urine during 24 hr.

IN SPITE of the broad substrate specificity of catechol-*O*-methyltransferase (COMT) from both animal and plant sources, all known substrates are characterized by a catechol configuration, regardless of other substituents on the aromatic nucleus.[1–8] The OH group attacked is situated either in the meta or in the para position in relation to the side chain.[2, 9–13] L-Ascorbic acid (L-AA) is believed to exist in the enediol form of 3-keto-L-gulonic acid, thus involving a 5-membered ring with two adjacent OH groups.[14]

L-Ascorbic acid



2-Methyl-L-ascorbic acid



In some of our experiments with COMT an inhibition of the formation of normetanephrine from norepinephrine in the presence of high amounts of L-AA was observed. The present investigation shows that L-AA serves as substrate for COMT.

EXPERIMENTAL

Preparation of rat liver COMT. The enzyme was prepared from the supernatant fraction of rat liver, free of microsomes, according to the method of Axelrod *et al.*,[1] with the following slight modifications: the ammonium sulphate precipitate was

* The results were briefly presented at a meeting of the Deutsche Pharmakologische Gesellschaft at Mainz on March 15–18, 1970.

dissolved in 0·05 M potassium phosphate buffer and dialyzed overnight against the same buffer. The final step of adsorption on calcium phosphate gel was omitted, but in some cases the preparation was run through a Sephadex 75 G column (0·01 potassium phosphate buffer pH 8 being used for elution). In one experiment a stepwise ammonium sulphate fractionation was accomplished in order to compare the enzyme activity of the individual fractions on different substrates. The amount of protein in the preparation was determined according to Warburg and Christian.[15]

Radioenzyme assay. The reaction mixture consisted of 0·3 ml 0·2 M phosphate buffer pH 8, 5 μmoles $MgSO_4$, 25 μl enzyme preparation (0·3 mg protein/ml), 22 nmoles S-adenosylmethionine-$^{14}CH_3$ ($^{14}CH_3$-SAME; Radiochemical Centre Amersham; sp.act. 50 mc/mM) and 2·8 μmoles L-AA (dissolved immediately before use), the reagents being mixed in the described order. The final pH was 7·8. In determining the K_m, with increasing concentrations of L-AA the pH of the reaction mixture was lowered to such an extent that the phosphate buffer had to be adjusted individually for each concentration of L-AA tested. The incubation was carried out at 37° in an atmosphere of nitrogen for at least 20 min. The reaction was stopped by addition of 0·4 ml 2·5 N HCl. The samples, following saturation with NaCl, were extracted at pH 1·0 with 8 ml ethylacetate.

Aliquots of 6 ml were transferred to counting vials, evaporated in an air stream and redissolved in 2 ml methanol. A toluene-scintillator solution (10 ml) was added and the radioactivity determined in a liquid scintillation spectrometer. Correction for quenching was made by using internal standards. Correction was also made for the extraction efficiency, the value of the extraction coefficient of the methylation product being 0·50. $^{14}CH_3$-SAME was not extracted into ethylacetate at the pH used.

Identification assays of the methylation product involved the use, on the one hand, of L-AA and $^{14}CH_3$-SAME, and, reciprocally, of unlabelled SAME (Boehringer Mannheim) and 1-^{14}C-L-AA (New England Nuclear, sp.act. 2·15 mc/mM) diluted with non-radioactive L-AA, in the reaction mixture.

Paper chromatography of methylated compounds. The ethylacetate extracts were evaporated *in vacuo*, redissolved in methanol and chromatographed by ascending paper chromatography on Whatman 1 or 3 MM in the following solvent systems: *n*-butanol–pyridine–water (6:4:3), isopropanol–25% ammonia–water (80:8:12), collidine–water (1:1; upper phase), *n*-butanol–propionic acid–water (50:20:15), *n*-butanol–butyric acid–water (7:7:3), *n*-butanol–glacial acetic acid–water (60:15:25), *n*-amylalcohol–glacial acetic acid–water (4:1:5; upper phase), ethylacetate–glacial acetic acid–water (3:1:3; upper phase) and phenol–0·1 N HCl (85:15). The following ethers of L-AA were run simultaneously: 1-methyl, 2-methyl, 3-methyl and 2,3 dimethylether. After being scanned the chromatographic strips were sprayed with 1% $FeCl_3$ or 1% $KMnO_4$.

Synthesis of methylethers of L-*AA.* 3-*O*-methyl-L-ascorbic acid (3-MeAA) was prepared by methylation of L-AA with diazomethane according to Haworth *et al.*[16,17] 1-*O*-methyl-L-ascorbic acid (1-MeAA) is a byproduct of this synthesis. Further extensive treatment of 3-MeAA with diazomethane for 16 hr yields 2,3-di-*O*-methyl-L-ascorbic acid (2,3 DiMeAA). 2-*O*-methyl-L-ascorbic acid (2-MeAA) was obtained by treatment of the 2,3 DiMeAA according to Haworth *et al.*[17] The four isomers are best separated by paper chromatography in alkaline systems, e.g. isopropanol–ammonia–water or *n*-butanol–pyridine–water. They give characteristic colours with

$FeCl_3$: 3-MeAA—a permanent blue, 1-MeAA—a fast disappearing, light red and 2-MeAA—a permanent reddish brown. 2,3 DiMeAA can be distinguished only by its yellow colour with 1% $KMnO_4$. It was possible to synthesize only the 3-MeAA in crystalline form. Indeed, the 2-MeAA has never been crystallized at all.

Experiments in vivo. Four rats were intravenously injected with 10 μc 1-[^{14}C]-L-AA dissolved in saline. The urine was collected in vials containing 0·2 ml 1% L-AA over a 24 hr-period. Aliquots of urine were extracted at pH 1 with ethylacetate as described above. Samples of untreated urine as well as the ethylacetate extracts were cochromatographed with synthetic methylethers of L-AA and scanned. The area corresponding to the synthetic 2-MeAA spot was cut out from the paper chromatogram, eluted with 0·01 HCl, the eluate extracted with ethylacetate, evaporated, redissolved in methanol and rechromatographed.

In order to determine the percentage contribution of 2-MeAA towards the total radioactivity excreted in urine, the radioactivity of urine aliquots was determined on addition of a dioxane scintillator solution in a liquid scintillation counter; following chromatography of aliquots of the same urine the radioactivity of the eluate from the area corresponding to 2-MeAA was measured in the same way.

RESULTS

The formation of methyl ascorbic acid by rat liver COMT. The data presented in Table 1 show the formation of methylated L-AA. The enzymic reaction has an absolute requirement for Mg^{2+} (the effect of divalent Zn, Mn, Fe ions was much less). No methylation of L-AA occurs in the presence of the chelating agent, EDTA. Since the partially purified enzyme preparation contained small quantities of various compounds which are also methylated, but without any requirement for Mg^{2+}, and are

TABLE 1. ENZYMIC METHYLATION OF L-ASCORBIC ACID BY COMT

System	Methyl. product (nmoles)
Complete system	4·9
L-Ascorbic acid omitted	0·7
COMT omitted	0·1
Mg^{2+} omitted	0·9
Compl. system + 6·7 μmoles EDTA	0·7
Compl. system + 27·3 μmoles NA	1·0

Reaction mixture consisted of 60 μmoles phosphate buffer pH 8, 5 μmoles $MgSO_4$, 21·6 nmoles ^{14}C-SAME, 25 μl COMT and 2·8 μmoles L-ascorbic acid. Final pH 7·8. Incubation at 37° under anaerobic conditions for 30 min.

extracted at the low pH into ethylacetate, the blank is rather high. Further purification of the enzyme by Sephadez G 75 lowered the stability of the preparation; hence it was found preferable to work with the partially purified one.

The optimum pH of the reaction lies between 7·6 and 8·0 (Fig. 1). Norepinephrine at a concentration 100 times lower than the L-AA concentration inhibits the reaction by 80 per cent. The inhibition occurs simultaneously with the formation of normetanephrine, which can be extracted only at an alkaline pH into isoamylalcohol–

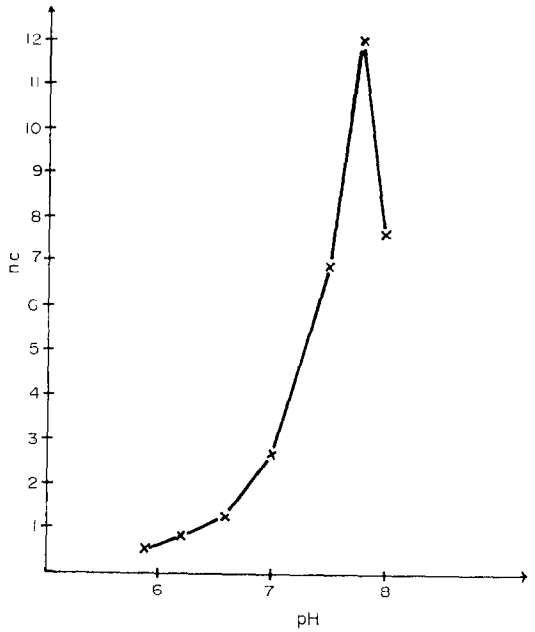


FIG. 1. Effect of pH on *O*-methylation of L-ascorbic acid. The reaction mixture contained 10 μmoles phosphate buffer pH 8·0, 5 μmoles $MgSO_4$, 25 μl enzyme preparation, 22 nmoles S-adenosyl-methionine-$^{14}CH_3$ and 2·8 μmoles L-ascorbic acid. The reaction time was 20 min.

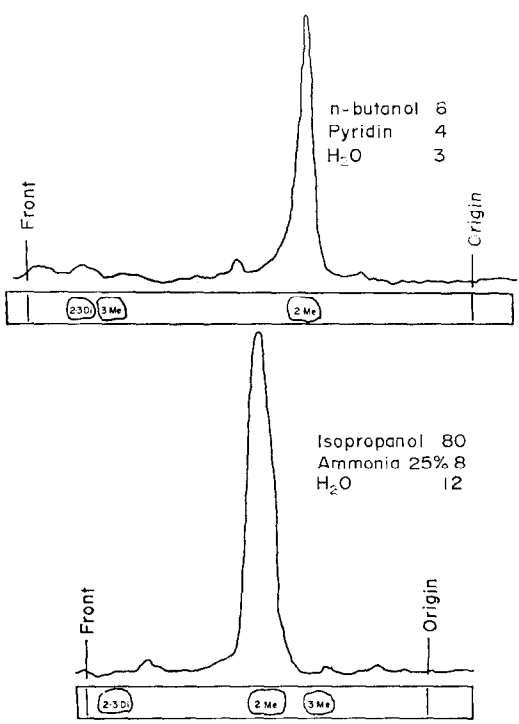


FIG. 2. Paper chromatogram of 2-*O*-[^{14}C]-methyl-L-ascorbic acid. L-ascorbic acid was incubated under anaerobic conditions with soluble catechol-*O*-methyltransferase from rat liver. S-adenosyl-methionine-$^{14}CH_3$ was used as methyl donor. Ascending chromatography on Whatman 1 in (a) *n*-butanol–pyridine–water (6:4:3) solvent (b) isopropanol–25% ammonia–water (80:80:12) solvent. Cochromatography with synthetic 2-*O*-methyl-L-ascorbic acid.

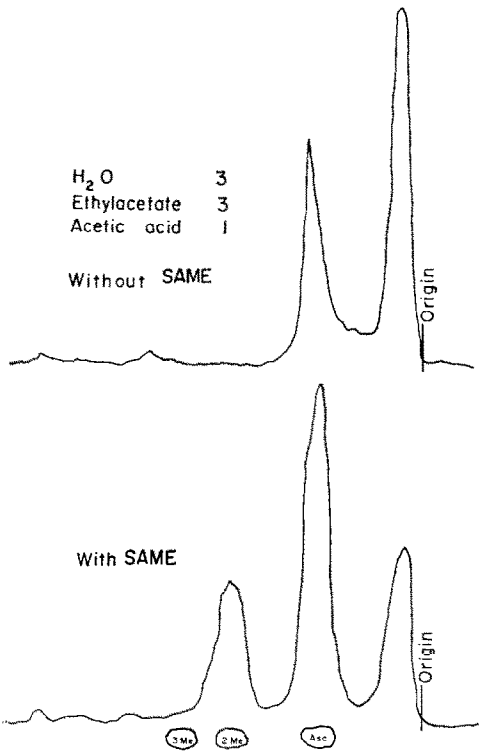


FIG. 3. Paper chromatogram of 2-*O*-methyl-1-[^{14}C]-L-ascorbic acid. (a) 1-[^{14}C]-L-ascorbic acid was incubated with catechol-*O*-methyltransferase without methyl donor. (b) The same assay with unlabelled S-adenosylmethionine as methyl donor. Ascending chromatography on Whatman 1 in ethylacetate–glacial acetic acid–water (3:1:3) solvent. Cochromatography with synthetic 2-*O*-methyl-L-ascorbic acid.

toluene. Enzymic activity with L-AA as substrate was found in the same fractions obtained by stepwise saturation of the COMT preparation with ammonium sulphate, i.e. 35–55 per cent, as in the presence of norepinephrine as substrate.

The K_m could only be roughly determined for reasons elaborated in the discussion; it lies between 1.10^{-3} and 3.10^{-3} M of L-AA. The Lineweaver–Burk plot [19] was used to derive the K_m.

Identification of the methylated product. Of the different solvents tested for the extraction of the methylated product of L-AA, ethylacetate was found to be the best, non-polar solvents being ineffectual. As the extraction curve with ethylacetate shows a maximum between pH 0 and 1 and a steep fall between pH 2 and 3, the product was considered to be a polar substance of acidic behaviour. When $^{14}CH_3$-SAME was used as methyl donor in the reaction mixture with L-AA and the assay was performed under anaerobic conditions, the chromatographic scan showed one peak which corresponded in all 9 chosen systems to the spot of the synthetic 2-MeAA (Fig. 2). When, on the other hand, L-AA in the C_1-labelled form was used as substrate, the scan showed not only the peak of MeAA, but also unchanged L-AA and a further metabolite, which were simultaneously extracted at low pH. L-AA and the unidentified metabolite were present even in assays without added methyldonor (Fig. 3). Hence, an

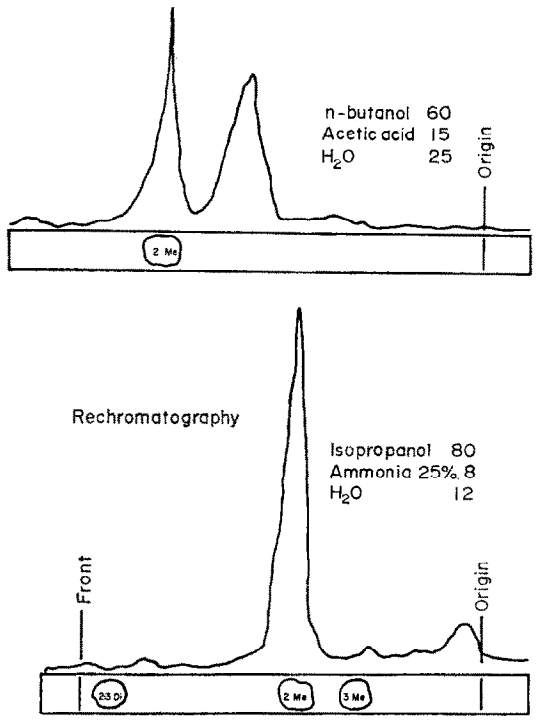


FIG. 4. Paper chromatogram of 2-*O*-[^{14}C]-methyl-L-ascorbic acid excreted in rat urine. (a) The ethylacetate extract at pH 1 of the urine excreted by rats given 1-[^{14}C]-L-ascorbic acid intravenously was chromatographed on Whatman 3 MM in *n*-butanol–glacial acetic acid–water (60:15:25) solvent. Cochromatography with synthetic 2-methyl-L-ascorbic acid. (b) The radioactive peak corresponding to synthetic 2-*O*-methylascorbic acid was cut out, eluted, extracted and rechromatographed in isopropanol–25% ammonia–water solvent system. Cochromatography with the reference compound.

assay with and without methyldonor had to be run on chromatograms simultaneously, because it was not possible to separate 2-MeAA from L-AA either on ion exchange or adsorption columns. The acidity of L-AA lies very close to the acidity of 2-MeAA since the highly acidic group at C_3 (pK 4·1) remains intact in 2-MeAA[18] and this easily explains the difficulties met with on attempting separation of them on ion exchange columns. The peak found in the extracts of the complete enzyme assay showed the same R_f value as synthetic 2-MeAA. On cochromatography of MeAA prepared by the enzymic methylation of unlabelled L-AA with ^{14}C-SAME on the one hand and of 1-^{14}C-L-AA with unlabelled methyldonor on the other hand, the obtained radioactive maxima of the methylated product both coincided with the synthetic 2-MeAA spot, thereby confirming the identity of the methylated product.

O-methylation of L-AA in vivo. The chromatograms of the acidic ethylacetate urine extracts showed four radioactive peaks on scanning. One of them coincided in all chosen systems with synthetic 2-MeAA. After elution and extraction of the appropriate area, rechromatography in a different system gave a single radioactive maximum, which again corresponded to synthetic 2-MeAA (Fig. 4). 2-MeAA accounts for approximately 5 per cent of the total radioactivity excreted in urine.

DISCUSSION

Although the structure of L-AA differs from the catechol nucleus, some features are common to both of them.[18,20] First of all, they are both classified as "reductones", on account of their reducing ability. Both possess the enediol group. The catechol configuration is, therefore, sometimes classified as an "aromatic enediol",[18] the mesomeric state with 6π electrons being responsible for its weaker reducing ability as compared to aliphatic enediols. A molecular model of L-AA built in agreement with the X-ray crystallographic data of Cox[21] and, more recently, of Hvoslef,[22] is flat, as is an established fact for the catechol nucleus.[31] At pH 8·0 the OH group at C_3 of L-AA in aqueous solution dissociates and the ionised system is subsequently transformed into a state of resonance[14,18] although not identical with the aromatic mesomeric state, since only 4π electrons are present. The similar features in the chemical behaviour of catechols and L-AA seem to be of great interest in view of the present findings demonstrating similar behaviour in respect to enzymic *O*-methylation.

The absolute requirement for Mg^{2+}, the characteristic optimum pH and the inhibition of the reaction by norepinephrine gives strong support to the idea that cytoplasmic COMT is the enzyme responsible for methylating L-AA.

The chromatographic scan of assays performed with the labelled methyl donor under aerobic conditions showed three peaks representing methylated compounds, whilst experiments with C_1-labelled L-AA showed only one peak corresponding to a methylated derivative of L-AA. The formation of different isomers of MeAA is, therefore, improbable. A COMT preparation purified twice on Sephadex was then used in the experiments with labelled methyl donor in order to exclude the presence of any contaminating low molecular substance capable of being methylated either directly, or following hydroxylation by L-AA in oxygen atmosphere (Udenfriend *et al.*[23]) but the three peaks remained unchanged. Under anaerobic conditions two of the three peaks disappeared; the remaining peak was identified as 2-MeAA. It is possible that the two unidentified peaks represent degradation products of 2-MeAA formed only in the presence of oxygen. This process must, moreover, involve C_1 degradation, in view of the above-mentioned findings with C_1-labelled L-AA. The use of a universally labelled L-AA would provide the answer to this question.

Whilst in non-enzymic reactions methylation of L-AA occurs first on the most acidic group, i.e. at C_3, the enzymic reaction leads to the formation of 2-MeAA. Stereostructural relations possibly tend to favour the C_2 position in the enzymic reaction. On viewing the formula of L-AA in an analogous manner to that of norepinephrine, the OH group at C_2 is in the "meta" position with respect to the "side chain". Since 1-MeAA is very rapidly hydrolyzed to L-AA even by water,[24] it cannot appear under the conditions used. There is no indication in the present investigation of the formation of 2,3-DiMeAA and, indeed, the only example in the literature of the formation of a dimethyl derivative of a polyhydroxyphenolic compound was described by Archer *et al.*[25] in the case of pyrogallol, but this finding was not confirmed by Masri *et al.*[11]

Two circumstances made it difficult to achieve greater accuracy in the estimation of the substrate K_m: the rather high blank value and the technical impossibility of maintaining strict anaerobic conditions during the mixing procedure. Nonetheless it can be seen that the K_m for L-AA is considerably higher than the K_m for e.g. dihydroxybenzoic acid (6.10^{-5} M)[26] or epinephrine ($1{\cdot}2 \cdot 10^{-4}$ M).[1] This observation is

not surprising if the L-AA levels in the organism are taken into consideration.[27,28] On the other hand, the use of L-AA in high amounts as antioxidant in reaction mixtures with COMT and catechols should be avoided, since it can falsely lower the methylating rate of the catechol compound.

In view of the previously-mentioned occurrence of 2-McAA in the urine of rats (Fig. 4), it would be of interest to elucidate whether this substance represents merely an inactive degradation product of L-AA or still possesses some antiscorbutic properties.

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