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Spontaneous N^{ϵ}-methylation and N^{ϵ}-formylation reactions between L-lysine and formaldehyde inhibited by L-ascorbic acid

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For the inhibition of spontaneous N^{ϵ}-methylation and N^{ϵ}-formylation reactions between L-lysine and formaldehyde, L-ascorbic acid proved to be most suitable. The inhibition was not complete unless the molar concentration of ascorbic acid exceeded that of formaldehyde. T.l.c., potentiometric titration, n.m.r. spectroscopy and radiometric analysis were applied in the study of the inhibition process. Formaldehyde was reduced by L-ascorbic acid to ethylene glycol.

Spontaneous methylation and formylation reactions proceeding simultaneously between L-lysine and formaldehyde have been reported, and similar reactions can occur with L-lysine in peptide linkage (for which N^{α}-acetyl-L-lysine methylamide may serve as a model compound) (Tyihák *et al.*, 1980; Trézl *et al.*, 1979). The reaction scheme for the model compound is indicated in Scheme 1.

Repetition of such reactions leads to the formation of various lysine derivatives, including N^{ϵ}-trimethyl-L-lysine, which has proved to be biologically active, increasing cell proliferation (Tyihák *et al.*, 1977; Suba *et al.*, 1980; Paik & Kim, 1980).

In biological systems N^{ϵ}-methyl-L-lysine can also be formed with formaldehyde derived from formaldehyde precursors [e.g. from dimethylnitrosamine (Turberville & Craddock, 1971; Johansson & Tjälve, 1978; Sawicki & Sawicki, 1978)].

Endogenous releasable formaldehyde is known to occur at low concentrations in biological systems. Thus in normal human blood 0.4–0.6 μg/ml and in urine 2.8–4.0 μg/ml have been detected with [^{14}C]-dimedone reagent (Szarvas *et al.*, 1981).

However, significant deviations have been observed in different tumorous conditions, with values ranging from 8–10-fold higher to 10-fold lower than the normal formaldehyde concentration having been reported. In blood samples from certain tumour-bearing humans, indeed, the concentration of formaldehyde was less than 0.01 μg/ml (Szarvas *et al.*, 1981).

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Also, the activity of the enzyme responsible for the demethylation of N^{ϵ}-methylated L-lysines was found to be practically zero in Novikoff hepatoma, i.e. no formaldehyde was released (Kim & Paik, 1974).

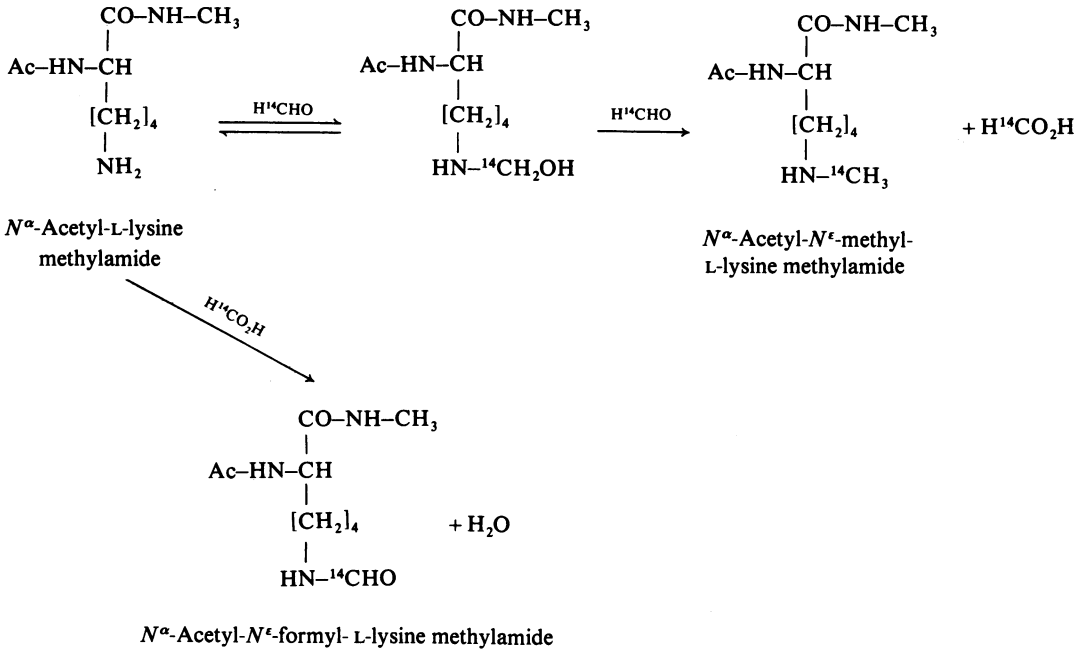
It has further been reported that N^{ϵ}-trimethyl-L-lysine glutamate induces blastic transformation of human peripheral blood cells (Suba *et al.*, 1980) in short-term culture. Remarkably, however, when the ascorbate derivative of N^{ϵ}-trimethyl-L-lysine was used under the same experimental conditions and in the same dosage (200 μg/ml), blastic transformation did not occur.

The inhibiting effect in spontaneous *N*-methylation reactions has not been mentioned in various papers dealing with the fundamental biological role of L-ascorbic acid (Lewin, 1976; Stone, 1976; Cameron *et al.*, 1979; Szent-Györgyi, 1980).

Results

During the potentiometric titration of L-lysine with formaldehyde, the pH of the system decreases in accordance with what may be expected on the basis of the Sörensen formol titration (Sörensen, 1908). After the neutralization point the pH of the system does not change any more with further increase in formaldehyde concentration. The pH does increase, however, if L-ascorbic acid is added to the system at the beginning of the titration.

Salt formation can be assumed between L-ascorbic acid and the ε-amino group of L-lysine (Lewin, 1976), leaving no free active sites to react with formaldehyde. As a consequence of this salt



Scheme 1. *Reactions between formaldehyde and N^{α}-acetyl-L-lysine methylamide*

formation the original pH value of the L-lysine solution, 9.1, decreased to 5.2.

The salt linkage splits as added formaldehyde is reduced by an equivalent amount of the combined L-ascorbic acid, and there is a simultaneous increase in the pH of the system, due to the released ε-amino groups.

N^{ε}-Methyl-L-lysine, N^{ε}-formyl-L-lysine, N^{ε}-dimethyl-L-lysine and N^{ε}-trimethyl-L-lysine were detected among the reaction products of L-lysine and formaldehyde by means of t.l.c. after the titration.

If, however, L-ascorbic acid was present in an equivalent amount to L-lysine in the system, N^{ε}-methyl or N^{ε}-formyl compounds could not be detected.

Similar conclusions can be drawn on the basis of n.m.r. studies on N^{α}-acetyl-L-lysine methylamide. The protons of the two methyl groups in N^{α}-acetyl-L-lysine methylamide are represented by two sharp singlet lines at 1.95 and 2.65 p.p.m. in the n.m.r. spectrum (Fig. 1*a*). After the reaction with formaldehyde, sharp singlet lines characteristic of N^{ε}-methyl groups (2.82 p.p.m.) and N^{ε}-formyl groups (8.20 p.p.m.) are obtained (Fig. 1*b*). If excess of L-ascorbic acid was present no singlet peaks characteristic of methyl and formyl groups could be detected in the n.m.r. spectrum (Fig. 1*c*). To study the reactions with extremely low concentrations (10 ng/ml), radiometric analysis was appropriate, with labelled formaldehyde and labelled L-lysine. If the concentration of L-ascorbic acid exceeded that of formaldehyde, no N^{ε}-methyl-L-lysine or N^{ε}-formyl-L-lysine could be detected.

On the basis of these various analytical data, it can be assumed that L-ascorbic acid inhibits the addition of formaldehyde to the ε-amino group of L-lysine, and the possibility that its reducing effect on formaldehyde is involved must be considered. The reaction between L-ascorbic acid and formaldehyde (above 30°C, pH 4–8) is accompanied by CO_2 formation (Reithel & West, 1948), with loss of L-ascorbic acid reducing capacity (Reithel & Wither, 1949). It could be suspected that methanol and ethylene glycol are the possible products, besides dehydro-L-ascorbic acid, in the reaction. In fact, no methanol could be detected by g.l.c. even after a reaction time of 24 h at 37°C, though at that time a significant amount of ethylene glycol was formed and unambiguously identified.

Discussion

It has been demonstrated that the spontaneous methylation and formylation of L-lysine with formaldehyde can be inhibited or completely eliminated by L-ascorbic acid. The inhibition is concentration-dependent, and is complete only when there is a

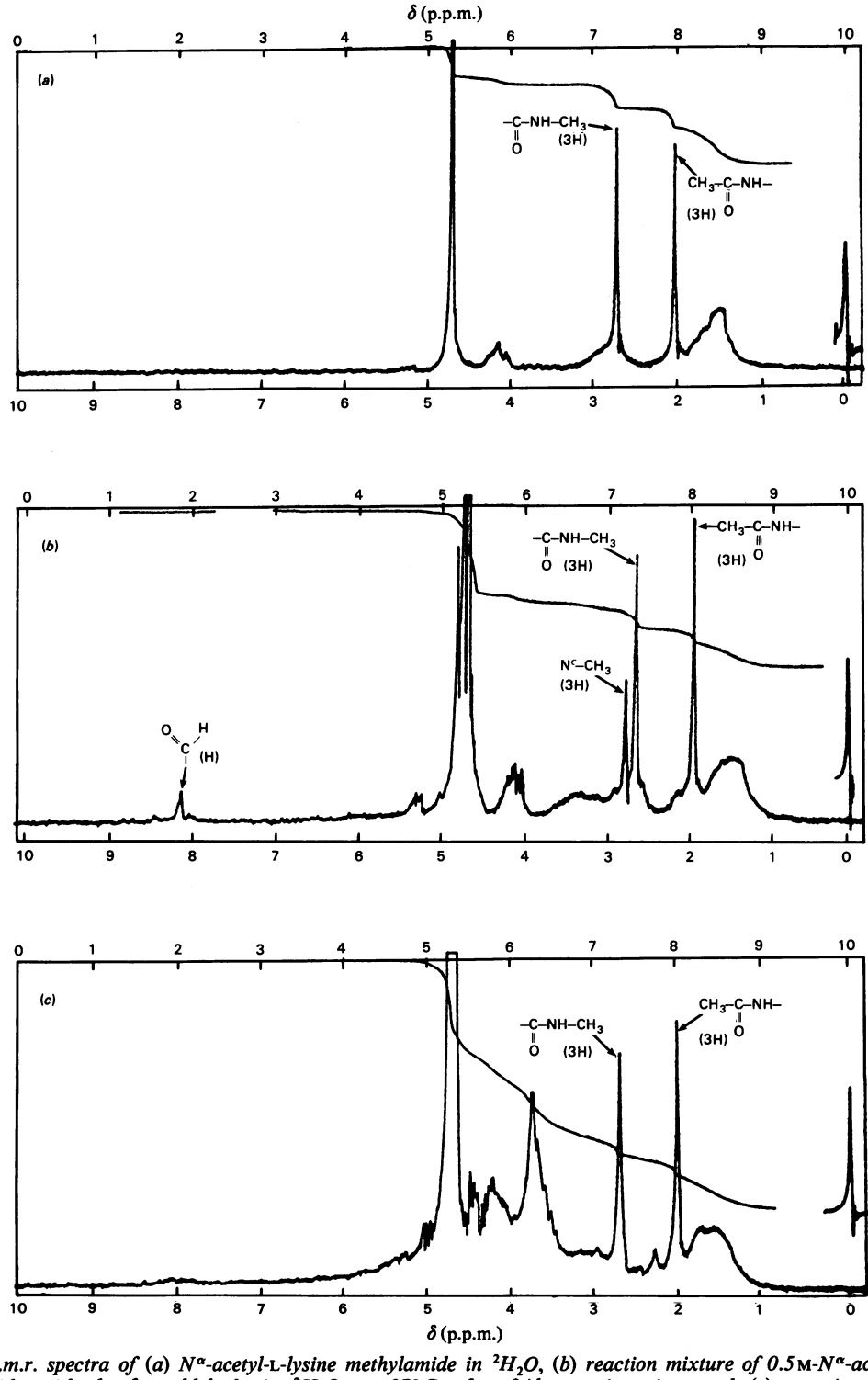


Fig. 1. *N.m.r. spectra of* (*a*) *N^{α}-acetyl-L-lysine methylamide in ^2H_2O,* (*b*) *reaction mixture of 0.5 M-N^{α}-acetyl-L-lysine methylamide with 1 M-formaldehyde in ^2H_2O at 37°C after 24 h reaction time and* (*c*) *reaction mixture of 0.5 M-N^{α}-acetyl-L-lysine methylamide and 1 M-L-ascorbic acid with 1 M-formaldehyde in ^2H_2O at 37°C after 24 h reaction time*

The ^{1}H-n.m.r. spectra were taken with a Perkin–Elmer R-12 60 MHz device.

molar excess of L-ascorbic acid over that of the formaldehyde. L-Ascorbic acid inhibits the reaction by reducing formaldehyde to ethylene glycol.

The inhibition by ascorbic acid of spontaneous methylation and formylation reactions may be significant in biological systems.

Materials and methods

Materials

L-Lysine (Reanal, Budapest, Hungary), L-ascorbic acid (Reanal), formaldehyde (Lachema, Brno, Czechoslovakia), paraformaldehyde (Merck, Darmstadt, West Germany), methanol (Reanal), ethylene glycol (Reanal), N^{α}-acetyl-L-lysine methylamide (CIBA–Geigy, Basel, Switzerland), N^{ϵ}-trimethyl-L-lysine glutamate and N^{ϵ}-trimethyl-L-lysine ascorbate (G. Richter, Budapest, Hungary), N^{ϵ}-monomethyl-L-lysine hydrochloride (Sigma Chemical Co., St. Louis, MO, U.S.A.), N^{ϵ}-dimethyl-L-lysine hydrochloride (Vega, Columbia, U.S.A.), N^{ϵ}-trimethyl-L-lysine dihydrochloride (Puskás & Tyihák, 1969), N^{ϵ}-formyl-L-lysine (Reanal) and ^2H_2O (Merck) were of analytical grade and were used without purification.

[^{14}C]Formaldehyde and labelled L-[6-^{3}H]lysine were prepared at the Institute of Isotopes of the Hungarian Academy of Sciences.

Potentiometric titration and t.l.c.

L-Lysine solution (10mM) was potentiometrically titrated with 3% (w/v) formaldehyde solution. Simultaneously with changes in pH, samples were taken from the reaction mixture and the ninhydrin-positive products were detected on FIXION 50 X8 cation-exchanging thin-layer sheet.

Similar titrations of 10mM-L-lysine with formaldehyde were performed in the presence of 1mM-, 5mM-, 10mM- and 50mM-L-ascorbic acid.

N.m.r. analysis

Formaldehyde (0.15g) (made from solid paraformaldehyde) was dissolved to 5ml in boiling ^2H_2O; 0.505g of N^{α}-acetyl-L-lysine methylamide was added to the solution after it had cooled. The reaction mixture was kept at 37 ± 1°C for 24h, and the n.m.r. spectrum was taken with a Perkin–Elmer R-12 60MHz device. The investigation was repeated also in the presence of 0.1M-, 0.5M- and 1M-L-ascorbic acid respectively.

Analysis with labelled compounds

L-Lysine (1mM) and [^{14}C]formaldehyde (1mM; 19.98kBq/ml) were allowed to react in 0.1M-sodium phosphate buffer, pH7, at 37 ± 1°C. The reaction mixture analysed by t.l.c. on a FIXION 50 X8 thin-layer sheet after 24h. The radioactivity distribution was determined by a thin-layer-chromatogram scanner.

The analysis was also performed with L-[6-^{3}H]lysine (1mM) and unlabelled formaldehyde (1mM). The reactions described were repeated also in the presence of 1mM- and 5mM-L-ascorbic acid.

G.l.c. analysis

Formaldehyde (0.1M) (made from solid paraformaldehyde) was mixed with an equal volume of 0.2M-L-ascorbic acid. The system was kept at 37 ± 1°C for 24h. Reaction products were analysed on a Hewlett–Packard gas chromatograph (5% Chromosorb W; 2m × 4mm column). Gases generated during the reaction were tested for CO_2 with a mass spectrograph.

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