DEGRADATION OF ^{14}C-L-HISTIDINE DURING CHROMATOGRAPHY AND ELECTROPHORESIS

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SUMMARY

The loss of histidine and the formation of additional by-products during the purification and storage of [^{14}C(U)]-L-histidine preparations on chromatographic carriers was followed. The product formed mainly on chromatographic paper was identified as the condensation product of histidine with formaldehyde; on thin layer of silica gel mainly degradation products of histidine were obtained. Possibilities to decrease the formation of these undesirable by-products were discussed.

Key Words: ^{14}C-L-Histidine, Transformation, Chromatographic Carriers, Condensation, Formaldehyde

INTRODUCTION

During paper chromatography and electrophoresis of small amounts of histidine we observed losses of histidine and formation of unknown by-products. Chromatographically prepurified samples of [^{14}C(U)] -L-histidine and[carboxyl-^{14}C] -D,L-histidine submitted to paper-electrophoretic control contained two further radioactive compounds in addition to histidine (Fig.1). The spot with lower radioactivity remained at the position of

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neutral substances (indicated as the fraction N) while the spot of higher radioactivity (indicated as the product X) migrated as an independent substance of less pronounced basic character than histidine.

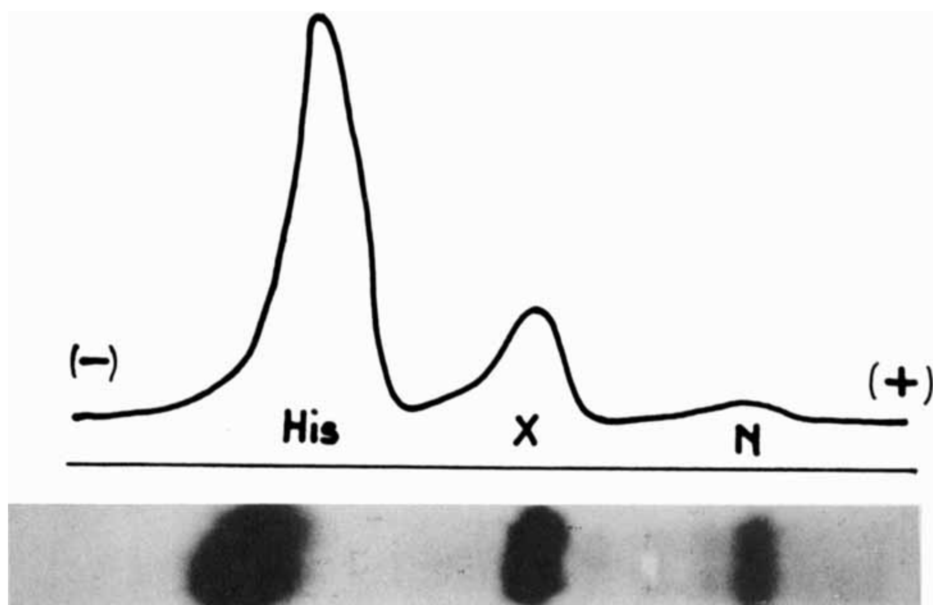


Fig.1 Electrophorogram of [^{14}C(U)] -L-histidine sample (specific activity 210 mCi/mM) prepurified by paper chromatography in n-butanol-acetic acid-water.

The aim of this paper was: 1.) to identify the main unknown by-product X and to explain its formation.

2.) to find the optimal conditions for chromatographic purification and storage of histidine preparations labelled with high specific radioactivity.

EXPERIMENTAL

[^{14}C(U)] -L-histidine preparations (UVVVR, Prague) were purified by paper electrophoresis (Whatman 3, pyridine-acetate buffer of pH 5.6, voltage drop 33 V/cm, 60 min). Pure samples of ^{14}C histidine were applied onto prewashed and unprewashed chromatographic paper Whatman 3 in 1 μg amounts and stored in various conditions. In some cases also [carboxy-^{14}C] -DL-histidine (The Radiochemical Centre, Amersham) was used. Strips of the chromatographic paper with applied spots were cut off at various intervals and submitted to electrophoretic separation. Simultaneously ^{14}C histidine samples were applied on a thin-layer of cellulose or silica gel (Lucefol or Silufol, Kavalier Company, Czechoslovakia), gradually eluted with water and analysed in a similar manner. The decrease in histidine concentration and the formation of by-products were determined radiometrically.

For mass spectra a JEOL-MS-D100 instrument was used. ^{1}H-NMR spectra (59.797 MHz, d_6-DMSO) and ^{13}C-NMR spectra (15.036 MHz, dioxane standard, δ 67.4) were measured with JEOL FX-60 FT mode, solvent deuterium lock system.

RESULTS AND DISCUSSION

As shown from the Table 1 the quantity of the by-products X and N increases with the time of standing of histidine on the chromatographic paper after its application. The conversion of histidine is substantially lower if higher amounts are applied; for example when 40 μg were applied and the sample allowed to stand on the paper for 9 days before electrophoretic separation (the mass of histidine was concentrated in narrow zones surrounding the applied spots), product X and fraction N were formed only in 4% and 1.5% respectively.

Table 1

The decrease of histidine and formation of by-products in dependence on the time of standing of ^{14}C-histidine samples applied in 1 μg amounts on the chromatographic carriers and stored in laboratory atmosphere (expressed in % radioactivity).

	Whatman 3 paper			Thin layer of cellulose				Thin layer of silica gel			
Days	His	X	N	His	X	N	A	His	X	N	A
0	99.3	0.4	0.3	99.3	0.4	0.3	0.0	99.3	0.4	0.3	0.0
2	91.0	6.5	2.5	94.9	2.4	2.7	0.0	79.3	3.8	6.1	10.8
9	77.0	17.3	5.6	87.5	4.5	4.4	3.6	29.8	3.6	29.9	36.7
14	62.0	27.7	10.4	75.0	8.6	9.1	7.3	19.5	3.7	34.5	42.3

When chromatographic papers were employed that were previously washed with water, the formation of product X - in comparison with an unemployed paper - was decreased by 70%. Simultaneously it was found that product X was formed even in an aqueous solution of histidine, to which eluates of unemployed chromatographic paper were added.

The formation of product X increases with the area occupied by the histidine on the chromatographic paper and with the homogeneity of its spreading. When a ^{14}C-histidine sample was electrophoretically or chromatographically purified and stored directly on paper strip cut off from the electrophorogram or chromatogram (the area of spot about 40 μg /4 cm^2), the formation of product X was distinctly pronounced (Table 2).

The formation of by-products can be partly limited be keeping the paper with the spots of histidine in evacuated vials.

Table 2

Stability of [^{14}C(U)] -L-histidine preparation purified by
1.) paper electrophoresis in pyridine-acetate buffer pH 5.6
2.) paper chrpmatography in n-butanol - n-propanol - ammonia - water (7:5:7:2)
(The cut out spots of histidine (40 μg/4 cm^2) were stored in the open laboratory atmosphere; they were eluted at selected intervals and reanalysed electrophoretically. Expressed in % of radioactivity).

1.)			
Days	His	X	N
1	95.0	2.5	2.5
3	84.0	12.6	3.4
6	70.0	27.0	3.0
10	62.0	34.3	3.7
15	47.0	49.3	3.7
15 (under vacuum)	82.8	13.8	3.4
2.)			
6	50.0	45.5	4.5

All these results indicate that the formation of by-products depends on the presence of impurities in chromatographic carriers or in the laboratory atmosphere.

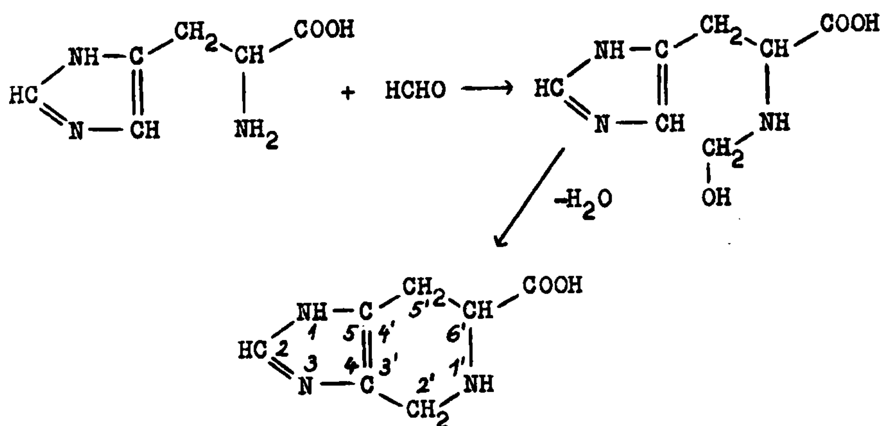
For identification purposes an aqueous solution of about 10 mg inactive L-histidine preparation (Calbiochem, a.g.) was applied over the whole surface of a Whatman 3 sheet; after 10 d standing the paper was eluted with water, the eluate concentrated by evaporation at elevated temperature and the product X was

separated from the unchanged histidine by paper electrophoresis. From the aqueous eluates of the paper strips containing the product X crystals (needles) were obtained after evaporation, that released water in two steps on drying in a vacuum (at 60-80°C, 100-150°C) and melted under decomposition at about 230°C. The substance obtained was optically active, $[\alpha]^{22}_D = 164°$ for anhydrous substance (c = 0.47 in water); its positive reaction with Pauly's reagent indicated the presence of imidazole nucleus, the negative reaction with ninhydrin indicated the absence of free amino group. An experiment with 1-^{14}C -DL-histidine proved that product X contained the radioactive carbon atom of the original carboxyl group. The R_F value of this product in a number of chromatographic systems (n-butanol - acetic acid - water, n-butanol - n - propanol - ammonia - water, phenol - ethanol - water) was the same or very close to that of histidine.

In the high-resolution mass spectrum the following main ionic species were recorded: m/e 167.0692 (38%; $C_7H_9N_3O_2$, + 0.3); 122.0721 (67%; $C_6H_8N_3$, - 0.3); 120.0560 (34%; $C_6H_6N_3$, - 0.2); 95.0606 (83%; $C_5H_7N_2$, + 0.3); 94.0530 (100%; $C_5H_6N_2$; + 0.1), further ions of m/e 58 (24%), 50 (20%) and 41 (38%). From the elemental composition of the ionized molecules and their ability to eliminate the carboxyl group it may be judged that compound X is a product of condensation of histidine with formaldehyde, and that it is the amino group and one of the hydrogens of imidazole heterocycle which undergo condensation. This hypothesis was confirmed and elaborated by comparison of the properties of product with the autenthic sample prepared synthetically, and by the study of its properties: when histidine was reacted with about a 10% excess of 7% formaldehyde solution (6 h, 50-60°C) the expected product was formed in almost theoretical yield. After triple crystallization from water, needle-like crystals were ob-

tained that had identical properties with product X.

Also ^{1}H-NMR spectra of the isolated and the synthetic sample were identical. They exhibited multiplets at δ 2.96, 3.73, and 4.02 ppm and a singlet at 8.29 ppm. However, they did not allow an unambiguous structure elucidation. The ^{13}C-NMR spectrum displayed signals of seven carbons (in D_2O, δ, off-resonance multiplicity): 17.5 t, 23.7 t, 41.7 d, 124.4 s, 125.5 s, 137.5 d, and 173.7 s. The assignment of the most downfield signal to the carboxyl carbon was straightforward. There were also present three sp^2-hybridised carbons (one carrying a hydrogen), two methylene and one methine group. A detailed examination of the off-resonance spectra indicated that the 23.7 signal was due to an isolated methylene, whereas the protons attached to the carbons at δ 17.6 and 41.7 formed a strongly coupled system $CHCH_2$[1]. The structure of the compound X fits these observed data well, and corresponds to the 1', 2', 5', 6'-tetrahydro- (pyridino-3', 4' : 4,5-imidazolo)-6'-carboxylic acid.



This compound is known as a natural substance, spinacine, occuring in the liver of some species of sharks[2] and its somewhat complex synthesis has also been described.[3]

The main source of formaldehyde necessary for the observed transformation of histidine during chromatography is probably not the chromatographic material itself. Samples applied on chromatographic paper and kept out of the laboratory atmosphere formed less condensation products, similarly as papers previously washed with water. Our results indicated that formaldehyde was present in the laboratory atmosphere in which it could also be detected by its conversion to 2,4-dinitrophenylhydrazone. From the atmosphere formaldehyde is adsorbed on chromatographic material. For example, 20 μg of formaldehyde could be eluted with water from one sheet of chromatographic paper Whatman 3 kept previously in laboratory atmosphere for 3 d. During the formation of product X with adsorbed formaldehyde the direct reaction of dissolved or moist histidine with formaldehyde from the air can also take place. It is necessary to mention that the chromatographies were carried out in laboratories where no work with formaldehyde had been done, and where it had not been even stored; formaldehyde was probably liberated into atmosphere from the plastics hardened with formaldehyde. It was also demonstrated that the condensation takes place intensively even when chromatograms were dried after their withdrawal from the chromatographic tanks, especially if the developing systems contained alcohols.

On thin layers of cellulose or silica gel the yield of condensation product X is lower than on paper, other conditions being identical; the decrease in the concentration of histidine on the cellulose layer is comparable to the paper, but on silica gel it is distinctly higher (Table 1). As was established by

parallel experiments with the chromatography of condensation product the reason for its low yield is the decomposition of both histidine and product X to the neutral fraction N taking place on silica gel and including also the formation of compounds having the character of week acids (Fraction A). Fractions N and A have not been investigated in this paper in greater detail. It was demonstrated chromatographically that the fraction N is a mixture of substances, among which a part corresponds to deamination products of histidine (imidazolyl-lactic and imidazolyl-acetic acids). In the acid fraction traces of aspartic acid could be detected.

This fact indicates that the storage of small amounts of histidine on thin layer of silica gel brings about a deep decomposition of histidine probably in consequence of oxidation reactions catalysed by admixture in chromatographic carriers, similarly as described in the case of pyrazolonic substances[4]. However, even on thin-layers the formation of condensation product and partly of other decomposition products too can be decreased by storage of chromatograms outside the laboratory atmosphere and under decreased temperature.

During chromatography of small amounts of ^{14}C-histidine or its storage on chromatographic carriers the following processes can take place: 1.) condensation of histidine with formaldehyde present in the laboratory enviroment or liberated from the residues of chromatographic solvents during drying of chromatograms; this reaction takes place mainly when histidine is stored and worked up on chromatographic paper.

2.) destruction of histidine in consequence of oxidative reactions, catalysed by admixture in chromatographic carriers. These reactions predominate especially when small amounts of histidine are purified or stored on thin layer of si-

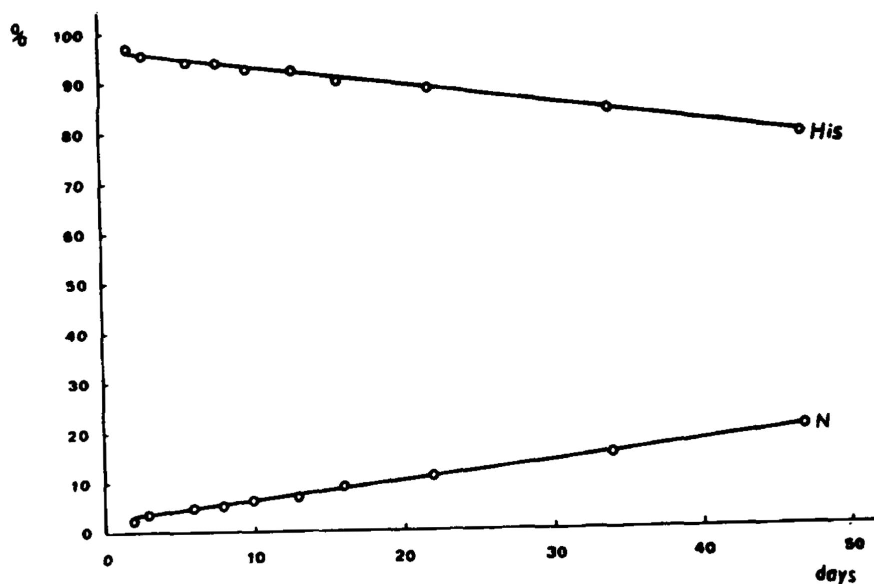


Fig.2 The stability of $[^{14}C(U)]$-L-histidine preparation stored in aqueous solution. (The sample of histidine after electrophoretic purification was eluted from the paper with water and stored in aqueous solution; at selected intervals, samples of this solution were analysed electrophoretically). No condensation product X was formed.

lica gel.

In order to restrict these reactions it may be recomended: for the preparation and purification of labelled substances of high specific activity to use chromatographic paper previously washed with water, and select suitable chromatographic systems. In the case of ^{14}C-histidine, paper electrophoresis at pH 5.6 was found to be the best method for separation and purification. Paper strips with the isolated substances should not be allowed to stand for long periods in the open laboratory atmosphere; elution should be carried out as soon as possible. The stability of ^{14}C-histidine samples obtained in this manner and stored in solutions (Fig.2) is substantially higher than the stability of labelled histidine stored freely on the paper. In cases when elution cannot be carried out immediately after separation it is recommended to store the paper strips with histidine spots in evacuated ampoules.

In view of the high reactivity of formaldehyde it may be assumed that similar reactions proved in the case of ^{14}C histidine may also take place during the chromatography of other labelled preparations with high specific radioactivity.

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