# THE FORMATION OF DOUBLE AND TRIPLE β-ALANINE STAINS DURING PAPER CHROMATOGRAPHY

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A series of recent investigations has shown that multiple stains may be formed by certain compounds during paper chromatography. These investigations show that great care is necessary when the method of chromatography is used for the identification of substances in biological materials of complex composition.

Multiple stain formation, which has been described for lysine, tryptophane, kynurenin and phenylalanine [1, 2, 4, 6], salicylic and sulfosalicylic acids [5], certain glucosides and antibiotics [3] and sulfuric acid, has not yet been explained. Various workers have put forward the hypothesis that these compounds may form complexes with the solvents, or that a hydrate membrane is formed around the ions, altering their mobility; no convincing experimental proof of these hypotheses has, however, been forthcoming. The most obvious case is that of duplication during chromatography of the optical isomers of certain amino acids, each possessing a different Rf.

## METHOD AND RESULTS

We demonstrated duplication of β-alanine stains during chromatography by means of the use of a preparation of this compound in which the carbon of the carboxyl group was labeled with $C^{14}$.

Trichloroacetic extracts of minced tissues (muscles, liver and kidneys), of which one was incubated at 37° with β-alanine-$C^{14}$ and the others were controls, were freed from trichloroacetic acid by repeated extraction with ether until a weakly acid reaction was given with congo red. The solutions were then evaporated to dryness on a water bath in porcelain dishes and the residue was dissolved in 0.5-1.0 ml of water. Chromatography was carried out in a mixture of butanol, acetic acid and water (5:1:1). After they had been dried, the amino acids gave the usual reaction with ninhydrin (0.2% solution in acetone) on heating. A parallel, undeveloped chromatogram strip was cut into sections, each of which was examined with an end-type counter. The distribution of radioactivity and of the colored stains was compared.

A characteristic picture is shown in Fig. 1. It is seen quite clearly that two or three stains have developed, sharply demarcated from each other, and radioactive. Radioactivity is distributed approximately uniformly between the two large stains; the third stain usually is smaller and less active. The same distribution pattern of stains was shown by both the incubated samples and the controls; the second stain could not, therefore, be a metabolic product of β-alanine. Subsequent experiments showed that two or three stains are also formed during chromatography of control solutions of β-alanine—$C^{14}$ in 2.5% trichloroacetic acid and in water, but only when large amounts (over 40 μg) of β-alanine were used. When smaller amounts of β-alanine—$C^{14}$ (5-20 μg) were applied to the paper, chromatography of pure solutions of the amino acid gave one stain at the level of the standard stain of unlabeled β-alanine. It must be pointed out that the preparation of β-alanine—$C^{14}$ which we synthesized was in the form of well-shaped crystals, with a melting point of 197-198°.

The relationship between duplication of the amino acid stains and concentration is shown in Fig. 2. In this case chromatography was carried out on a solution of β-alanine in a phosphate-saline mixture. Two stains were formed, differing slightly from the usual shape.

Since β-alanine has no optical isomers, it might at first be thought that the additional stains are the result of contaminants, found only with high concentrations of the amino acid. However, the substance responsible for the additional stain was not, in any case, a decarboxylation product of β-alanine (the radioactivity of the carboxyl group was preserved), or a deamination product (staining with ninhydrin). Furthermore, the chemical properties of the β-alanine—$C^{14}$ preparation described above gave no grounds for concluding that any contaminants were present. It was further shown that the "principal" (at the level of the standard solution in a low concentration) and the large "additional" stain could be reduced to a single stain by a change in the reaction of the solution applied to the chromatogram.

In Fig. 3 is shown the chromatogram of β-alanine—$C^{14}$ solutions of different pH. It can clearly be seen that, as the pH increases, the size of the "additional" stain decreases, and finally it disappears altogether, while at the

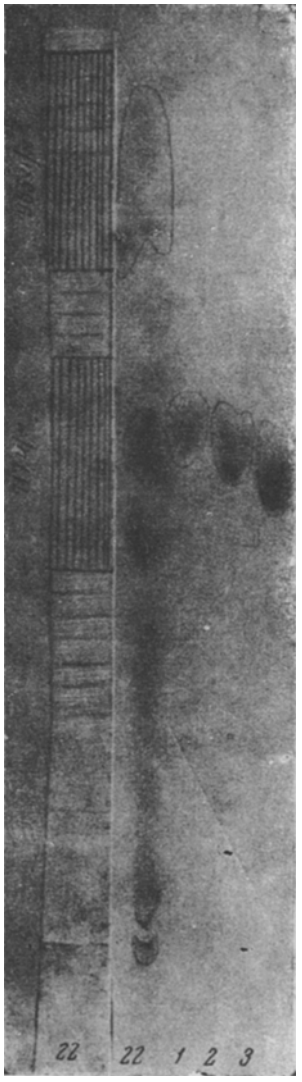


Fig. 1. Chromatogram of a trichloroacetic extract of rat muscle tissue containing β-alanine–$C^{14}$. 22) trichloroacetic extract + β-alanine–$C^{14}$; 1,2,3) standard solutions of β-alanine–$C^{14}$; on the left) chromatogram strip, corresponding to 22, examined with the counter.

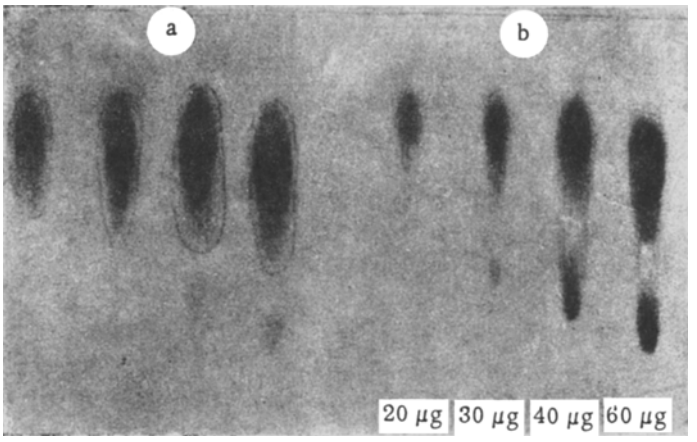


Fig. 2. Chromatogram (a) and the corresponding autoradiogram (b) on x-ray film (exposure 20 days). 20 μg, 30 μg, 40 μg, 60 μg) amounts of β-alanine–$C^{14}$ applied to the chromatogram.

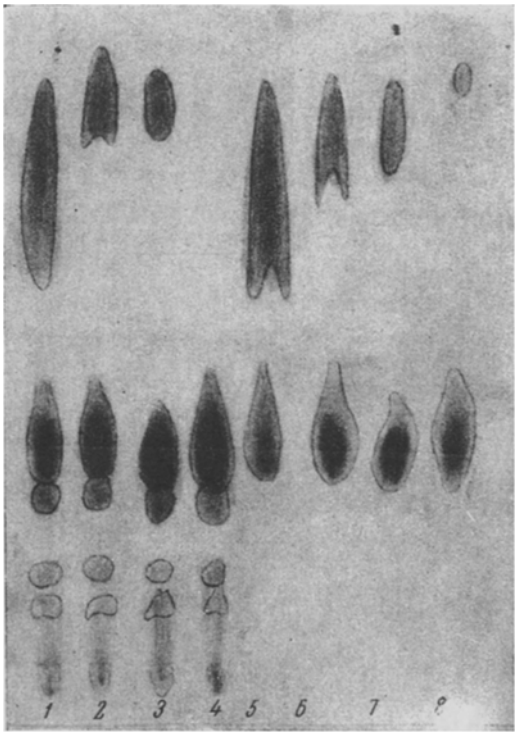


Fig. 3. Relationship between duplication of stains of β-alanine–$C^{14}$ and pH of the amino acid solution applied. 1-4) trichloroacetic filtrate of tissue + β-alanine–$C^{14}$; 5-8) trichloroacetic acid + β-alanine–$C^{14}$; 1,5) pH≈3; 2, 6) pH≈5; 3, 7) pH ≈7; 4, 8) pH≈11.

same time the intensity of the "principal" stain increases sharply. The change in pH was brought about by the addition of a solution of NaOH to the weakly acid β-alanine–$C^{14}$ solution.

We cannot yet give an explanation of the duplication of the β-alanine stain which we observed. We must point out, however, that the results of our research, in conjunction with reports in the literature, suggest that uncontrollable conditions may often arise in the process of chromatography, which may distort the results of the determination of the qualitative composition of the mixture undergoing analysis or of the quantitative content of its individual ingredients.

## SUMMARY

The formation of double and triple β-alanine–$C^{14}$ spots is seen during chromatography in butanol-acetic mixture; a relationship between this phenomenon and the pH of the β-alanine solution applied on the paper is also apparent. The author discusses the importance of this phenomenon for evaluation of the results of qualitative and quantitative investigations of the composition of complex biological mixtures.

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