The identical behaviour of some peptides and amino-acids in paper chromatography

Paper chromatography is generally recognised as a powerful tool for both qualitative and quantitative determinations of amino-acids. However, it has now been found that the technique, when used for the identification of free amino-acids in natural products, may give completely erroneous results unless certain precautions are observed. The difficulty arises from the fact that the behaviour of peptides in two-dimensional chromatography can be almost identical with that of known amino-acids. Consequently, in the absence of any further tests an incorrect assignment of structure is possible.

The discovery of this behaviour of peptides was made during an investigation into the composition of suint (the water-soluble material contained in raw wool). That the phenomenon may be more widespread is indicated by similar findings for human sweat.

Suint is a complex mixture of inorganic and organic substances which may be conveniently classified into four groups:

A. inorganic anions and cations; B. organic acids; C. organic ampholytes; D. neutral organic substances.

One and two-dimensional paper chromatograms of Fraction C, isolated by means of ion-exchange resins, and of crude suint were identical. Eleven amino-acids were tentatively identified, and confirmation was sought by making a mixture of the eleven compounds, adding it to Fraction C, and running the product in two dimensions. The results are recorded in Table I.

TABLE I

R_F VALUES IN TWO-DIMENSIONAL CHROMATOGRAMS

Standard amino-acid mixture	*Butanol-Acetic acid-Water, 4:1:5*			*Phenol-Water, 4:1 (0.1% ammonia)*		
	Standard	*Fraction C + Standard*	*Fraction C*	*Standard*	*Fraction C + Standard*	*Fraction C*
Aspartic acid	0.10	0.12	0.11	0.03	0.05	0.02
Glutamic acid	0.16	0.20	0.20	0.09	0.13	0.10
Asparagine	0.12	0.14	0.13	0.16	0.21	0.17
Serine	0.07	0.09	*	0.20	0.27	*
Glycine	0.13	0.15	0.15	0.25	0.31	0.26
Ornithine	0.18	0.21	0.20	0.30	0.35	0.32
Threonine	0.05	0.07	0.05	0.33	0.46	0.36
Alanine	0.25	0.27	0.27	0.45	0.50	0.47
Tyrosine	0.30	0.36	*	0.44	0.45	*
Arginine	0.09	0.11	0.09	0.56	0.69	0.64
Methionine	0.47	0.47	0.43	0.65	0.65	0.72

* Insufficient to give a ninhydrin reaction.

It will be seen that in both solvents there is a tendency for the known amino-acid to run slightly more slowly than the corresponding compound from Fraction C, while the mixture of known amino-acid + compound from Fraction C tends to run faster than either component alone. The mixture of amino-acids + Fraction C did not give any spots not given by Fraction C alone.

Although the chromatograms of suint and of suint after acidic hydrolysis were very similar, they were not identical. The differences might have been attributable to slight variations in the experimental conditions, but in view of certain other properties of suint solutions [for example, the variations of their surface tensions with pH, and also the fact that non-proteins can be precipitated from them with tungstic acid] the possibility that peptides were present could not be ignored. A more rigorous test was therefore carried out.

Crude suint (2.5 mg) and Fraction C (2.2 mg) were run with aqueous butanol-acetic acid on Whatman No. 3MM, thick paper. After development of representative strips with ninhydrin eleven spots appeared from each mixture. The undeveloped parts of the sheet were cut into sections corresponding to the visible components, and each section was extracted with warm (35° C) water. A portion of each extract, which corresponds to a single spot, was transferred to Whatman No. 1 paper; the remainder was hydrolysed for 24 h with 6 *N* hydrochloric acid at 110° C in a sealed tube. After removal of the water and acid under reduced pressure at room temperature, the residues were taken up in water, transferred to the Whatman No. 1 paper, run in butanol-acetic acid, and developed with ninhydrin. The result showed that every spot given

by crude suint and by Fraction C in the first separation was, in fact, a peptide containing between eight and thirteen amino-acids. A composite diagram of some of the results is given in Fig. 1.

Although the ninhydrin sensitivity of the peptides is relatively weak, they behave in a consistent manner and can easily be distinguished. Table II records the R_F values of the peptides of Fig. 1, together with the R_F values of the same spots from the previous run on thick paper.

TABLE II

R_F VALUES OF PEPTIDES IN AQUEOUS BUTANOL-ACETIC ACID

*Spot No.**	*Original R_F*	*Rerun R_F*
1	0.02	0.03 – 0.07
2	0.14	0.14
3	0.16	0.14
4	0.24	0.28
5	0.30	0.34
6	0.57	0.56
7	0.56	0.53 – (0.15)
8	0.10	0.11 – 0.22
9	0.39	0.32

* As in Fig. 1. Nos. 1–6 from crude suint; Nos. 7–9 from Fraction C. Nos. 6 and 7 are the same peptide. Values in parentheses are for very weak spots.

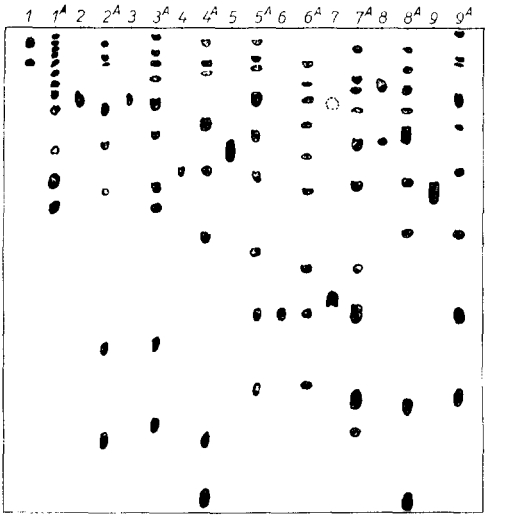

Fig. 1. Composite chromatogram of the individual peptides before and after hydrolysis: Nos. 1–6 from crude suint; Nos. 7–9 from Fraction C.

In order to ensure that these observations were not unique for suint, a second substance, human sweat, which has been reported to contain free amino-acids[1], has been examined in the same way. The study was made on a composite sample of sweat obtained by extracting cleaned cotton lint which had been used to wipe male torsos after strenuous exercise.

A one-dimensional run in butanol-acetic acid gave eight spots, each of which had an R_F value which corresponded to one of the amino-acids reported by ROTHMAN AND SULLIVAN[1]. After elution and hydrolysis of the compounds from the undeveloped parts of the sheet, followed by chromatography in butanol-acetic acid, each compound was found to consist of between six and nine amino-acids. In some instances the original, unhydrolysed material showed traces of contamination, but the presence of peptides was beyond all doubt.

The usual method[2] for obtaining free amino-acids from natural products is to extract them with 70–80% ethanol. They are then identified by paper chromatography. The free amino-acids in growing roots[3], marine algae[4,5], potato tissue[2], and amphibian embryos[6], have been identified in this manner.

Extraction of dried suint with 80% ethanol removes all the ninhydrin-sensitive material, and, as has been shown above, this consists largely of peptides. It is obvious, therefore, that an unequivocal identification is not obtained by this simple method. In the reports referred to above, a transition from amino-acid to protein is taking place so that the possibility that peptides are present cannot be ignored. *Proof* of the identification of free amino-acids requires at least one more test. The minimum requirement is that the chromatograms before and after hydrolysis should be identical; preferably, the single components should be hydrolysed and examined separately.

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