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# THE UTILISATION OF 1-DIMETHYLAMINONAPHTHALENE-5-SULPHONYL CHLORIDE FOR QUANTITATIVE DETERMINATION OF FREE AMINO ACIDS AND PARTIAL ANALYSIS OF PRIMARY STRUCTURE OF PROTEINS*

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SUMMARY

A dansylation method has been developed for the rapid partial determination of primary protein structures at the nanomole level. Modifications of published methods for the purification and chromatography of dansyl-amino acids and dansyl-peptides were necessary for quantitative analysis. The amino acid composition and N-terminal amino acids of proteins can be determined by this method. After fingerprinting on a preparative scale, the amino acid composition and N-terminal amino acids of each dans-peptide can also be characterised.

## INTRODUCTION

1-Dimethylaminonaphthalene-5-sulphonyl chloride (dans-Cl) covalently binds to free amino, phenol, imidazole and sulphhydryl groups[1]. Dansyl derivatives of amino acids (dans-AA) can be easily produced for all the amino acids. Since these derivatives are intensely fluorescent and are highly resistant to acid and alkaline hydrolysis, GRAY AND HARTLEY[2-4] have used them for the identification of the N-terminal amino acids of proteins. Other authors have used dansylation for revealing low quantities of amino acids and peptides separated by thin-layer chromatography[5-17]; but all these methods are qualitative, except for that of GROS AND LABOUESSE[14] in which the N-terminal amino acids are determined quantitatively.

After a preliminary study which established the optimal conditions for the dansylation of amino acids, and for the purification, chromatography and measurement of the fluorescence of dans-AA, we set up a quantitative method for the partial structural analysis of proteins on a microscale. The method has been used to determine

* Abbreviations used: dansyl or dans: 1-dimethylaminonaphthalene-5-sulphonyl. dans-Cl: 1-dimethylaminonaphthalene-5-sulphonyl chloride. dans-OH: 1-dimethylaminonaphthalene-5-sulphonic acid. dans-$NH_2$: 1-dimethylaminonaphthalene-5-sulphonamide. dans-AA: 1-dimethylaminonaphthalene-5-sulphonyl amino acid.

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the amino acid composition and N-terminal amino acids of a given protein and of the dans-peptides obtained from a peptide map after enzymatic hydrolysis of the protein on a preparative scale.

The method is rapid and inexpensive and requires only $\mu$g quantities of protein.

## MATERIALS

### *Reagents*

Dans-Cl (98 %), dimethylformamide (for UV spectroscopy) and amino acids (Chr., puriss.) were obtained from Fluka (Buchs, Switzerland), and dans-AA (A grade) from Calbiochem (Lucerne, Switzerland). Bovine serum albumin (Fraction V powder) and bovine pancreatic ribonuclease A (Type I A) were from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Trypsin (3 × crystallised) was from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.). Other chemical reagents were Analytical grade.

### *Chromatographic materials*

Silica gel (Kieselgel G nach Stahl) was from Merck A.G. (Darmstadt, G.F.R.) and polyamide sheets were purchased from Chang Chin Trading Co. Ltd., (Taipei, Taiwan, Republic of China). Silica gel plates were coated by means of a Camag (Muttens, Switzerland) apparatus to a thickness of 0.3 mm.

### *Apparatus*

The apparatus used was the following: a Cary 14 recording spectrophotometer (quartz cuvettes 1 cm path length); a Zeiss spectrofluorometer with two monochromators (Ref. PM QII + ZFM 4), (cuvettes Hellma QS, volume 1.5 ml).

## METHODS

### *Dansylation of amino acids and purification of dans-AA*

To avoid loss of fluorescence of dansylated proteins, peptides or amino acids, all operations from the dansylation to the last spectrofluorometric step, were carried out rapidly and as much as possible in the dark. Amino acid samples (between 1 and 50 nmole of each amino acid) were dissolved in 300 $\mu$l of 0.2 *M* sodium phosphate buffer (pH 8.85), and an equal volume of freshly prepared dans-Cl in acetone (10 mg/ml) was added, the tubes were tightly stoppered, shaken and left to stand at room temperature. After 120 min the acetone was evaporated under a light stream of air, then 2 ml of 0.1 *M* ammonium formate buffer (pH 3.5) was added to precipitate the excess dans-Cl. The precipitates were separated by low speed centrifugation, the supernatants decanted, and the pellet washed twice with 1 ml of buffer. The pooled supernatants were lyophilised and the dans-AA's were desalted by three successive extractions of the dans-AA's from the residue into 500 $\mu$l of acetone–1 *M* HCl (19:1). The pooled supernatants were rapidly dried in a stream of cold air while the tube was kept at 50°. The residues were taken up in 100 $\mu$l of acetone–1 *M* HCl (19:1) for chromatography.

### *Dansylation of peptides and purification of dans-peptides*

The peptide mixture (between 1 and 20 nmole of each peptide) was dansylated

as described for the amino acids. The excess dans-Cl was converted to dans-OH by the addition of a few drops of 3 *M* KOH until the orange colour, typical of dans-Cl, had disappeared. The pH was then adjusted to approximately 3.5, by addition of glacial acetic acid.

Salts and dans-OH were then eliminated according to SCHMER AND KREIL[15] by adsorption onto columns of Dowex 50 X4 (2.0 × 0.5 cm) equilibrated with 0.01 *M* acetic acid. The blue-green fluorescence, typical of dans-OH, was washed from the column with 0.01 *M* acetic acid, then the dans-peptides were eluted with water–acetone–25 % ammonia (80:20:4) until all yellow fluorescence had disappeared from the column. This eluate was lyophilised and the residue dissolved in a small volume of acetone–water (1:1) for chromatography.

*Dansylation and hydrolysis of proteins for N-terminal amino acid identification*

From among several published methods for the determination by dansylation of N-terminal amino acids[2,13,14] we have chosen the technique of GROS AND LABOUESSE[14] with certain technical modifications, important for the quantitative estimations.

The protein (between 5 and 10 nmole) was dissolved in 200 $\mu$l of water. Then 250 mg urea, 150 $\mu$l of 0.2 *M* sodium phosphate buffer (pH 8.85), 250 $\mu$l of dimethyl formamide and 100 $\mu$l of dans-Cl in acetone (10 mg/ml) were added successively. The tubes were stoppered and left at room temperature. After 120 min, 5 ml of 10 % TCA was added, the tube was shaken and centrifuged (1000 g for 10 min). The precipitated dans-protein was washed twice with 1 ml of 1 *M* HCl, then twice with 1 ml of acetone.

Hydrolysis of the dansylated protein was performed with 250 $\mu$l of 6 *M* HCl at 115° in a sealed tube for 4 h. After the HCl had been removed *in vacuo*, the dry residue was taken up quantitatively in acetone–1 *M* HCl (19:1) for chromatography.

*Bidimensional thin-layer silica gel chromatography of dans-AA*

A homogeneous suspension of 54 g of silica gel in 125 ml of water was spread as a 300 $\mu$ layer over 20 × 20 cm glass plates. After standing until the plates became opaque, they were placed in an oven at 110° for at least 30 min, then kept at room temperature. The sample of dansylated amino acids was applied dropwise under a stream of warm air so that the spot diameter was kept less than 5 mm. Before development the plates were placed in an oven at 110° for 5 min, followed by 5 min at room temperature.

Chromatograms were developed in the first dimension with toluene–pyridine–acetic acid (150:50:3.5)[13] until the solvent front reached the edge of the plate (about 45 min). The plates were then dried under a stream of warm air until opaque, then at 110° for 5 min. The plates were then examined under UV light (365 m$\mu$) to locate slightly fluorescent impurities eluted from the silica gel which migrated as a large band close to the solvent front. A horizontal groove was scratched on the silica gel to separate these impurities which interfere with the migration in the second solvent and the subsequent measurement of fluorescence of dans-leu, dans-ile, dans-val and dans-pro. Residual pyridine was eliminated by exposing the plates to an atmosphere of ammonia for 1 min. The chromatogram was then developed in toluene–chloro-2-ethanol–25 % ammonia (100:80:6.7) until the solvent front was 4 cm from the edge of the plate (about 45 min). The plates were then dried rapidly in warm air. The spots were marked under UV light and immediately eluted.

*One-dimensional chromatography of dans-AA on polyamide sheets*

Sheets of polyamide (5 × 5 cm) were divided by pencil marks, into a maximum of 10 lanes. To obtain acceptable resolutions each completely desalted sample must be applied as a spot less than 1 mm in diameter. The solvents used[8] were either benzene–acetic acid (9:1) (development 6 min) or formic acid–water (1.5:100) (development 4 min).

*Bidimensional silica gel thin-layer chromatography of dans-peptides*

The technique was identical to that used for the separation of dans-AA except for the solvents. After 45 min development in methyl acetate–isopropanol–25 % ammonia (9:6:4), the plates were dried in warm air, then placed in an oven at 110° for 5 min, then under a hood at room temperature for 10 min. It was necessary to observe these conditions strictly before proceeding to the second dimension, namely isobutanol–acetic acid–water (15:4:2) for 120 min.

*Elution of dans-AA and dans-peptides and measurement of fluorescence*

The spots of dans-AA were eluted from the silica gel by means of the technique of GROS with chloroform–methanol–acetic acid (7:2:2). Dans-peptide spots were eluted in the same manner with acetone–water (1:1). The completeness of elution was controlled by UV examination.

Five volumes of absolute ethanol were added to the eluate, and the fluorescence of this mixture was read at 340/510 mμ. A known quantity of a control dans-AA (dans-glu) was chromatographed and eluted in parallel to the sample. An area of silica gel equal to the spots was eluted to serve as a silica gel blank.

If the fluorescence measured for the standard is $F^0$ (the apparatus was adjusted so that $F^0 = 100$) the silica gel blank is $F_b$ and that of the sample $F_s$, then:

$$F_s^0 = F_s - F_b \qquad (1)$$

where $F_s^0$ is the "real" fluorescence of the sample. This value is then corrected for the fluorescence yield of each dans-AA, and for losses during separation, by the application of a "recovery factor relative to dans-glu" ($\alpha_s$). The determination of this factor is discussed later. This correction allows calculation of the fluorescence equivalent of the sample $F_s'$:

$$F_s' = \alpha_s \times F_s^0 \qquad (2)$$

The percentage of each amino acid in a mixture can be calculated from the formula:

$$\text{amino acid } \% = \frac{F_s'}{\Sigma F_s'} \times 100 \qquad (3)$$

The absolute quantity of each amino acid in nmole ($n_s$) is given by the equation:

$$n_s = n \times \frac{F_s'}{F^0 - F_b} \qquad (4)$$

$n$ being the number of nmole of standard dans-glu used.

*Performic oxidation of proteins*

This process was carried out according to the technique of HIRS[18]. It was necessary to observe the conditions specified strictly, in particular with regard to the temperature and the use of fresh hydrogen peroxide in order to prevent the rupture of certain peptide bonds.

## RESULTS AND DISCUSSION

*Determination of the composition of a mixture of amino acids by dansylation*

The conditions for dansylation of amino acids reported in the literature[2–13] are extremely variable. GROS AND LABOUESSE[14] have studied this problem recently and we have confirmed that, in general, their conditions are satisfactory. However, we preferred to use a longer reaction time of 120 min, instead of 30 min because a kinetic study showed that the dicarboxylic amino acids, which react more slowly, were not completely dansylated within 30 min (Fig. 1). Complete dansylation of these amino acids occurs in 120 min without significant formation of dans-OH which interferes with later chromatographic steps. Significant formation of dans-OH occurs with the longer reaction times suggested by certain authors[2–4].

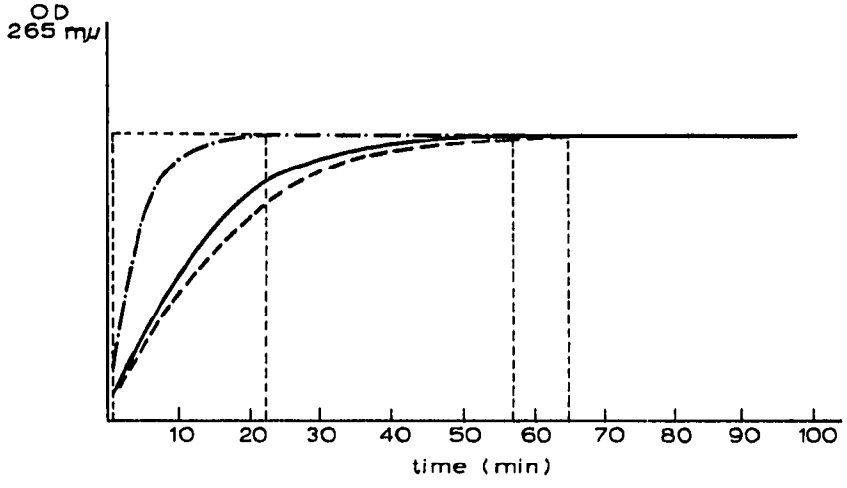


Fig. 1. Kinetics of the dansylation of some amino acids (———, glu; – – – –, asp; –·–·–, gly). 100 nmole of each amino acid in 300 μl of 0.2 *M* sodium phosphate buffer (pH 8.85) was mixed with 300 μl of dans-Cl in acetone (10 mg/ml) in a quartz cuvette. The absorption was followed at 265 mμ with a recording spectrophotometer against a blank containing the same mixture without amino acid.

The reaction medium must be kept as free as possible from salts other than disodium phosphate. The excess of dans-Cl (approximately 50 moles per mole of amino acid), the optimal amount for dansylation, strongly interferes with the chromatographic separation and therefore must be removed before chromatography. All other authors have suggested hydrolysis of the excess dans-Cl by alkali or strong acid[9,13,14], but both methods produce large amounts of dans-OH which then must be removed by chromatography on a Dowex 50 column[15]. This procedure is time-consuming, difficult to apply to a large number of samples simultaneously and causes a significant loss of fluorescence as a result of the UV irradiation necessary to follow the elution. An alternative technique[9,14] is to extract the dans-AA's into ether. However, dans-arg,

dans-his, ε-dans-lys, dans-$CySO_3H$ etc. remain in the aqueous phase with the dans-OH. The distribution of the dans-AA into two phases necessitates two chromatographic steps. Moreover, the dans-OH in the aqueous phase interferes with the chromatography.

We found that 0.1 *M* ammonium formate buffer (pH 3.5) will completely precipitate excess dans-Cl and this simple method can be used on several samples simultaneously. The formate precipitation produces very little dans-OH, provided that the operations are carried out rapidly. Desalting was then performed by extracting the dans-AA's from the residue, after lyophilisation, into acetone–1 *M* HCl (19:1). Under the conditions that we have described (1–50 nmole of each amino acid), all the dans-AA's were completely extracted except for dans-trp. An additional wash, necessary for complete extraction when dans-AA's were present in quantities larger than 50 nmole, will also quantitatively extract dans-trp (if present in quantities less than 20 nmole). Additional washes were required for larger amounts of dans-trp. The procedure described gives an over-all yield of amino acids, recovered as dans-AA's, of 92 ± 2 % in a salt-free solution which contains very little dans-OH and no dans-Cl. The dans-AA's are now ready for quantitative chromatography on a single plate.

Bidimensional silica gel TLC of a standard mixture of amino acids treated by our procedure (Fig. 2) indicated that, with the solvents used, all dans-AA spots were

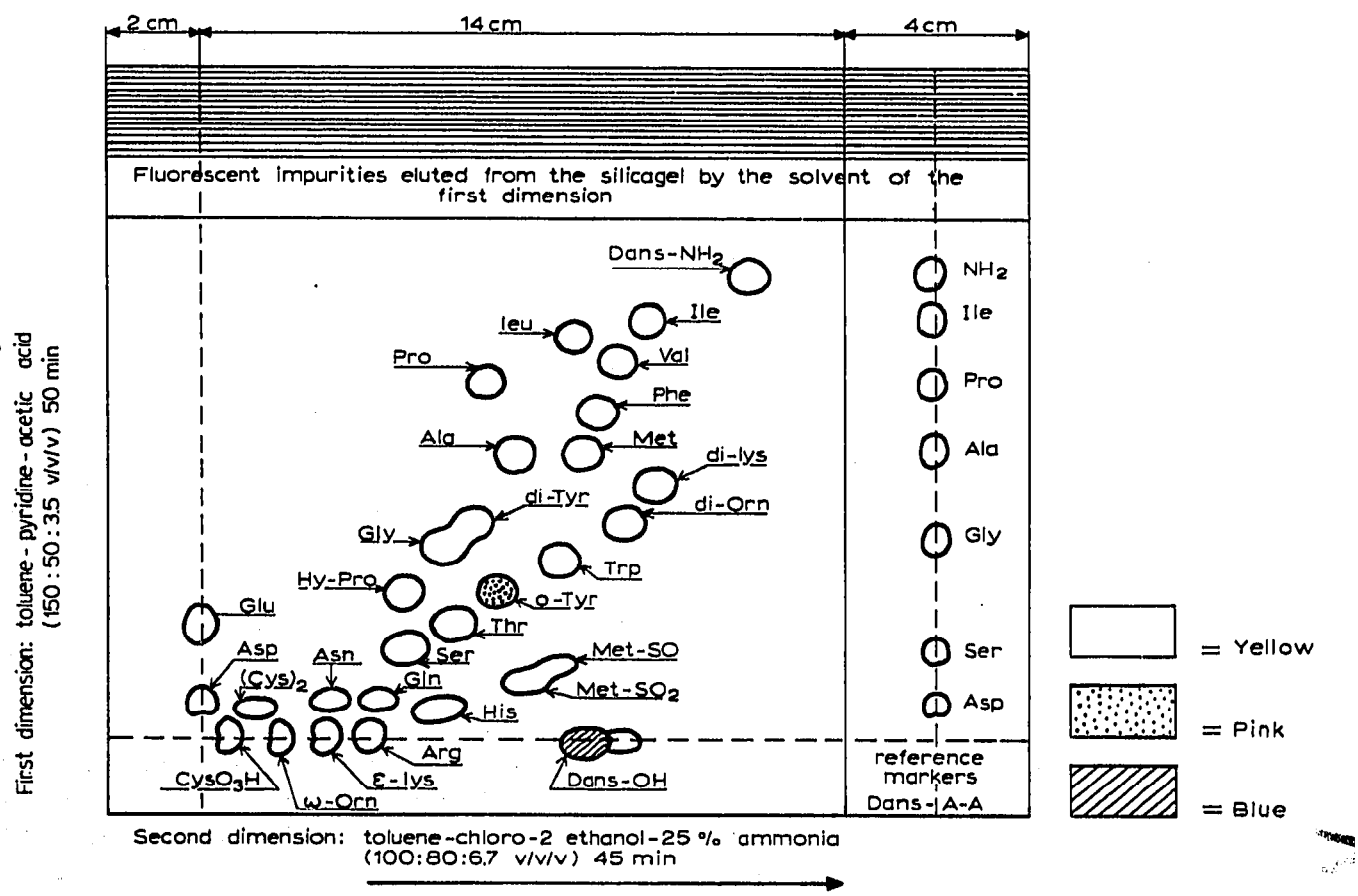


Fig. 2. Tracing of a two-dimensional separation of dans-AA's on a silica gel thin-layer plate. 5 nmoles of each dans-AA were applied.

well defined, with practically no trailing and, except for dans-gly and di-dans-tyr, completely separated.

The method reported has overcome the problem found by other[5–14] of overlapping of the dansyl derivatives of the branched chain amino acids with dans-pro, of dans-phe with dans-met, and among the dansyl derivatives of the basic amino acids.

TABLE I

$R_{DANS.NH_2}$ VALUES × 100 OF DANS-AMINO ACIDS, DANS-$NH_2$ AND DANS-OH ON SILICA GEL THIN LAYERS

Values are the mean ± S.D. of 20 determinations. Solvent I: toluene–pyridine–acetic acid (150:50:3.5); solvent II: toluene–chloro-2-ethanol–25% ammonia (100:80:6.7).

| *Dans-AA* | *Solvent I* | *Solvent II* | *Dans-AA* | *Solvent I* | *Solvent II* |
|---|---|---|---|---|---|
| Dans-$NH_2$ | 100.0 | 100.0 ± 1 | Dans-thr | 25.0 ± 0.5 | 45.0 ± 1 |
| Dans-ile | 90.5 ± 1 | 83.5 ± 1 | Dans-glu | 24.0 ± 0.5 | 0.5 ± 0.1 |
| Dans-leu | 87.5 ± 1 | 67.5 ± 1 | Dans-ser | 19.0 ± 0.5 | 36.0 ± 0.5 |
| Dans-val | 82.5 ± 1 | 75.0 ± 1 | Dans-met-SO | 15.0 ± 0.5 | 58.0 ± 1 |
| Dans-pro | 78.5 ± 1 | 51.5 ± 1 | Dans-met-$SO_2$ | 14.0 ± 0.5 | 57.0 ± 1 |
| Dans-phe | 73.0 ± 1 | 71.0 ± 1 | Dans-asp | 8.0 ± 0.5 | 0 |
| Dans-met | 66.0 ± 1 | 69.0 ± 1 | Dans-asn | 8.0 ± 0.5 | 20.0 ± 0.5 |
| Dans-ala | 62.0 ± 1 | 56.0 ± 1 | Dans-gln | 8.0 ± 0.5 | 29.0 ± 0.5 |
| Di-dans-lys | 55.0 ± 1 | 82.0 ± 1 | Di-dans-$(Cys)_2$ | 5.0 ± 0.5 | 9.0 ± 0.2 |
| Di-dans-orn | 49.0 ± 1 | 79.0 ± 1 | Di-dans-His | 4.0 ± 0.5 | 42.0 ± 1 |
| Di-dans-tyr | 47.0 ± 1 | 49.0 ± 1 | Dans-arg | 0 | 29.0 ± 0.5 |
| Dans-gly | 44.0 ± 1 | 46.0 ± 1 | ε-Dans-lys | 0 | 20.0 ± 0.5 |
| Dans-trp | 41.0 ± 0.5 | 64.5 ± 1 | Dans-orn | 0 | 14.0 ± 0.5 |
| O-dans-tyr | 30.0 ± 0.5 | 50.0 ± 1 | Dans-$CySO_3H$ | 0 | 4.0 ± 0.2 |
| Dans-hyp | 30.0 ± 0.5 | 36.0 ± 0.5 | Dans-OH | 0 | 67.0 ± 1 |

The method is reproducible (without any requirement for further purification of "Analytical grade" solvents), as shown by the migration characteristics of each dans-AA in the two solvent systems used (Table I). All amino acids can be unambiguously identified. Chromatography on polyamide (Fig. 3) is useful as a confirmation of the nature of certain dans-AA's eluted from the silica gel chromatogram. It is particularly useful for the unambiguous identification of dans-gly and di-dans-tyr, since their migrations on polyamide are quite different. Dans-AA's (1–50 nmole) are clearly separated on a single silica gel plate and can be quantitated reliably down to 0.5 nmole.

Ready-made commercially available silica gel thin-layer plates are not suitable for quantitative analysis since the material which strongly binds the silica powder to the support plates interferes with the fluorescence measurements, makes the recovery of dans-AA spots very difficult and limits the scale of operation to less than 10 nmoles of a given amino acid.

To demonstrate the reproducibility and the linearity of the method, four mixtures of amino acids were taken (Table II), mixture I contained equimolar amounts of each amino acid; in mixture II the concentrations of the amino acids were from 1–5 times greater and in mixture III from 1–10 times greater. Mixture IV contained amino acids in the proportions found in oxidised bovine RNAase[19]. Different quantities of each mixture were dansylated and purified and analysed by chromatography

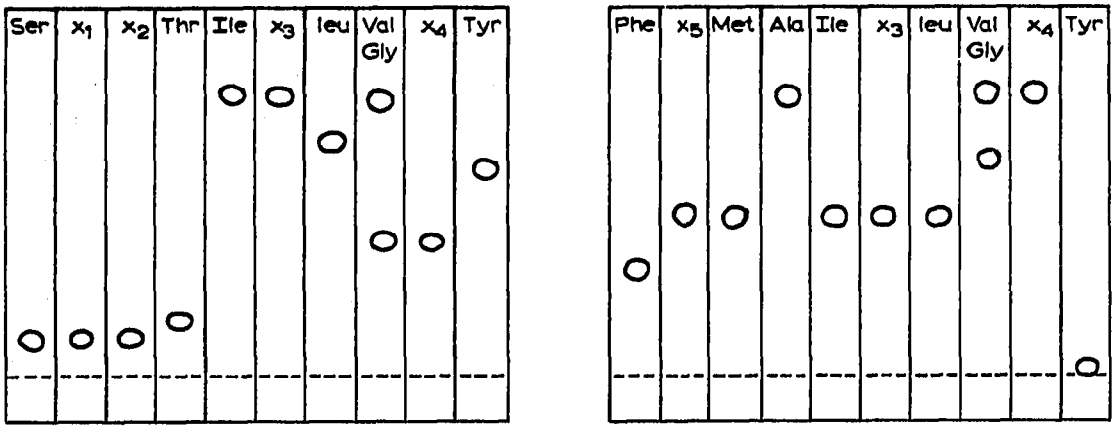


Fig. 3. Tracings of one-dimensional separation of dans-AA's on polyamide sheets. 0.01 nmole of each standard dans-AA was applied. The spots $x_{1-5}$ were dans-AA's eluted from a silica gel thin-layer chromatogram to confirm their identity. A = benzene–acetic acid (9:1); B = formic acid–water (1.5:100).

TABLE II

COMPOSITION OF THE STANDARD MIXTURES OF AMINO ACIDS USED (nmole/100 $\mu$l)

The amino acids were dissolved in 0.2 *M* sodium phosphate buffer (pH 8.85).

| *Amino acid* | *I* | *II* | *III* | *IV* |
|---|---|---|---|---|
| Ile | 1 | 1 | 10 | 3 |
| Leu | 1 | 5 | 2 | 2 |
| Val | 1 | 2 | 10 | 9 |
| Pro | 1 | 2 | 5 | 4 |
| Phe | 1 | 2 | 2 | 3 |
| Met | 1 | 5 | 10 | 0 |
| Ala | 1 | 5 | 10 | 12 |
| Lys | 1 | 5 | 10 | 10 |
| Orn | 1 | 0 | 0 | 0 |
| Tyr | 1 | 0 | 10 | 6 |
| Gly | 1 | 5 | 0 | 3 |
| Thr | 1 | 2 | 10 | 10 |
| Ser | 1 | 5 | 5 | 15 |
| Glu | 1 | 5 | 10 | 12 |
| Met-$SO_2$ | 1 | 2 | 0 | 4 |
| Asp | 1 | 5 | 10 | 15 |
| Asn | 0 | 2 | 0 | 0 |
| Gln | 0 | 1 | 0 | 0 |
| $(Cys)_2$[a] | 1 | 2 | 2 | 0 |
| His | 1 | 5 | 10 | 4 |
| Arg | 1 | 5 | 10 | 4 |
| $CySO_3H$ | 1 | 5 | 0 | 8 |

[a] Expressed in nmole of cysteine/100 $\mu$l.

(Table III). It is clear that the method is satisfactory both with respect to the reproducibility and the linearity. However, dans-met was sometimes partially degraded during spotting, and also during migration, to dans-methionine sulphoxide and methionine sulphone. In practice, it is convenient to oxidise all the methionine to methionine sulphone before dansylation.

TABLE III

REPRODUCIBILITY AND LINEARITY OF THE METHOD FOR THE DETERMINATION OF THE COMPOSITION OF AMINO ACID MIXTURES

The fluorescence values ($F_s^0$, see eqn. 1) were expressed for 10 nmole of dans-AA. I, II, III and IV denote the standard amino acid mixtures whose composition is given in Table II.

| *Dans-AA* | *IA* | *IB1* | *IB2* | *IIA* | *IIB* | *IIIA* | *IIIB* | *IVA* | *IVB* |
|---|---|---|---|---|---|---|---|---|---|
| Dans-ile | 132 | 121 | 116 | 132 | 114 | 127 | 132 | 121 | 132 |
| Dans-leu | 150 | 150 | 140 | 150 | 140 | 140 | 140 | 140 | 140 |
| Dans-val | 170 | 162 | 173 | 170 | 165 | 172 | 170 | 165 | 161 |
| Dans-pro | 387 | 320 | 355 | 387 | 370 | 346 | 354 | 354 | 346 |
| Dans-phe | 183 | 180 | 168 | 173 | 168 | 175 | 168 | 178 | 171 |
| Dans-met | traces | traces | 295 | 275 | 245 | 255 | 325 | 0 | 0 |
| Dans-ala | 259 | 246 | 259 | 263 | 254 | 259 | 263 | 250 | 269 |
| Di-dans-lys | 300 | 272 | 286 | 272 | 300 | 272 | 293 | 293 | 286 |
| Di-dans-orn | 288 | 295 | 292 | 0 | 0 | 0 | 0 | 0 | 0 |
| Di-dans-tyr | 840 | 780 | 790 | | | 505 | 462 | 810 | 825 |
| Dans-gly | | | | 302 | 318 | | | | |
| Dans-thr | 252 | 252 | 257 | 243 | 245 | 257 | 252 | 252 | 257 |
| Dans-ser | 288 | 288 | 288 | 288 | 288 | 288 | 288 | 288 | 288 |
| Dans-glu | 168 | 178 | 187 | 183 | 178 | 183 | 183 | 178 | 180 |
| Dans-met-$SO_2$ | 236 | 193 | 220 | 325 | 330 | traces | traces | 236 | 225 |
| Dans-asp | 204 | 204 | 223 | 207 | 203 | 204 | 205 | 203 | 207 |
| Dans-asn | 0 | 0 | 0 | 190 | 195 | 0 | 0 | 0 | 0 |
| Dans-gln | 0 | 0 | 0 | 195 | 195 | 0 | 0 | 0 | 0 |
| Di-dans$(Cys)_2$ | 295 | 272 | 295 | 295 | 300 | 276 | 295 | 0 | 0 |
| Dans-his | 420 | 378 | 397 | 403 | 407 | 427 | 412 | 412 | 425 |
| Dans-arg | 280 | 276 | 293 | 290 | 280 | 276 | 276 | 287 | 280 |
| Dans-$CySO_3H$ | 276 | 287 | 300 | 285 | 295 | 0 | 0 | 280 | 275 |

| *Sample* | *Volume dansylated (ml)* | *Aliquot chromatographed* |
|---|---|---|
| Ia | 0.5 | 100 % |
| Ib | 2 | 25 % |
| Ic | 2 | 75 % |
| IIa | 1 | 50 % |
| IIb | 0.5 | 50 % |
| IIIa | 0.5 | 100 % |
| IIIb | 1 | 20 % |
| IVa | 0.5 | 100 % |
| IVb | 1 | 25 % |

To determine the quantities of Gly and Tyr, when both were present in a mixture, the incompletely separated spots were eluted together after silica gel chromatography and the total fluorescence measured. After evaporation, the sample was rechromatographed (with two migrations in each dimension). Dans-gly and di-dans-tyr, now clearly separated, were eluted and the relative amounts determined (see eqn. 3). Then the absolute quantity of each amino acid was calculated from the total fluorescence value after the first chromatography.

To prevent the rapid loss of fluorescence (50 % in 30 min) which occurs when the dans-AA's remain on dry thin layers, the elution of dans-AA's must be done rapidly. It is possible to elute twenty dans-AA's before the plaque is dry (about 3 min).

TABLE IV

RECOVERY FACTOR ($\alpha_s$) RELATIVE TO DANS-GLU OF VARIOUS DANS-AMINO ACIDS

Fluorescence values ($F_s^0$, see eqn. 1) expressed for 10 nmoles of dans-AA, are the means ± S.D. of 9 determinations.

| *Dans-AA* | *Fluorescence value* | $\alpha_s$ | *Dans-AA* | *Fluorescence value* | $\alpha_s$ |
|---|---|---|---|---|---|
| Dans-ile | 125.2 ± 7.3 | 1.44 ± 0.08 | Dans-thr | 251.9 ± 4.0 | 0.71 ± 0.0 |
| Dans-leu | 143.3 ± 4.5 | 1.26 ± 0.04 | Dans-ser | 288.0 ± 0.5 | 0.63 ± 0.0 |
| Dans-val | 167.6 ± 1.5 | 1.08 ± 0.01 | Dans-glu | 179.8 ± 4.0 | 1.00 ± 0.0 |
| Dans-pro | 357.7 ± 15.4 | 0.50 ± 0.02 | Dans-met-$SO_2$ | 230.0 ± 16.0 | 0.78 ± 0.0 |
| Dans-phe | 173.8 ± 4.5 | 1.03 ± 0.03 | Dans-asp | 206.6 ± 8.0 | 0.87 ± 0.0 |
| Dans-met | 290.0 ± 35.0 | 0.64 ± 0.08 | Dans-asn | 192.5 ± 2.5 | 0.94 ± 0.0 |
| Dans-ala | 258.0 ± 6.6 | 0.70 ± 0.02 | Dans-gln | 195.0 ± 0.5 | 0.92 ± 0.0 |
| Di-dans-lys | 286.0 ± 11.0 | 0.63 ± 0.03 | Di-dans$(Cys)_2$ | 289.7 ± 10.5 | 0.62 ± 0.0 |
| Di-dans-orn | 291.7 ± 3.5 | 0.62 ± 0.01 | Dans-his | 409.0 ± 14.4 | 0.44 ± 0.0 |
| Di-dans-tyr | 483.5 ± 21.5 | 0.37 ± 0.02 | Dans-arg | 282.0 ± 6.0 | 0.64 ± 0.0 |
| Dans-gly | 310.0 ± 8.0 | 0.58 ± 0.02 | Dans-$CySO_3H$ | 285.4 ± 9.6 | 0.63 ± 0.0 |

The dans-AA's in the elution mixture are stable in the dark and do not lose fluorescence after several hours.

Two factors must be taken into account when calculating the quantities of dans-AA's in these experiments; the different molar fluorescence of the dans-AA's[14] and fluorescence losses during chromatography. The "recovery coefficients" relative to dans-glu ($\alpha_s$) of the amino acids, determined under our experimental conditions, are given in Table IV.

*Application of the method to the determination of the amino acid composition of a protein*

Two proteins of known composition (bovine serum albumin, bovine pancreatic RNAase) were chosen to illustrate the validity of the method. Five nmole of native or performic acid oxidised proteins were hydrolysed in sealed tubes for 24 h at 115° with 6 *M* HCl. The HCl was removed *in vacuo* and the dry residue treated as described for a mixture of free amino acids. The results are given in Table V and are compared to literature data obtained using the classical method of MOORE AND STEIN.

This method, without the use of an expensive instrument, is of a precision comparable to that of the classical method, with the advantages that it is possible to carry out several determinations in parallel, quickly, and requires 50–100 times less protein. On the other hand, contaminants reacting with dans-Cl, even in trace amounts, can be a significant source of error at this level of sensitivity; for this reason particular care must be taken in handling of the sample.

*Determination of N-terminal amino acids by dansylation*

The method of GROS AND LABOUESSE[14] was modified as follows: the time of dansylation was extended to 120 min, as indicated by the results for serum albumin (Table VI) and the excess dans-Cl was eliminated by washing the precipitate of dansylated protein with dilute HCl and with acetone. Therefore no dans-OH was formed during protein hydrolysis. This procedure allows direct chromatography of the dans-AA's, without the need for additional purification as previously described in the literature[9,14].

TABLE V

APPLICATION OF DANSYLATION TO THE DETERMINATION OF THE AMINO ACID COMPOSITION OF PROTEIN

These results are expressed in terms of the percentage of each amino acid and are the means of three analyses on 5 nmoles of protein.

| *Amino acid* | *Bovine ribonuclease* | | | *Bovine serum albumin* | | |
|---|---|---|---|---|---|---|
| | *Native* | *Oxidised* | *Literature data*[d] | *Native* | *Oxidised* | *Literature data*[e] |
| Ala | 10.20 | 10.09 | 9.68 | 9.30 | 8.50 | 8.20 |
| Arg | 2.94 | 2.98 | 3.23 | 3.62 | 3.54 | 3.95 |
| Asp | 11.45 | 11.55 | 12.10 | 9.58 | 9.62 | 9.50 |
| Cys | 6.92 | 7.14[b] | 6.45 | 6.94 | 6.80[b] | 6.30 |
| Gly | 6.96 | 6.88 | 2.42 | 5.50 | 5.50 | 2.84 |
| Tyr | | | 4.84 | | | 3.25 |
| Glu | 10.24 | 10.04 | 9.68 | 12.30 | 12.30 | 13.10 |
| His | 2.64 | 2.58 | 3.23 | 2.94 | 2.60 | 3.00 |
| Ile | 2.06 | 2.30 | 2.41 | 2.32 | 2.30 | 2.35 |
| Leu | 1.10 | 1.13 | 1.61 | 10.90 | 10.80 | 10.90 |
| Lys[a] | 8.71 | 8.71 | 8.06 | 10.85 | 10.60 | 10.70 |
| Met | 3.59 | 3.43[c] | 3.23 | 0.90 | 0.50[c] | 0.63 |
| Phe | 2.76 | 2.94 | 2.42 | 4.02 | 4.72 | 4.55 |
| Pro | 2.92 | 2.82 | 3.23 | 3.57 | 3.61 | 4.80 |
| Ser | 11.37 | 11.42 | 12.10 | 4.70 | 4.75 | 4.70 |
| Thr | 8.51 | 8.56 | 8.06 | 5.50 | 5.50 | 5.65 |
| Val | 7.63 | 7.43 | 7.25 | 5.80 | 5.80 | 5.80 |

[a] Recovered as di-dans-lys.
[b] Found as cysteic acid.
[c] Found as methionine sulphone.
[d] According to SMYTH *et al.*[19].
[e] According to WEBER AND YOUNG[21].

Certain authors recommend longer times for the hydrolysis of the protein when the N-terminal amino acid is Ile, Leu or Val[14]. Under our experimental conditions all the Ile N-terminal of trypsin (Table VI) was released after 4 h hydrolysis.

The effect of performic acid oxidation on the N-terminal amino acid determination of three known proteins was studied (Table VI). The results for native or oxidized RNAase and trypsin were similar. For serum albumin a preliminary performic oxidation was necessary in order to obtain a quantitative yield of the N-terminal amino acid. Since the presence of urea in the dansylation medium was insufficient for certain proteins to overcome steric hindrances, we recommend systematic rupture of disulphide bonds by performic acid oxidation prior to all quantitative determinations.

For the quantitative determination, the absolute amount ($n_s'$) of each N-terminal amino acid, in nmole is given by:

$$n_s' = n_s \times l \tag{5}$$

where $n_s$ is defined by eqn. 4 and $l$ is the recovery coefficient after acid hydrolysis as measured by GROS AND LABOUESSE[14].

As a control for the quantitative recovery of the N-terminal amino acids, we have carried out the following calculations for the three proteins tested (Table VI).

J. Chromatog., 51 (1970) 441–458

TABLE VI

QUANTITATIVE DETERMINATION OF N-TERMINAL AMINO ACIDS AND OF LYS CONTENT OF SOME PROTEINS

| *Protein* | *N terminal AA* | *Dansylation time (min)* | *Moles N-terminal amino acid/mole protein* | | *Theoretical value* | *Moles lys/mole protein* | | *Theoretical value* | *Mol. wt.* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | *Without performic oxidation* | *With performic oxidation* | | *Without performic oxidation* | *With performic oxidation* | | *Calculated* | *Literature data* |
| Ribonuclease | Lys | 30 | 0.92 | 0.93 | 1 | 8.62 | 9.43 | 10 | 15 000 | 14 500 |
| | | 120 | 0.95 | 0.97[b] | | 8.65 | 9.77 | | | |
| Trypsin | Ile | 120 | 0.90 | 0.92[b] | 1 | 12.60 | 12.90 | 14 | 24 400 | 22 500 |
| Serum albumin[a] | Asp | 30 | 0.08 | 0.65 | 1 | 24.00 | 38.50 | 55 | 76 800 | 66 000 |
| | | 120 | 0.16 | 0.86[b] | | 51.20 | 46.00 | | | |

[a] Cohn fraction V powder.

[b] Values used to calculate mol. wt.

Since Lys is dansylated in the $\varepsilon$ position, it is possible to determine the molar quantity of Lys in the sample[14] by using eqn. 5; the molar quantity of N-terminal amino acids relative to that of Lys can be easily calculated. By knowing the number of Lys residues per mole of protein it is easy to verify whether one mole of N-terminal amino acid corresponds to one mole of protein or less than one, thus giving an indication of the number and approximate size of the polypeptidic chains. The same argument is valid for the sum of the N-terminal amino acids if more than one is detected. On the other hand, the ratio between moles of N-terminal amino acid and moles of protein can indicate the presence of masked N-terminal amino acids. The principal advantage of this method is that quantitative determinations of unmasked N-terminal amino acid are possible with 1–10 nmole of protein.

*Fingerprinting of proteins using dans-peptides*

Elimination of excess dans-Cl was not performed by formate precipitation, for the purification of dans-peptides produced after enzymatic hydrolysis of a protein, since certain dans-peptides were also precipitated by this reagent. However, since the disadvantages of the alkaline hydrolysis method for elimination of dans-Cl do not apply in this case, given a limited number of hydrolysates to be handled and the fact that the hydrolyses are, themselves, not absolutely quantitative, this method was applied.

Several supports and solvent systems for chromatographic separation of dansylated peptides for the fingerprinting of proteins, have been described[15–17]. Peptides produced after tryptic digestion[20] of native and oxidised RNAase and serum albumin were dansylated as previously described and used to test the different methods.

Thin-layer silica gel chromatography was preferred to polyamide layers because of the low capacity and the difficulty of elution from the latter. Furthermore, the peptide maps are difficult to reproduce and trailing of spots is difficult to avoid on polyamide sheets.

All highly volatile solvents reported in the literature were rejected due to difficulties in getting reproducible maps. Solvent systems with low dielectric constants (around 10) like the chloroform–ethanol–acetic acid (38:4:3) mixture used by ATHERTON AND THOMSON[17] did not resolve the spots sufficiently, leaving a large number of dans-peptides at the origin. On the other hand, most of the peptides migrated toward the solvent front in chromatograms developed in solvents with a dielectric constant of the order of 50. We obtained the best results with solvents of dielectric constants of the order of 20–25.

Fig. 4 shows the map obtained for a tryptic hydrolysate of bovine pancreatic RNAase. The number of dans-peptides obtained corresponds to the number of Lys and Arg residues present in the molecule allowing for the fact that two of the "Lys-X" peptide bonds are not hydrolysed by trypsin[19]. Fig. 5 shows the map obtained for bovine serum albumin. 61 spots were clearly separated, whereas only 35 were observed in the system according to ATHERTON AND THOMSON[17]. The number of spots expected for bovine serum albumin would be around 70 assuming that all the "Lys-X" and "Arg-X" peptide bonds were hydrolysed.

Under our conditions the peptide maps obtained show good resolution, and thus the method can be used for proteins of high mol. wt. The dans-peptides can be detected at levels as low as 0.01 nmole; a reliable map can be obtained from 0.1–1

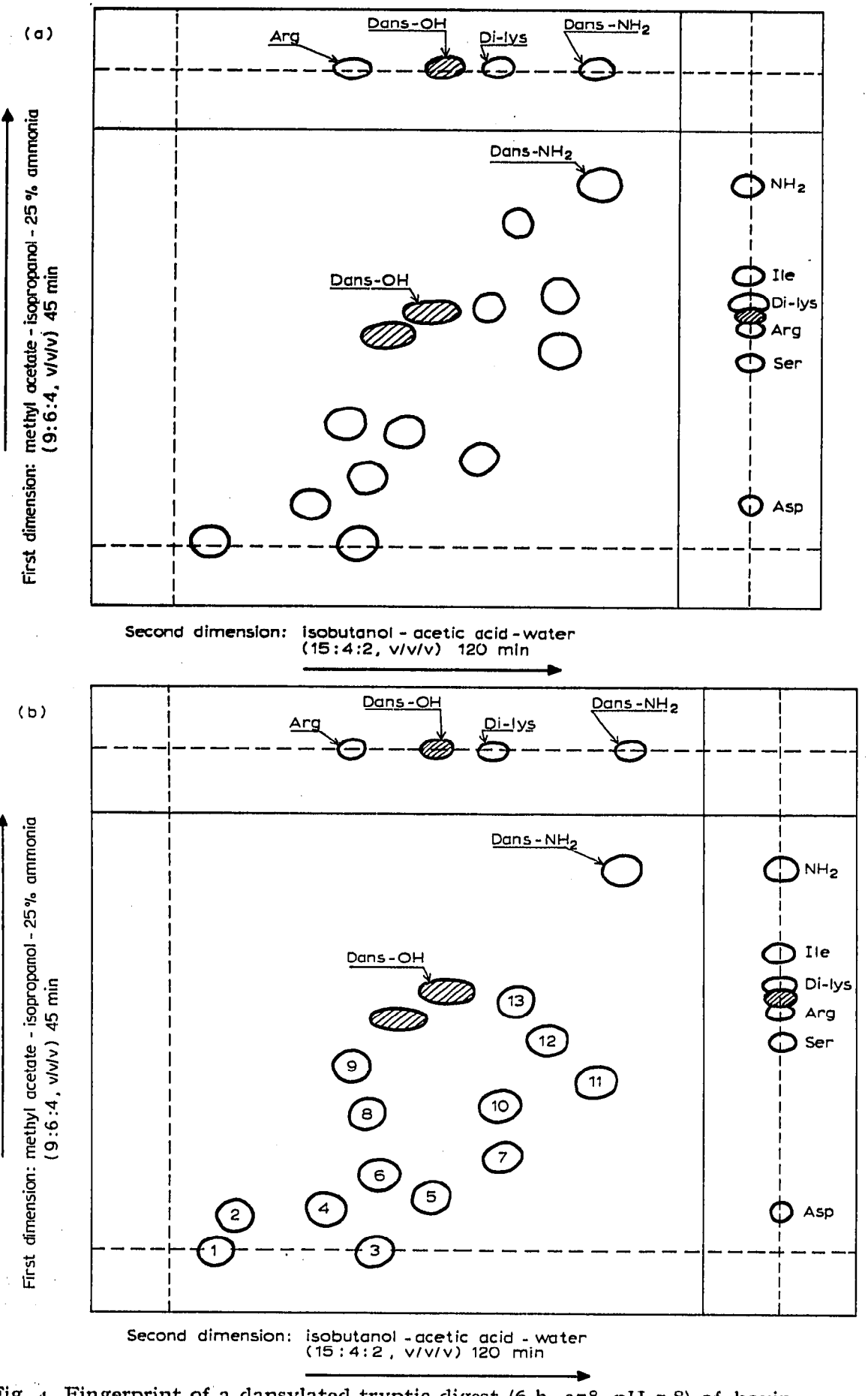


Fig. 4. Fingerprint of a dansylated tryptic digest (6 h, 37°, pH 7.8) of bovine pancreatic ribonuclease on a silica gel thin-layer. Spotted material corresponds to 1 nmole of protein. A = native

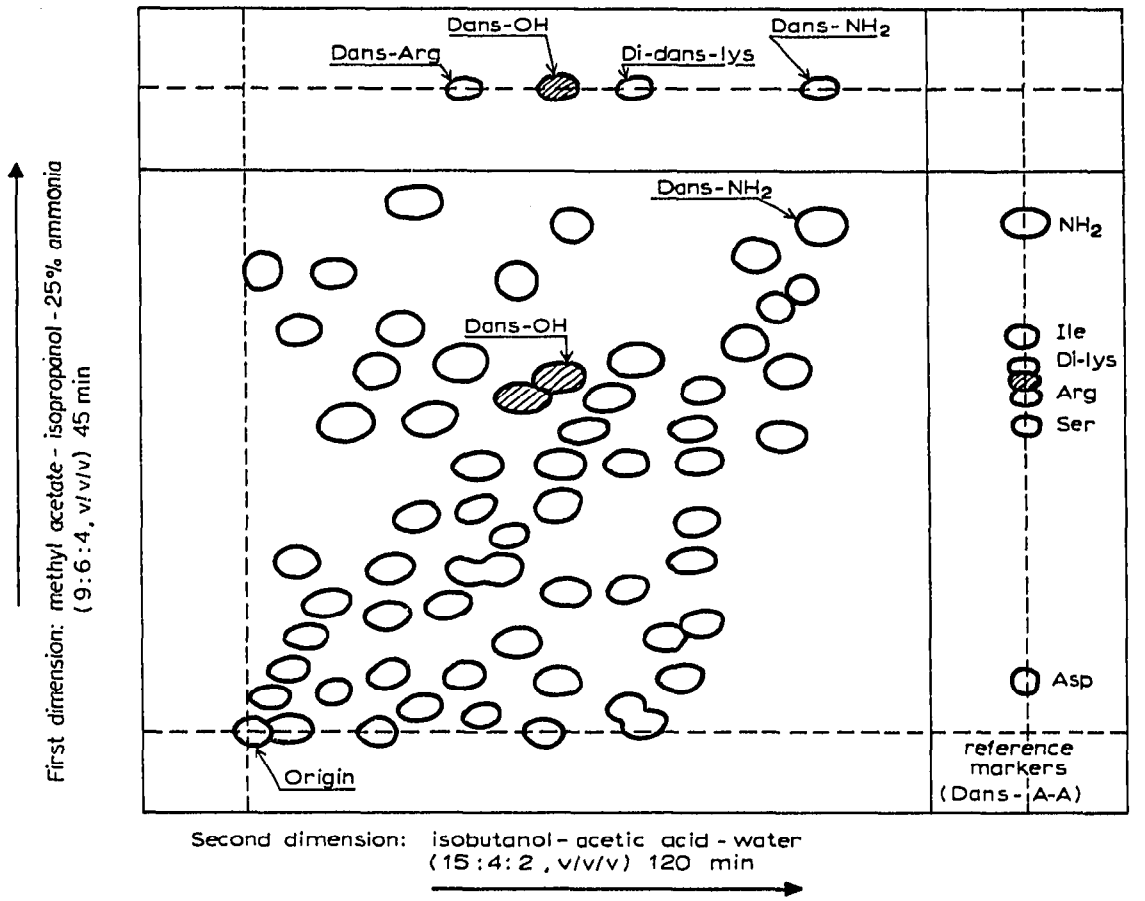


Fig. 5. Fingerprint of a dansylated tryptic digest (24 h, 37°, pH 7.8) of oxidised bovine serum albumin on a silica gel thin-layer. Spotted material corresponds to 1 nmole of protein.

nmole of protein. Peptides obtained from up to 20 nmole of protein can be separated on a single silica gel plate and higher quantities if necessary can be used for proteins of low mol. wt. Given the reproducibility of the chromatograms, corresponding spots eluted from several plates can be pooled in order to obtain sufficient quantities of dans-peptides for subsequent analysis. This technique which has been used to prepare dans-peptides is much quicker than the classical methods of fractionation of enzymatically hydrolysed proteins.

*Partial determination of the primary structure of a protein*

A combination of the various techniques described in this paper can be used to determine partially the primary structure of a protein on a microscale. Two aliquots were used for the quantitative determination of N-terminal amino acids and for the total amino acid composition of the protein. A third aliquot was used for the preparative fingerprinting.

The N-terminal amino acids and ε-dans-lys were determined on an aliquot of each eluted dans-peptide containing 2.5–7.5 nmole. After the lyophilisation of the acetone–water eluate, the residue was hydrolysed in sealed tubes in 250 μl of 6 *M* HCl for 4 h at 115°. HCl was removed *in vacuo* and the residue taken up in 50 μl of acetone–1 *M* HCl (19:1). 25 μl were then chromatographed on the silica gel thin layer

TABLE VII

DETERMINATION OF AMINO ACID COMPOSITION AND TERMINAL GROUPS OF DANSYLATED TRYPTIC PEPTIDES OF PERFORMIC ACID OXIDISED RIBONUCLEASE (20 nmole)

$T_1$–$T_{13}$ denote the dans-peptides eluted from the peptide map (see Fig. 4).

| *AA* | $T_1$[c] | $T_2$ | $T_3$ | $T_4$[f] | $T_5$ | $T_6$ | $T_7$ | $T_8$ | $T_9$[g] | $T_{10}$[g,h] | $T_{11}$ | $T_{12}$ | $T_{13}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ala | 0.40 | 2.25 | | 2.10 | | | | 1.10 | 2.15 | 3.10 | 1.10 | | 1.15 |
| Arg | 1.00[d] | | 1.00[d] | | 1.00[d] | 1.00[d] | | | | | | | |
| Asp | 2.90[e] | 2.55 | | 2.45 | | 0.80[e] | | 0.95 | 1.90 | | 0.95 | 0.85[e] | 0.65[e] |
| Cys[a] | 2.30 | 1.10 | | 0.75 | | | | 1.05 | 1.75[e] | | | | 1.10 |
| Gly | 0.95 | | | 0.75 | | | 1.05 | | | | | | |
| Tyr | 2.40 | 1.20 | | 1.05 | | | | 1.65[e] | | | | | |
| Glu | 2.40[e] | 1.65[e] | 0.95 | 1.00 | | | 0.65[e] | | 2.45 | 0.95 | 0.95 | | |
| His | 0.20 | 1.25 | | 2.25[e] | | | | | 1.10 | | | | |
| Ile | 0.95 | | | 1.75 | | | | | | | | | |
| Leu | | | | | | | | | 0.85 | | | 0.82 | |
| Lys | 0.20[d] | 1.00[d] | | 0.25 | | | 1.00[d] | 1.00[d] | 2.00[d] | 2.00[d,e] | 1.00[d] | 1.00[d] | 1.00[d] |
| Met[b] | 1.20 | 1.75 | | | | | | | | | | | |
| Phe | | | 0.78[e] | 0.85 | | | | | 0.85 | | | | |
| Pro | | | | 1.55 | | | | 1.15 | 1.15 | | | | |
| Ser | 4.50 | 7.27 | | 1.25 | 0.80[e] | | 2.15 | | 1.75 | | | | |
| Thr | 3.45 | 1.15 | | 0.15 | | | 1.05 | | 0.85 | 0.90 | 1.75[e] | 0.95 | |
| Val | | | | 3.85 | | | | | 3.65 | | | | 0.90 |

[a] Found as cysteic acid.
[b] Found as methionine sulphone.
[c] $T_1$ was slightly contaminated with $T_2$ because of the high quantity of protein used.
[d] C-terminal amino acid.
[e] N-terminal amino acid.
[f] $T_4$ corresponds to the C-terminal peptide of the protein.
[g] Peptide containing a Lys-X peptide bond not cleaved by trypsin.
[h] $T_{10}$ corresponds to the N-terminal peptide of the protein since it is the only peptide which has the same N-terminal amino acid as the protein.

and the 25 $\mu$l remaining was kept in case it proved necessary to confirm the nature of the N-terminal amino acids by polyamide chromatography. From the yield of N-terminal amino acids of a given spot it was possible to determine whether the peptide resulted from a specific enzymatic cleavage or from a non-specific lysis. A high molecular yield of N-terminal amino acid relative to protein used indicates a specific enzymatic hydrolysis. This is particularly helpful when two N-terminal amino acids are detected in the same spot. For tryptic fingerprinting, lysine containing peptides yield, upon acid hydrolysis, two major fluorescent spots: one due to the N-terminal amino acid of the peptide, the other to the C-terminal-lys. An N-terminal amino acid/$\varepsilon$-dans-lys molar ratio of 1 indicates that the peptide results from specific enzymatic cleavage. Since the $\omega$-dansylation of arginine is very slow, it is not possible to carry out similar calculations for arginine containing peptides. The chromatograms of the acid hydrolysate of these peptide spots are characterised by absence of $\varepsilon$-dans-lys, and a weak spot of $\omega$-dans-arg. The C-terminal peptide of the protein lacks both $\varepsilon$-dans-lys, and $\omega$-dans-arg.

Another aliquot of 2.5–7.5 nmole of each dans-peptide was used to determine the amino acid composition of the peptide. The acetone–water eluate was lyophilised and the residue treated as described for the determination of the amino acid composition of a protein. Eqn. 4 gives the absolute quantity of amino acid in nmole ($n_s$). It was necessary however to introduce an additional correction for fluorescence losses due to partial destruction of $\varepsilon$-dans-lys during acid hydrolysis of the dans-peptide. The absolute amount of $\varepsilon$-dans-lys is given by:

$$n_{lys} = 1.6 \times n_s \qquad (6)$$

A similar correction is required for the N-terminal amino acid of the peptide.

The analysis of bovine pancreatic RNAase (Tables V–VII) shows that our results are in good agreement with the published data obtained by other methods[19].

The dansylation method allows the rapid partial determination of the primary structure on 15–35 nmoles of protein; *i.e.* 5–10 nmoles for N-terminal group determination, 5–10 nmoles for the determination of the amino acid composition and 5–15 nmoles for the tryptic fingerprinting and the subsequent analysis of the peptides. Using these quantities, all operations can be carried out with precision and give quantitative data. With smaller quantities (0.5–5 nmole), semiquantitative results can be obtained only if scrupulously clean glassware and reagents are used.

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