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Note

A new fluorescence method for the detection of hexosamines and their separation by means of thin-layer chromatography

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A number of methods have been developed for the determination of amino sugars: the method of Elson and Morgan[1,2] and its modifications[3–5] for measuring their total content, the method of Dische *et al.*[6], the ninhydrin method[7] and gas chromatographic[8] and ion-exchange chromatographic methods[9]. Kelleher *et al.*[10] proposed a new method for the quantitative determination of glucosamine only by means of column chromatography and radio isotope dilution. More recent spectrophotometric and colorimetric methods, based on colour reactions with various reagents, such as trinitrobenzene-1-sulphonic acid[11], *p*-nitrobenzenediazonium salts in alkaline medium[12], *p*-nitrobenzaldehyde[13] and N-methylbenzothiazolone hydrazone[14] have also been described. Methods based on the 2,4-dinitrophenyl derivatives of amino sugars and their separation by means of paper (PC)[15] and thin-layer[16] chromatography (TLC) are also known.

The separation of the individual amino sugars is most often achieved by PC (ref. 17) and TLC (e.g., ref. 18), detection being effected with different reagents. Amounts of 10–100 μg of each individual sugar are needed for these methods. Moczar *et al.*[19] suggested a more sensitive method (1.5 μg).

The most sensitive methods involve fluorescence techniques. Galoyan *et al.*[20] described a sensitive method ($3–5 \times 10^{-9}$ mole) for the detection of amino sugars as 5-dimethyl aminonaphthalene-1-sulfonyl (Dns) derivatives and for their separation by means of TLC. Cho Tun *et al.*[21] described an automatic spectrofluorimetric determination of the formaldehyde released by periodate oxidation of amino sugars as 3,5-diacetyl-1,4-dinitro-2,6-dimethylpyridine. Maeda *et al.*[22] proposed a highly sensitive method for the determination of amino sugars by using pyridoxal and zinc(II) ions. The last two methods, however, do not provide the possibility of determining individual sugars, but only their total content.

In previous papers, we showed that Dis-chloride (diphenylindenone sulphonyl-chloride or 2-*p*-chlorosulphophenyl-3-phenylindenone) reacts with amines[23] and amino acids[23–25] by forming the corresponding Dis derivatives and that under the action of sodium ethylate, the latter are transformed into strongly fluorescent derivatives of diphenylisobenzofuran[26]. This reaction offered the opportunity of determining amino acids in amounts of 10^{-12}–10^{-13} mole. On this basis, a highly sensitive method

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for the detection of the N-terminal groups in proteins and peptides was developed[26], and we have attempted to establish whether we could also detect small amounts of hexosamines using Dis-chloride, and also to find conditions for the separation of Dis derivatives of hexosamines from each other and from the Dis derivatives of amino acids.

EXPERIMENTAL

α-D-Glucosamine hydrochloride (Koch-Light, Colnbrook, Great Britain) and D-galactosamine hydrochloride (Loba-Chemie, Vienna, Austria) were used.

Preparation of Dis derivatives of glucosamine and galactosamine

The procedure described[26] for the preparation of the Dis derivatives of amino acids was used with certain modifications: 40 μl of an acetone solution of Dis-chloride (1 mg/ml) was added to 2×10^{-11} mole of glucosamine or galactosamine dissolved in 20 μl of a 0.1 *M* solution of sodium hydrogen carbonate. After leaving the mixture for about 2.5 h at room temperature, the samples were evaporated to dryness in a vacuum desiccator, and the dry residue was dissolved in acetone and applied to the chromatographic plate.

Chromatography

The separation of the Dis derivatives was achieved by TLC using silica gel G (E. Merck, Darmstadt, G.F.R.) as the carrier. Before each chromatographic run, the plates were activated by heating them for 20 min at 105°. The following solvent systems were used:

(A) *n*-butanol–toluene–25% ammonia (80:10:10)[28]
(B) chloroform–ethyl acetate–acetic acid (50:66:2.5)[27]
(C) chloroform–ethyl acetate–methanol–propionic acid (70:40:22.5:0.5)
(D) toluene–ethylene chlorohydrin–25% ammonia (30:50:20)

After the chromatography, the plates were dried at 105° for 5 min in order to remove trace amounts of the solvents, cooled to room temperature and sodium ethylate solution (5 g of Na per 100 ml of 96% ethanol) was poured over them. The plates were immediately observed under UV light (365 nm), and the spots of the Dis derivatives of the hexosamines, as well as those of Dis-acid, Dis-amide and Dis-chloride, showed yellow-green fluorescence.

RESULTS AND DISCUSSION

The solvent systems used for the separation of the corresponding Dns derivatives[20], as well as those for the separation of Dis amino acids[27], were tested for the separation of the Dis derivatives of glucosamine and galactosamine.

The solvent systems recommended for use with Dns derivatives in the preliminary application of buffers to the plates proved to be unsuitable. A relatively good separation was achieved by using systems C and D with preliminary impregnation of the plates with borate buffer (pH 8.6) and a single development in one direction (Table I).

A considerably improved and simplified separation, without preliminary

TABLE I

R_F VALUES OF Dis DERIVATIVES OF α-D-GLUCOSAMINE (GLA) AND D-GALACTOSAMINE (GAA) IN THE SOLVENT SYSTEMS USED

Carrier: silica gel G (0.25 mm layer thickness).

Dis derivative	*Solvent system*			
	A	*B*	*C*	*D**
GLA	0.61	0.45	0.45	0.27
GAA	0.53	0.29	0.28	0.20
OH	0.23	0.00	0.14	0.55
NH_2	0.86	0.86	0.90	0.92
Cl	0.90	0.97	0.96	0.00

* with borate buffer, pH 8.6.

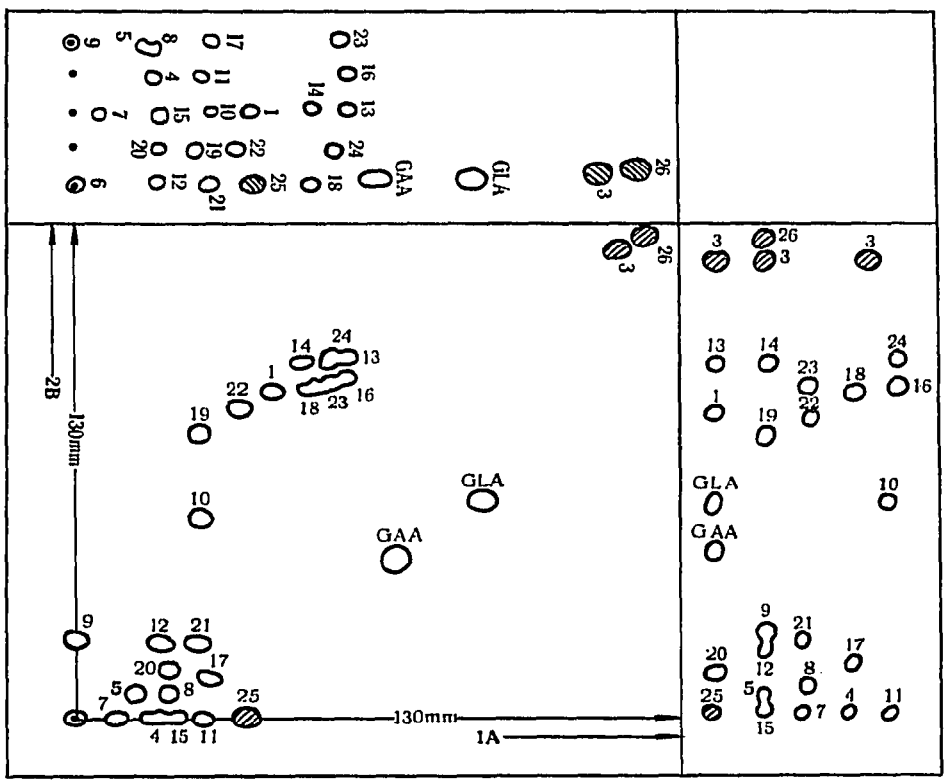

Fig. 1. Two-dimensional separation of the Dis derivatives of α-D-glucosamine (GLA), D-galactosamine (GAA) and common amino acids. (1) Dis-α-alanine; (3) Dis-amide; (4) Dis-arginine; (5) Dis-asparagine; (6) Dis-aspartic acid; (7) Dis-cysteic acid; (8) Dis-glutamine; (9) Dis-glutamic acid; (10) Dis-glycine; (11) di-Dis-histidine; (12) Dis-hydroxyproline; (13) Dis-isoleucine; (14) Dis-leucine; (15) ε-Dis-lysine; (16) di-Dis-lysine; (17) Dis-methionine-sulphone; (18) Dis-phenylalanine; (19) Dis-proline; (20) Dis-serine; (21) Dis-threonine; (22) Dis-tryptophan; (23) di-Dis-tyrosine; (24) Dis-valine; (25) Dis-OH; (26) Dis-Cl. Carrier: silica gel G (0.5 mm layer thickness). First run: *n*-butanol–toluene–25% ammonia (80:10:10) (A), developed once. Second run: chloroform–ethyl acetate–acetic acid (50:66:2.5) (B), developed once. Dimensions of plates: 200 × 200 mm.

impregnation of the plate, was achieved by the use of systems A and B with a single development (Table I). A reliable separation not only of the Dis derivatives of glucosamine and galactosamine from each other, but also of the Dis derivatives of the amino acids contained in the proteins, was obtained by using two-dimensional chromatograms in systems A and B (Fig. 1). Moreover, under these conditions, almost all Dis derivatives of amino acids were separated from each other. Only Dis-isoleucine and Dis-valine, Dis-arginine and ε-Dis-lysine, and also Dis-phenylalanine, di-Dis-tyrosine and di-Dis-lysine, remained unseparated. All these Dis derivatives of amino acids, however, can be separated from each other by using the solvent systems proposed by us[27].

The proposed fluorescence method makes possible the detection of amino sugars in amounts as low as 2×10^{-11} mole, which means that amounts of hexosamines 100 times lower than those detected by the method of Galoyan *et al.*[20] can be determined.

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