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CHROMATOGRAPHY OF DINITROPHENYLAMINO ACIDS AND HETEROCYCLIC BASES ON THIN LAYERS OF PROTEIN

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SUMMARY

The properties of thin layers of protein prepared from wool cortical cells are further investigated. Thin-layer chromatographic separations of dinitrophenylamino acids and of certain heterocyclic compounds (substituted purines and pyrimidines) are described.

INTRODUCTION

Recently[1] we reported that wool cortical cells, the product of enzymic digestion of keratin fibres, could be used to form thin layers of protein that were suitable for chromatography. The potential of such layers in resolving certain types of dyestuff was outlined. Wool protein has free side-chain amino, carboxyl, hydroxyl, disulphide and sulphydryl groups[2]; it could be expected that chemical modification of these would cause subtle variations in chromatographic behaviour. This expectation has been confirmed[3] in studies of the ion-exchange properties of both native and modified keratin. The present study of dinitrophenyl (DNP) amino acids and heterocyclic bases (purines and pyrimidines) was undertaken to extend the range of substances chromatographed on thin layers of protein and to further compare the properties of unmodified and methyl-esterified cortical cells.

Thin-layer chromatographic (TLC) procedures on other supports have been described for DNP amino acids[4–9] and heterocyclic bases derived from nucleic acids[10–14].

EXPERIMENTAL

Materials

Cortical cells were isolated, methyl-esterified and formed into thin layers as described[3]. The dinitrophenylamino acids were obtained from the Sigma Chemical Company.

Chromatographic procedure

All layers were eluted in the relevant solvent and then air-dried at least once, before chromatographing the test substances. This procedure ensured the removal

of impurities (especially from layers of unmodified cells) that migrated as a broad band in the solvent front. Failure to observe this precaution resulted in poor resolution and irregular solvent fronts. All development was effected in closed tanks containing wicks to provide solvent-saturated vapour. Depending on their solubility, the DNP amino acids were applied from either aqueous or ether solutions. Preliminary spotting of the plates with ethanol facilitated subsequent application of aqueous solutions.

The DNP amino acids were detected either visually or, particularly with esterified cells where background absorption was low, by inspection under UV light (254 nm or 366 nm sources). The purines and pyrimidines were detected only with the UV lamp. The R_F values were measured after a single development of 12 cm.

RESULTS AND DISCUSSION

Dinitrophenylamino acids

Originally we had hoped to study the chromatography of amino acids on the protein support and to obtain data on amino acid interactions with protein. However, it was found that visualisation of the amino acids on cortical cell layers was subject to interfering reactions between the support and common chromogenic reagents. Consequently the coloured and readily observable DNP amino acids were employed. Of several solvents described for the TLC of DNP amino acids[4] only the base-containing toluene system (S_3 here) was found suitable for use with cortical cell layers. In this work an additional acidic (S_1) and a basic (S_2) solvent were established.

Table I records the R_F values of some DNP amino acids on layers prepared from unmodified and methyl-esterified cortical cells with various solvent systems. On both substrates the range of R_F values suggests that these solvents would be suitable for analytical purposes. With the exception of N-DNP-L-arginine, N,S-di-DNP-L-cysteine, N,N'-di-DNP-L-ornithine and N,O-di-DNP-L-tyrosine, the DNP amino acids migrated in approximately the same order irrespective of differences in support and developing solvent. Nevertheless, with S_1, R_F values were significantly higher on esterified cells indicating decreased affinity of the DNP amino acids for the modified support.

Solvent S_3 has been widely used in the thin-layer chromatography of DNP amino acids on silica and its use with methyl-esterified keratin layers permits comparison of the two types of layer. The R_F values on the modified keratin were lower than published[4] values for silica chromatography, although the relative order of migration was essentially the same. Significant exceptions were N-DNP-L-tryptophan and N,N'-di-DNP-L-histidine. Relatively, the former compound was more firmly retained and the latter more rapidly eluted on keratin than on silica. It is noteworthy that the aromatic amino acid derivatives N-DNP-L-tryptophan and N-DNP-L-phenylalanine which were poorly separated on silica gel[4] were better resolved on the protein layer.

In comparing the relative effects of the acidic (S_1) and basic (S_2) solvents, used with unmodified cortical cells, several patterns were observed:

(*i*) Most compounds had R_F values which were slightly higher (uniformly about 10%) in S_2 than those in S_1.

(*ii*) The bis-DNP derivatives (N,S-di-DNP-L-cysteine; N,N'-di-DNP-L-histi-

TABLE I

R_F VALUES OF SOME DINITROPHENYLAMINO ACIDS ON THIN PROTEIN LAYERS

R_F values are means of at least four determinations. Solvent systems are: S_1 = *n*-butanol–water–glacial acetic acid (30:20:10); S_2 = *tert.*-amyl alcohol–0.88 ammonia (50:10); S_3 = toluene–pyridine–ethylene chlorhydrin–0.88 ammonia (100:30:60:60), this is the well known "toluene system" which separates into two phases. Only the upper phase was used. Pretreatment of the layers with the lower phase did not influence the R_F values subsequently obtained with the top phase.

DNP amino acid	*R_F values × 100*			
	Unmodified cortical cells		*Methyl-esterified cortical cells*	
	S_1	S_2	S_1	S_3
N-DNP-D,L-α-amino-*n*-butyric acid	49	58	70	33
N-DNP-D,L-α-aminocaprylic acid	87	84	87	57
N-DNP-L-arginine	92	28	86	20
N-DNP-L-asparagine	23	30	42	8
N-DNP-L-aspartic acid	14	7	34	1
N-DNP-L-cysteic acid	1	4	1	1
N,S-di-DNP-L-cysteine	9	72	16	42
N,N'-di-DNP-L-cystine	1	s[b]	1	s[b]
N-DNP-D,L-glutamic acid	24	10	49	2
N-DNP-L-glutamine	26	32	44	9
N-DNP-glycine	27	38	48	19
N,N'-di-DNP-L-histidine[a]	47	66	84	40
N-DNP-L-isoleucine	71	69	83	37
N,N'-di-DNP-L-lysine	38	67	48	52
N-DNP-D,L-methionine	44	65	60	36
N-DNP-D,L-norleucine	64	74	82	41
N-DNP-D,L-norvaline	59	68	76	39
N,N'-di-DNP-L-ornithine[a]	29	55	40	46
N-DNP-L-phenylalanine	43	61	67	39
N-DNP-L-proline	45	41	69	24
N-DNP-L-threonine	35	47	56	18
N-DNP-L-tryptophan	45	58	58	25
N,O-di-DNP-L-tyrosine	40	82	48	64
N-DNP-L-valine	62	67	80	34

[a] These substances tended to elongate with solvent S_1 and unmodified cells.
[b] "s" indicates that this substance streaked badly.

dine; N,N'-di-DNP-L-lysine and N,O-di-DNP-L-tyrosine) all had significantly higher R_F values in the basic solvent (S_2). Apparently this behaviour is due to increased lipophilic properties conferred by a second dinitrophenyl ring.

(*iii*) The aspartic and glutamic acid derivatives had slightly lower R_F values in S_2. With the exception of N,N'-di-DNP-L-cystine (see *v*) these were the only dicarboxylic acids studied.

(*iv*) N-DNP-L-arginine was the only compound which showed a large decrease in R_F value in the basic solvent (S_2). Unlike most of the other DNP amino acids, the arginine derivative has a free basic group; its guanidino function, in the protonated form, can associate with keratin carboxylate anions and, presumably, it is this effect which causes the lower R_F value in S_2.

(*v*) N,N'-di-DNP-L-cystine was immobile in S_1, and streaked badly in S_2. This effect was not due to poor solubility in S_1 or S_2 and it contrasts with N,S-di-DNP-L-

cysteine which chromatographed normally. The possibility that this disulphide has reacted chemically with the sulphydryl groups of the protein substrate requires examination.

Bidimensional separations

Protein layers can be used to effect bidimensional separations. For this purpose, unmodified cell layers were most suitable because they gave the sharpest resolution of compounds. Of the two solvents studied with unmodified cells, S_1 provided the most compact spots and was therefore used first (normal to the layering direction), followed by S_2. Fig. 1 represents a tracing of an actual bidimensional resolution of

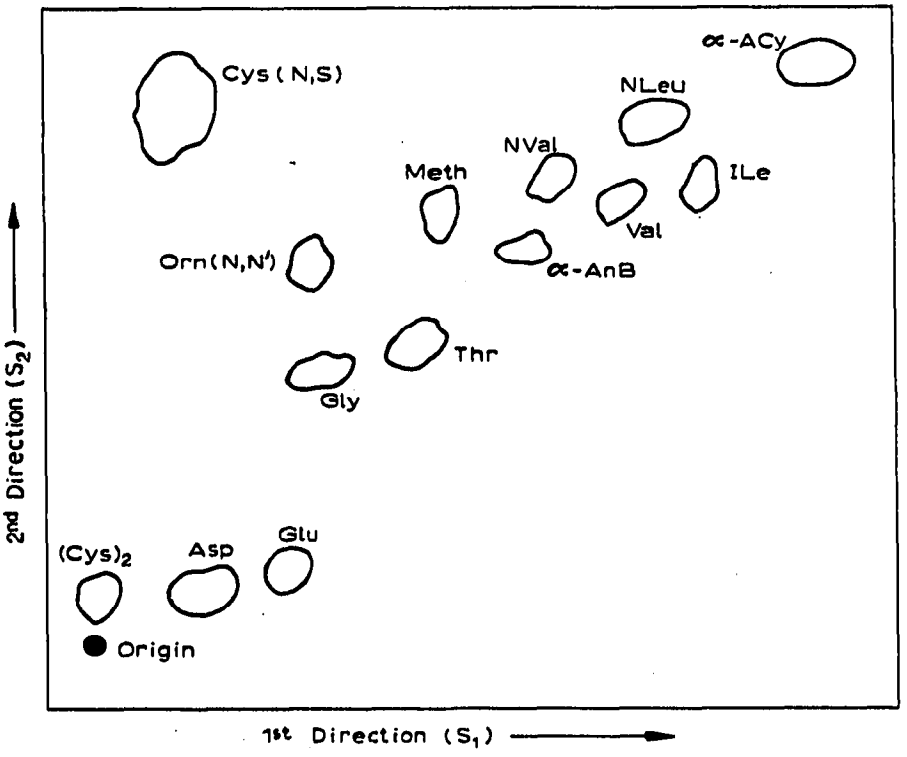

Fig. 1. Tracing of bi-dimensional resolution of fourteen dinitrophenylamino acids. 1st dimension (normal to layering direction), solvent S_1; 2nd dimension, solvent S_2.

fourteen selected compounds with single developments of 10 cm in each direction. It is clear that satisfactory finger-printing was obtained on the layers.

Pyrimidines and purines

Because layers of methyl-esterified cortical cells are slightly fluorescent, detection of pyrimidines and purines under UV light is more satisfactory than on layers of untreated cells. Esterified layers were therefore selected for chromatography of heterocyclic compounds and Table II records the R_F values of a number of pyrimidine and purine derivatives. The substances chosen are commonly encountered as synthetic intermediates. With both types of heterocyclic compound a neutral solvent (S_4) was found which provided satisfactory resolution. The acidic solvent (S_5) which resolved the pyrimidine derivatives proved ineffective for the separation of purines.

TABLE II

R_F VALUES OF PYRIMIDINES AND PURINES ON METHYL-ESTERIFIED PROTEIN LAYERS

R_F values are means of three determinations. Solvent systems are: S_4 = *n*-butanol saturated at 25° with water; S_5 = *n*-butanol–water–glacial acetic acid (70:20:10).

Compound	*R_F values × 100*	
	S_4	S_5
Pyrimidine		
4-Amino-5-bromo-	83	91
4-Amino-5-bromo-2-hydroxy-	57	74
4-Amino-2,6-dihydroxy-5-nitro-	3	34
4-Amino-2-mercapto-	55	67
4,5-Diamino-	66	77
4,5-Diamino-2,6-dimercapto-	10	45
4,5-Diamino-6-hydroxy-	57	70
4,5-Diamino-6-hydroxy-2-mercapto-	28	61
4,5-Diamino-6-mercapto-	51	67
4,5-Diamino-6-methylmercapto-	77	85
4,6-Dihydroxy-5-nitro-	4	10
2-Hydroxy-4-mercapto-	63	72
Purine		
2-Amino-	74	
6-Amino-	59	
2-Hydroxy-	34	
6-Mercapto-	60	
8-Mercapto-	68	
Purine	81	
8-Azaadenine	52	

Even though S_5 is, generally, a non-ionising solvent, to properly understand the significance of the R_F values of the pyrimidines in acidic media, information about the formation of ionic species is required. Data of this kind are not available, nevertheless, some comments on the influence of chemical structure on R_F are possible. In the 4,5-diaminopyrimidine series, further substitution with groups capable of forming hydrogen bonds (OH and SH) produced compounds whose R_F values were lowered; the reduction depended on the type and number of substituting groups. Conversely, a decrease in the number of substituent groups capable of forming hydrogen bonds (as in the replacement of the 5-amino function of 4,5-diamino pyrimidine with bromine) had the effect of increasing the R_F value. The introduction of a methylmercapto function, which presumably decreased hydrophilic character also resulted in an increased R_F value. These trends were apparent with both solvents although the acidic system (S_5) furnished higher R_F values than the neutral system (S_4). The two highly polar dihydroxy-5-nitropyrimidines migrated to an useful degree with system (S_5) only. The substituted purines chromatographed in a manner similar to the pyrimidines. The introduction of hydrogen-bonding groups (OH, SH, NH_2) produced derivatives whose R_F values were lower than the parent compounds.

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REFERENCES

1 P. R. BRADY, J. DELMENICO AND R. M. HOSKINSON, *J. Chromatog.*, 38 (1968) 540.
2 ANONYMONS, *Wool Science Review*, 21 (1962) 27.
3 P. R. BRADY AND R. M. HOSKINSON. *J. Chromatog.*, 54 (1971) 55.
4 M. BRENNER, A. NIEDERWIESER AND G. PATAKI, in E. STAHL (Editor), *Thin-Layer Chromatography, a Laboratory Handbook*, Academic Press, New York, 1965, p. 391.
5 D. WALZ, A. R. FAHMY, G. PATAKI, A. NIEDERWIESER AND M. BRENNER, *Experientia*, 19 (1963) 213.
6 J. M. DELLACHA AND A. V. FONTANIVE, *Experientia*, 21 (1965) 351.
7 K. T. WANG, J. M. K. HUANG AND I. S. Y. WANG, *J. Chromatog.*, 22 (1966) 362.
8 K. T. WANG AND I. S. Y. WANG, *J. Chromatog.*, 24 (1966) 460.
9 J. M. GHUYSEN, D. J. TIPPER AND J. L. STROMINGER, *Methods Enzymol.*, 8 (1966) 685.
10 H. K. MANGOLD, in E. STAHL (Editor), *Thin-Layer Chromatography, a Laboratory Handbook*, Academic Press, New York, 1965, p. 440.
11 J. E. CIARDI AND E. P. ANDERSON, *Anal. Biochem.*, 22 (1968) 398.
12 G. PATAKI AND A. KUNZ, *J. Chromatog.*, 23 (1966) 465.
13 G. PATAKI, *J. Chromatog.*, 29 (1967) 126.
14 G. PATAKI AND A. NIEDERWIESER, *J. Chromatog.*, 29 (1967) 133.