DIAZOREACTION FOLLOWING CHLORAMINE-T-OXIDATION AS A SENSITIVE AND SPECIFIC TEST FOR ASPARTIC ACID

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When gelatin and bovine albumin hydrolyzates on paper chromatograms were sprayed with Chloramine-T and Ehrlich aldehyde reagents[1] as a means of identifying hydroxyproline, aspartic acid was found to give a weak pink color after several days. Even though 10 γ aspartic acid/cm^2 were visible, attempts to improve this reaction failed.

In his investigation of the action of Chloramine-T upon aspartic acid, DAKIN[2] obtained dichloroacetaldehyde as the final oxidation product and noted that when the aspartic acid–Chloramine-T reaction mixture or dichloroacetaldehyde and ammonia were distilled, an unidentified compound was obtained which gave a highly colored product when coupled with diazotized sulfanilic acid[3].

We have found that, when aspartic acid on paper chromatograms is oxidized with Chloramine-T and coupled with diazotized sulfanilic acid, 1 γ/cm^2 is recognizable. Attempts to employ this series of reactions as a basis for a quantitative determination were unsuccessful. However, this is the only known test which permits a rapid differentiation of glutamic from aspartic acid, and with reference to the latter, the most specific and sensitive one on paper.

MATERIALS AND METHOD

Reagents

Chloramine-T reagent. N/50 Chloramine-T in 50 % methanol.

Sodium carbonate. 10 % solution.

Diazo reagent. 0.5 g semi-dry diazotized sulfanilic acid (if stored in a deepfreezer, it remains in good condition over a period of at least two years) is suspended in 5 ml cold water prior to application. The suspension should appear quite milky and is usable at least three days, if stored in a deepfreezer.

Spraying technique

The dried chromatogram is heavily sprayed with the Chloramine-T reagent with the aid of a mechanical sprayer. The correct amount of reagent has been applied when the moist paper requires 30 to 45 min to dry in the air. No heat may be applied. The

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diazo reagent is applied within 45 min to 3 h after application of the Chloramine-T reagent. No moisture from the Chloramine-T reagent may remain on the paper. The sodium carbonate solution must be very sparingly applied as fine droplets under 150 to 200 mm Hg pressure, preferably at a distance of approximately 50 to 60 cm. The paper should not become more than just barely moist during application. After 5 min the diazo reagent is applied in the same manner. If the paper becomes too moist, unreacted Chloramine-T will bleach the color in 3 to 5 min, or if too much diazotized sulfanilic acid is applied, the background will become yellow. The manner of spraying the diazo reagent is especially critical. When properly applied, the diazo reagent appears as innumerable fine dots on a white background. Aspartic acid yields immediately a bluish-red or red-orange spot. The relative amounts of the two colors are principally dependent upon the time interval between application of the two reagents. More bluish-red color is obtained with shorter and more red-orange color with longer intervals. The red-orange coupled product is the more stable of the two.

RESULTS

The comparison of the two products obtained from aspartic acid after oxidation and coupling with diazotized sulfanilic acid is given in Table I.

TABLE I

COMPARISON OF THE TWO COUPLED PRODUCTS OBTAINED FROM ASPARTIC ACID

	Bluish-red	*Red-orange*
Sensitivity (γAsp/cm^2)	1	1
Aqueous solution	unstable	very stable
Alkaline solution (1 *N* NaOH)	unstable	very stable
Spectral curve (aqueous solution)	—	sharp maximum at 480 mμ

TABLE II

REACTION OF AMINO ACIDS WITH THE CHLORAMINE-T AND THE DIAZO SPRAY REAGENTS

Amino acid	*Color*			*Sensitivity γ/cm^2*
	Diazo only	*Chloramine-T only*	*Chloramine-T and diazo*	
Aspartic acid	none	none	bluish-red/red-orange	1
Asparagine	none	none	bluish-red	5
Glutamic acid	none	none	none	
Glutamine	none	none	none	
Cysteine	none	none	orange-red	1
Cystine	none	none	orange-red	5
Histidine	red	none		1
			brown	1
Methionine	none	none	pink	1
Methionine sulfone	yellow	—	—	1
Tyrosine	red			1
		yellow		1
			remains yellow	
Tryptophan		violet	brown	
Creatine	none	none	none	
Creatinine	yellow	none	yellow	10
Glycocyamine	none	none	none	
Glycocyamidine	brown	yellow	yellow	100

The spectral curve was determined in a Unicam Sp. 500 in aqueous solution. A 50 γ spot of aspartic acid, treated with the Chloramine-T and diazo reagents and showing only the red-orange color, was washed with methanol to remove any excess Chloramine-T and extracted in 10 ml water. A blank was prepared in the same manner. The color intensity of the aqueous solution decayed at the constant rate of 2.5 %/h over a period of 8 h.

Table II summarizes the interfering amino acids. Other amino acids give no colored products with the Chloramine-T and diazo reagents and it should be pointed out, that neither glutamic acid nor glutamine form a colored diazo-product.

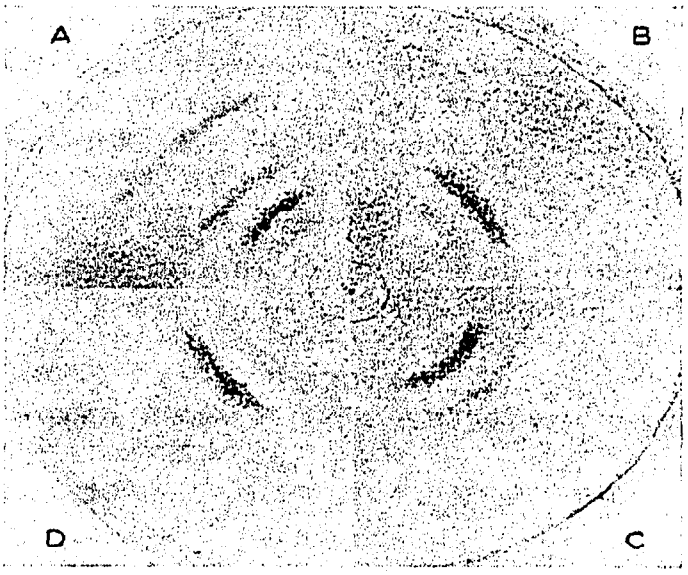

Fig. 1. Paper chromatogram on Schleicher u. Schüll 2043 b mgl. Additional explanations see Table III.

The chromatogram pictured in Fig. 1 was developed 48 h in freshly prepared *n*-butanol–glacial acetic acid–water (4:1:1)[4]. After drying, the Chloramine-T reagent was applied followed by the diazo reagent 3 h later. The identity of the bands is given

TABLE III

IDENTITY OF BANDS (See Fig. 1)

Substance spotted	*Band*	*Identity*	*Relative R_F Asp*	*Color*	*Absorption at 366 mμ*	*Amino acid present[b] in hydrolyzates γ*
A. Bovine albumin hydrolyzate 46γ	I	Unknown	49	Pink	—	?
	II	Histidine	69	Brown	+	2
	III	Aspartic acid	100	Red-orange	+	4
	IV	Tyrosine	172	Yellow	+	2
B. Gelatin hydrolyzate 40γ	I	Aspartic acid	100	Red-orange	+	2
C. Amino acid mixture 10γ each of Hist, Asp, Glu	I	Histidine	68	Brown	+	
	II	Aspartic acid	100	Red-orange	+	
D. Aspartic acid 5γ			100	Red-orange	+	

in Table III. Both bovine albumin* and gelatin were hydrolyzed 24 h in 20 % HCl at 105°.

DISCUSSION

Synthetic dichloroacetaldehyde gives a bluish-red color when coupled with diazotized sulfanilic acid on paper. This color is unstable in aqueous or alkaline solution. Thus the bluish-red coupled product given by aspartic acid shortly after oxidation is probably due to dichloroacetaldehyde. The time interval required for the appearance of the red-orange coupled product with the concommitant gradual disappearance of the bluish-red coupled product would indicate that the compound responsible for the bluish-red color further reacts, condensing with ammonia derived from the $-NH_2$ group in a manner similar to glyoxal[6] to yield an imidazole as suggested by DAKIN[2]. Imidazole coupled with diazotized sulfanilic acid on paper is reported to give a red-orange product[7].

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SUMMARY

A differentiation of aspartic acid from other amino acids, especially from glutamic acid, is possible when paper chromatograms have been sprayed with Chloramine-T and diazotized sulfanilic acid.

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