

chromatography. Using the pressureless apparatus with radial development for centrifugal chromatography, which has been described elsewhere⁷, it is possible to obtain perfect results within 20 min.

The bovine collagen (2.5 g) destroyed partially by alkali was extracted with 20 ml of acetate buffer pH 4.8 ($\mu = 0.1$) at 38° for 24 h. The extract was centrifuged and the supernatant liquid applied directly on the chromatogram. Whatman paper No. 3 was found to be the most suitable. Samples of the collagenous mixture were applied in quantities of 10 μ l to the start, which was 3 cm from the center of the chromatographic disc. Up to six samples could be developed at a time.

The mobile-phase gradient was made by running glacial acetic acid into 5 ml of amyl alcohol-acetic acid mixture (2:1). The necessary mobile-phase inlet was 1.2 ml per min.

Fig. 1 is a chromatogram on which the separation of two collagenous fractions is shown. In order to identify the separated fractions with those isolated by PIEZ⁵ on carboxymethyl-cellulose we made parallel separations on the carboxymethyl-cellulose column. The results obtained by means of ion-exchange chromatography agreed with our results and therefore we designated the first fraction ($R_F = 0.9$) as the α fraction of collagen and the second ($R_F = 0.45$) as the β fraction. Further investigation being made in our laboratory is necessary for the identification of soluble collagens from various sources.

Thanks are due to Dr. MILOŠ CHVAPIL of the Institute of Industrial Hygiene and Occupational Diseases, Prague, for his help with the classification of soluble collagens.

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Received July 20th, 1962

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J. Chromatog., 11 (1963) 110-111

A method for diazotising (2,4-dinitro-5-aminophenyl)-amino acids on thin layer chromatograms

Recently, BERGMANN AND BENTOV¹ introduced 2,4-dinitro-5-fluoroaniline as a reagent for the formation of amino acid derivatives. Its behavior is similar to that of 2,4-dinitrofluorobenzene and it can be used in similar circumstances. The dinitroaminophenyl (DNAP) derivatives have a free amino group which can be diazotised and coupled with phenols to form intensely colored azo compounds.

J. Chromatog., 11 (1963) 111-112

BERGMAN AND BENTOV suggested spray reagents for conducting the diazotisation and coupling directly on paper chromatograms. When their aqueous spray reagents were tried on silica gel G thin layer chromatograms, the water soluble diazonium salts diffused to produce large irregular spots. For this reason, the non-aqueous system reported here was developed.

The thin layer chromatograms of the DNAP amino acids were first sprayed with a toluene solution of nitrogen trioxide (N_2O_3) and then with a toluene solution of phenol and diethylamine. The DNAP amino acid spots, which were originally light yellow turned rusty brown following this treatment. Since neither the DNAP amino acids, nor their diazonium salts nor the azo dyes were eluted from silica gel by toluene, no diffusion of the spots was noticed. This reagent system permitted the detection of 10^{-9} moles of DNAP amino acid.

Toluene solution of nitrogen trioxide: 10 ml of toluene was layered on 10 ml of 6 N HCl and 10 ml of 3 M $NaNO_2$ was added. The aqueous solution turned green and gas was evolved vigorously. Nitrogen trioxide and other materials were extracted into the toluene by swirling. The upper layer was used immediately as the spray reagent and discarded after use. Toluene solution of phenol and diethylamine: 9.4 g (0.1 mole) of solid phenol and 14.6 g (0.2 mole (20.6 ml)) of diethylamine were mixed with toluene to make 100 ml of solution. The mixture was initially a pale yellow but darkened on standing for a few weeks.

This method is similar to that used by GRIESS² in the original discovery of diazonium salts. In his synthesis, "nitrous acid gas" was prepared by the reaction of nitric acid with arsenious oxide and bubbled into the amine salt in ethanol; the diazonium salt crystallized out as it formed.

When hydrochloric acid and sodium nitrite solutions are mixed, nitrous acid is produced but it decomposes almost immediately to nitrogen trioxide which can be extracted into toluene. The solution so obtained will diazotise aromatic amines. Diazonium salts couple with salts of phenols. Here, the diethylamine salt of phenol was used although other phenols and other organic bases are suitable. Neither phenol nor diethylamine are strong electrolytes and toluene is not a good ionizing solvent but nevertheless there is a small equilibrium concentration of phenoxide ion to couple with the diazonium salt.

This work was performed under a grant from the Surgeon General of the U.S. Army.

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Received September 6th, 1962