in double peaks located near the position of each of the smaller peptides in the partial acid hydrolysate. At least two homologous polylysine series were present in the lower molecular weight fractions which probably differ in their amino or carboxyl terminal end. The lower molecular weight series contained peptides with from one to about twenty lysine residues per molecule. The degree of polymerization of the higher series was estimated at 250 from the intrinsic viscosity in dimethyl formamide of the recarbobenzoxylated fraction[10,11]. Such analyses have shown a bimodal molecular weight distribution in the products of polymerization of ε-carbobenzoxy-L-lysine N-carboxy anhydride initiated in dioxane solution with ammonia at molar ratios of monomer to initiator of from 6 to 50[12]. We believe that the new principles illustrated in this study will be of value in the isolation and analysis of polyamino acids, polynucleotides, proteins, and other polyelectrolytes.

Acknowledgement

This work was supported in part by research grants (No. E-101) from the National Microbiological Institute of the National Institutes of Health, U.S. Public Health Service. Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

Department of Biochemistry, University of Wisconsin, Madison, Wisc. (U.S.A.)

JOHN W. STEWART
MARK A. STAHMANN

1 E. A. PETERSON AND H. A. SOBER, *J. Am. Chem. Soc.*, 78 (1956) 751.
2 S. G. WALEY AND J. WATSON, *Biochem. J.*, 55 (1953) 328.
3 S. G. WALEY AND J. WATSON, *J. Chem. Soc.*, (1953) 475.
4 H. G. KHORANA AND J. P. VIZSOLYI, *J. Am. Chem. Soc.*, 83 (1961) 675.
5 W. G. MILLER, *J. Am. Chem. Soc.*, 83 (1961) 259.
6 A. TISELIUS, *Arkiv Kemi*, 7 (1954) 443.
7 S. MOORE AND W. H. STEIN, *Advan. Protein Chem.*, 11 (1956) 191.
8 R. R. PORTER, in C. LONG, *Biochemists' Handbook*, D. Van Nostrand Co. Inc., Princeton, N. J., 1961, p. 115.
9 R. R. BECKER AND M. A. STAHMANN, *J. Am. Chem. Soc.*, 74 (1952) 38.
10 J. B. APPLEQUIST, *Ph. D. Thesis*, Harvard University, 1959.
11 A. BERGER AND A. YARON, in M. A. STAHMANN (Editor), *Polyamino Acids, Polypeptides and Proteins*, University of Wisconsin Press, Madison, Wisc., 1962, p. 13.
12 J. W. STEWART AND M. A. STAHMANN, in M. A. STAHMANN (Editor), *Polyamino Acids, Polypeptides and Proteins*, University of Wisconsin Press, Madison, Wisc., 1962, p. 95.

Received May 21st, 1962

J. Chromatog., 9 (1962) 233–235

Detection of ketosteroids on chromatograms

The detection of ketosteroids may be carried out on untreated strips by means of contact photography in U.V.-light at about 253 mμ. This method, however, is specific for Δ^4-3-ketosteroids and therefore saturated ketosteroids are not detectable in this way. The strips may also be treated with various, more or less specific reagents; in this case fluorescence in U.V. light or colouring of the spots occurs. However, treated chromatograms cannot be used for further quantitative evaluation, owing to the

stained background. Using fluorescence methods the strips usually undergo complete destruction. Moreover, the nonsteroid substances, such as neutral lipids and phospholipids, which are still present in the chromatographed extracts, give similar reactions with some reagents[1] and can therefore cause misinterpretation of the results.

For the detection of ketosteroids 2,4-dinitrophenylhydrazine (DNPH) was chosen as one of the most general reagents for ketones; an aqueous solution was used, since an alcoholic one may elute steroids from the chromatogram before the reaction with DNPH takes place.

In order to eliminate most, if not all, of the sources of error due to staining, the possibility was investigated of removing the unspecific chromogens and an excess of DNPH, without destroying the steroid hydrazones formed. Since it is well known, that various organic hydrazines are easily decomposed by mild oxidants, such as the Benedict reagent[2], several inorganic salts with oxidative properties were tested.

The best results were obtained with a solution of $KMnO_4$ made slightly alkaline with Na_2CO_3. It was proved by densitometry at 380 mμ, that almost all the colour due to phospholipids and any excess of the reagent disappeared after alkaline permanganate treatment during 10–30 min at room temperature in the pH range 7.5–10, while the steroid hydrazone spots remained unchanged after a similar treatment during 6 hours. A solution of ascorbic acid was used to remove the MnO_2 formed.

Pure steroids and corpus luteum extracts alone, or with steroids added, were chromatographed in the Bush system A (heptane–80% methanol) at 34° on Whatman No. 1 paper, using the "Keilstreifen" method of MATTHIAS[3] and the ascending technique. The results are given in Table I.

TABLE I

	PGS	*TSN*	*ADN*	*PGN*	*DHA*	*17-OH-PGS*	*PDN*
Final colour of the spot	orange	red-orange	purple-orange	lemon yellow	light lemon yellow	red-orange	yellow
Amount visually detectable $\mu g/cm^2$ (*ca.*)	0.5	0.5	0.5	1	5	0.5	1

Abbreviations: PGS = progesterone; TSN = testosterone; ADN = Δ^4-androstene-3,17-dione; PGN = Δ^5-pregnen-3β-ol-20-one; DHA = dehydroepiandrosterone; 17-OH-PGS = 17α-hydroxyprogesterone; PDN = pregnane-3,20-dione.

Reagents

(1) 0.1% solution of DNPH in 2 *N* HCl; stable for 2–3 weeks if stored in a cool and dark place.
(2) 1% $KMnO_4$.
(3) 10% $Na_2CO_3 \cdot 10H_2O$.
(4) Neutralizing fluid: dilute 10 ml of (3) to 100 ml with water.
(5) Decolorizing fluid: mix 2 ml of (2) and 1 ml of (3); dilute to 100 ml with water. Stable for one day.
(6) Reducing fluid: 0.2% solution of ascorbic acid; add one drop of concentrated hydrochloric or sulphuric acid per 100 ml of the fluid. Stable for one day.

All operations should be carried out at room temperature.

Procedure

The chromatograms are drawn through the DNPH solution and held in the air for 10 min. The wet strips are then washed for 1 min in the neutralizing fluid, 10 min in the decolorizing fluid, 5 to 10 min in the reducing fluid and finally in tap water.

If the decolorization is not complete, decolorizing with subsequent washings repeated once or twice will be helpful. After the last washing in water the strips are air-dried and can be stored for further use.

The procedure described increases the sensitivity of the DNPH qualitative test about 5 times, as compared with the data of NEHER[4], when non-impregnated papers are used.

Owing to its simplicity and satisfactory results, the method described seems to be a convenient basis for quantitative analysis of ketosteroid hydrazones. These applications are now under detailed investigation and will be published later.

Institute of Animal Physiology and Nutrition, Jablonna near Warsaw (Poland)

R. STUPNICKI
ELZBIETA STUPNICKA

[1] R. STUPNICKI AND E. STUPNICKA, unpublished observations.
[2] K. HINSBERG, H. PELZER AND A. SEUKEN, *Biochem. Z.*, 328 (1956) 117.
[3] W. MATTHIAS, *Züchter*, 24 (1954) 313.
[4] R. NEHER, *J. Chromatog.*, 1 (1958) 205.

Received May 22nd, 1962

J. Chromatog., 9 (1962) 235–237

Two-dimensional paper chromatography of cholesteryl esters

A new method for the separation of critical pairs

During recent years many authors have studied various chromatographic systems for the separation of cholesteryl esters[1-4]. Although in several cases good results were obtained no useful resolution of critical pairs of these substances by paper chromatography was described.

A two-dimensional technique in which adsorption and reversed-phase partition systems are combined enabled us to solve this problem.

Experimental

Cholesteryl esters. Esters of saturated fatty acids were synthetized by the method of KAUFMANN, MAKUS AND DEICKE[5]. Unsaturated esters were prepared enzymically according to MAHADEVAN AND GANGULY[6].

Impregnation of paper with silica gel. Whatman No. 3 paper (18 × 46 cm) was immersed in a mixture of about 2.5 % (v/v) solution of sodium silicate (prepared by dilution of a stock solution 36–38° Bé) and 5 % ammonium chloride in the ratio 100:30 (v/v). The excess of impregnating agent was removed by rubbing both sides of the paper with a glass rod. After drying in the air overnight the papers were washed three times in distilled water (*e.g.* using 1000 ml for six sheets of 18 × 46 cm) for