PAPYROGRAPHIC STUDIES ON PEPTIDES

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RANGA RAO AND SREENIVASAYA[1] have shown that the non-protein nitrogen (N.P.N.) fraction of the body fluids of the lac insect (*Laccifer lacca*) contains simple crystalloidal peptides, non-precipitable by trichloracetic acid. Milks obtained from different types of mammals[2] and pulses[3] have also been shown to be associated with high percentages of N.P.N. whose presence therein is believed to be responsible for the ease with which they are assimilated. Peptides are widely distributed and are intimately associated with all active and proliferating tissues—both plant and animal—and owe their existence to the continual breakdown and resynthesis of proteins which characterise living tissues and body fluids. Particularly rich is their concentration in the body fluids of animals and the saps of plants, since their role is one of providing tissues with an easily mobilisable source of nitrogen exceptionally adaptable for rapid tissue formation.

Special physiological significance is attached to some of the peptides ; they have been found to act as co-enzymes or activators, essential growth factors or antibiotics. Glutathione,[4,5] the well-known tripeptide, for example, is a co-enzyme of methyl glyoxylase, an activator of papain[6] and an effective stabiliser of ascorbic acid.[7] Its unfailing presence in actively proliferating tissues is suggestive of the suspicion that glutathione may have other functions yet undiscovered. More recently, glutathione, γ-glutamyl, and acyl peptides have been shown to participate in the enzymatic transpeptidase reactions.[8,9] Strepogenin[10] which was discovered by Woolley in 1944, and which has been shown to be present in most of the proteins of high biological value,[11] stimulates the growth of certain bacteria. Subsequently other investigators have sought to isolate other peptides from enzymatic digests of proteins and determine their growth-promoting potency. Ågren[12] has found significant increases in the growth of children fed with peptides resistant to the action of catheptic enzymes. Dunn[13] has recorded the stimulating effect of partially hydrolysed digests of casein and of the albumin of bovine plasma, on *L. casei* resulting in a higher rate of acid production. It was shown that the organism utilised the essential amino acid more readily when provided in a peptide-bound form. Simonds and Fruton[14] have also observed that a mutant of *E. coli* utilises for growth peptides of proline at a faster rate than proline. A genus of "alcaligens" utilised leucine peptides only when leucine was present at the amino end of the unsubstituted peptide.

Another entirely new group of peptides, which has received considerable attention, is derived from the culture filtrates of bacteria, *e.g.*, polymyxins, gramicidin, tyrocidine and Tyrothricin.[15] The structure of these antibio-

tically active peptides have been thoroughly elucidated and in the accomplishment of this task, papyrographic[16] and column chromatographic methods[17,18,19] have been extensively employed.

Fractionation, isolation and characterisation of such peptides present in enzymatic digests of proteins, body fluids, bacterial filtrates, etc., have presented problems difficult to solve but the recent techniques of papyrography and column chromatography promise to provide the means of achieving the objective with exceptional ease and elegance. Stein and Moore,[17,18,19] have extended the use of starch columns for a fractionation of the protein hydrolysates and more recently, Ottesen and Villee[20] have employed starch column chromatography for fractionating the peptides released during the enzymatic transformation of ovalbumin to plakalbumin. Papyrographic technique has been employed by Jones[21] and Cutch, *et al.*,[22] in a study of the separation and amino acid make-up of the polymyxins.

The present study is devoted to a determination of the Rf values of some nine peptides and three chloracetyl derivatives of amino acids employing two solvent systems and describes special methods for the location and characterisation of certain aromatic amino acid peptides. These methods have been developed mainly with the objects of applying them to a study of the nitrogenous constituents associated with hæmolymph and the silk gland of the silkworm.

Experimental

The synthetic peptides employed in our studies were all obtained from Hoffman La Roche (Switzerland). Aliquots of 1 μl. of a 1·0 per cent. aqueous solution of the peptide were employed for spotting along a line drawn 2 cm. above the edge of a rectangular sheet (28 cm. × 22 cm.) of Whatman No. 1 filter sheet. Spacing the spots 2 cm. apart, 13-14 spots could be accommodated on the sheet for a one-dimensional development of the papyrogram. The "spotted" sheet was rolled into a cylinder, which was then left in the developing chamber containing the solvent system, butanol—acetic acid—water (10 : 2·5 : 10) or pyridine—water (4 : 1), for a couple of hours with a view to have the cylinder equilibrated with the vapour phase of solvent system. At the end of this period, the cylinder was carefully lowered into the flat-bottomed dish containing the developing solvent. The development of the papyrogram, which was conducted at the room temperature (24° C.) takes 5-6 hours. The cylinder was then removed from the chamber, unfolded and the sheet air dried.

First an ultra-violet print (U.V.P.) was taken by the method of Markham and Smith;[23,24] then the paper was sprayed with 0·2 per cent. solution of ninhydrin in water-saturated butanol. The paper was then air dried and after making observations, the paper was further dried in a hot-air-oven (80° C.) for 10 minutes and again examined for the ninhydrin positive spots. The Rf values of amino acid derivatives and the peptides have been determined in the usual way, making use of both the ninhydrin positive and the ultra-violet opaque spots obtained in the U.V. print in the case of some of the "aromatic" peptides (see Table I). Observations made during the period of drying of the ninhydrin sprayed papyrogram, showed that a few of the spots appeared earlier than others. For example, alanyl glycine, glycyl glycine and diglycyl glycine were among the first to show the colours. The colours developed by glycyl glycine and di-glycyl glycine to begin with, are yellow which, later develops into a brownish pink.

TABLE I

Peptides	Butanol water	Ultra-violet print	Pyridine water
Alanylglycine ..	0·33	..	0·4
Glycylglycine ..	0·26	..	0·25
Diglycylglycine ..	0·22	..	0·30
Leucylglycine ..	0·60	..	0·63
Leucylglycylglycine ..	0·52	..	0·68
Glycyl-tryptophane ..	0·54	0·54	0·60
Glycylleucine ..	0·66	..	0·55
Glycyltyrosine ..	0·46	0·46	0·65
Glutathione ..	0·19	..	0·13
Chloracetyltyrosine ..	..	0·97	..
Chloracetyltryptophane	..	0·98	..
Chloracetylleucine ..	..	..	..

It will be seen (Table I) that the Rf values of the peptides obtained with the two solvent systems do not overlap; they are sufficiently wide apart to permit discrete separations of peptides in mixtures. Further, the individual spots of the peptides are smaller and more compact, a circumstance which adds to the efficiency of discrete separations and effective locations of spots either by the U.V.P. or the ninhydrin method. The U.V. printing of the pyridine-water developed papyrogram, is not possible in view of the fact that the residual pyridine which is difficult to eliminate from the

papyrogram itself absorbs the ultra-violet radiation.

Cf the peptides and amino acid derivatives which have been studied, the chloracetyl derivatives of leucine, tryptophane and tyrosine do not react with ninhydrin, since they have no free amino group. The location of two of the "aromatic" amino acid derivatives on the papyrogram was carried out by the U.V.P. method while the location of the leucine derivative is not possible. The spots of the aromatic amino acid derivatives and peptides could also be detected by reacting the papyrogram with dimethyl *p*-aminobenzaldehyde for the characterisation of the tryptophane peptide which yields a violet spot while the tyrosine derivative could be located by the brick-red colour developed with Millon's reagent.

The peptide spots were individually excised from the papyrogram, extracted with hot water by means of a microfilter, and the extracted peptide concentrated to a small volume in vacuum over silica gel. The solution was then hydrolysed in a sealed tube with 5 N hydrochloric acid for 2·5 hours at 100° C. The hydrolysate was freed of its HCl and concentrated by evaporating the solution in vacuum over caustic potash and sulphuric acid placed separately in a desiccator. The hydrolysate, when spotted and developed, gives the constituent amino acids of the peptide.

It will be seen from the preceding discussion that papyrographic methods when coupled with U.V.P. and micro-hydrolysis techniques, are useful for a separation and characterisation of the peptides and amino acids when they occur in mixtures as they do in enzymatic digests of proteins, plant saps, body fluids and tissue extracts. These methods, together with two-dimensional papyrographic methods, are being extended to a study of the hæmolymph and the silk gland of the silkworm.

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