ISOLATION OF AN AMINO ACID-LIPID COMPLEX FROM A PROTEOLYTIC DIGEST OF LIPOVITELLIN

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Lipovitellin isolated from the hen's egg has been extensively studied by Cook (1961) and his group, who developed procedures for the isolation of the pure lipoprotein completely separated from lipovitellenin and phosvitin (Burley and Cook, 1961) and of separating lipovitellin into two similar proteins, α-lipovitellin and β-lipovitellin, which differ primarily in phosphorus content and in electrophoretic behavior (Bernardi and Cook, 1960).

PROCEDURE

Lipovitellin was prepared essentially by the procedure of Burley and Cook (1961). Ether-extracted freshly prepared lipovitellin was suspended in 0.02 M phosphate buffer pH 7.8, 0.1 mg. of trypsin[2] (2 x recrystallized, 50% $MgSO_4$) per ml. was added, and the mixture was incubated at 37°C. with shaking for 24 hours. The trypsin digest was separated into soluble and insoluble fractions by centrifugation at 15,000 r.p.m. for 30 minutes in a MSE 1700 refrigerated centrifuge. The insoluble fraction was suspended in 0.02 M phosphate buffer pH 7.8, and 1.8 mg. of ethylenediaminetetraacetic acid per ml., 0.73 mg. cysteine per ml., and 0.13 mg. of papain[3] (suspension of 2 x

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crystallized papain) per ml. were added, and the mixture was incubated with shaking at 37°C. for 24 hours. The papain digest was also separated into soluble and insoluble fractions, and the insoluble fraction was suspended in 0.03 M phosphate buffer pH 7.4, 0.1 mg. of Pronase[4] per ml. was added, and the mixture was incubated with shaking at 37°C. for 48 hours. The Pronase digest was separated into three fractions by centrifuging; insoluble fraction R, soluble fraction S, and a rising fraction T.

Fraction S was separated into seven fractions by filter paper electrophoresis using the Shandon ridgehole tank based on the apparatus developed by Durrum (1950) with 1% ammonium carbonate pH 8.9 as the buffer. Separations were carried out on acetic acid extracted Whatman No.3 filter paper sheets (17 x 36 cm.) with 6v./cm. for 5 to 7 hours. One 1-cm. marker strip was dipped in 0.25% (w/v) ninhydrin solution in acetone and heated at 80 to 100°C. for 5 min. to detect peptides, and another was placed in a container with Sudan Black B solution (saturated solution in 50% ethanol) for at least 30 minutes and washed once with 50% ethanol and twice with 40% ethanol to locate lipids. Each band was marked on the original filter paper sheet, and after cutting the band from the sheet the band was then eluted with distilled water. After drying, the water-eluted lipid-containing band was extracted with chloroform. Water was removed by lyophylization and chloroform by evaporation at room temperature. The eluates and chloroform extract were hydrolyzed with redistilled constant boiling hydrochloric acid in a sealed tube for 24 hours in a boiling water bath. Acid and water were removed over sulfuric acid and sodium hydroxide in an evacuated desiccator. The residue was dissolved in two drops of distilled water and applied as a spot on one corner of a

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4. Kingsley and Keith (Chemicals) Ltd., London (contained 45,000 p.u.k. per gram).

22 1/2 x 18 inch sheet of Whatman No.1 filter paper, drying with a stream of hot air between additions. The sheet was chromatographed in the long direction with phenol-ammonia followed by n-butanol-acetic acid-water (4:1:5:, v/v/v) in the short direction. The dried paper was sprayed with a 0.1% solution of ninhydrin in n-butanol and the colour was developed by heating for 5 minutes at 80-100°C. Amino acids present were determined by comparison with a map previously prepared using known amino acids.

RESULTS

One of the seven bands from the paper electrophoretic separation of fraction S did not migrate and was the only band that reacted positively to the test for lipid. Results of amino acids present in each band as determined by paper chromatography are presented in Table I. Band 1 migrated towards the negative electrode, band 2 was the stationary one, and the other bands migrated towards the positive electrode with increasing speed from band 3 to band 7. From the large number of amino acids found in each band, it appears that each of the bands obtained was either a large peptide or a mixture of smaller peptides. The exception is the water insoluble, chloroform soluble lipopeptide (or amino acid-lipid) which contained but three amino acids; glutamic acid, serine, and glycine.

The eluates and chloroform extract from another electrophoretic separation of fraction S were reacted with fluorodinitrobenzene (FDNB) by the procedure of Sanger and Thompson (1953), the DNP-peptides were hydrolyzed for 16 hours, and the DNP-amino acids obtained were separated by filter paper chromatography using the tert.-amyl alcohol-phthalate buffer system of Blackburn and Lowther (1951). The large number of N-terminal amino acids (marked with an asterisk in Table 1) found in the water eluted bands show the presence of several peptides in each band. No N-terminal amino acids were detected in either the

TABLE I

Amino acids in fractions of fraction S.

Band 1	Band 2	Band 3	Band 4	Band 5	Band 6	Band 7	Chloroform ext. of band 2
Asp	Asp	Asp	Cys	Cys	Asp	Cys	Glu
Glu*	Glu*	Glu*	Asp*	Asp*	Glu*	Asp*	Ser
Ser	Ser*	Ser	Glu*	Glu*	Ser*	Glu*	Gly
Gly	Gly	Gly	Ser	Ser*	Gly*	Ser*	
Ala	Ala*	Thr*	Gly	Gly*	Thr*	Gly*	
Lys	Leu	Ala*	Thr	Thr*	Ala*	Thr*	
Arg	Ileu	Lys	Ala*	Ala*	Arg	Ala*	
Val*		Arg	Tyr	Tyr	Val*	Val*	
Met		His	His	His	Met	Met	
Leu		Val*	Lys	Lys	Leu*	Leu*	
Ileu		Met	Arg	Pro	Ileu	Ileu	
		Phe*	Pro	Val*			
		Leu*	Val*	Met			
		Ileu	Met	Leu*			
			Phe	Ileu			
			Leu*				
			Ileu				

* N-Terminal amino acids.

water or the ether soluble products of reaction of FDNB with the lipopeptide from fraction S.

Another experiment was carried out in the same way except that the water soluble amino acid fraction from the hydrolyzed DNP-lipopeptide was chromatographed for amino acids rather than for DNP-amino acids. There were no DNP-amino acids obtained from the lipopeptide, and the water soluble portion contained glutamic acid, serine, and glycine

confirming the previous observation that no amino acids had reacted with FDNB (Sanger and Tuppy, 1951).

DISCUSSION

The presence of three amino acids in the lipids of fraction S is of interest. Fraction S is composed of potassium and sodium phosphates from the buffer, lipids, and peptides that were insoluble after trypsin and papain digestion but were solubilized by digestion with Pronase, which breaks most peptide bonds (Nomoto, et al., 1960). Just how these particular amino acids are bound to the lipids is not known at present, nor is it known whether the amino acids are connected together as a peptide or if they are combined with lipid as individual amino acids similar to those found in hen oviduct lipids by Hendler (1961). The absence of a terminal amino group suggests that the amino acids are connected to the lipid through the amino group of one or all amino acids.

SUMMARY

Lipovitellin, isolated from yolk of the hen's egg, was broken down by progressive proteolytic hydrolysis, first by trypsin, a highly specific enzyme, then by papain, an enzyme of intermediate specificity, and finally by Pronase, a highly non-specific enzyme.

Fraction S, the water-soluble fraction obtained by Pronase hydrolysis of the trypsin and papain resistant core of lipovitellin, contained peptide, lipid, and potassium and sodium phosphates from the buffer. All of the lipid was concentrated in one non-mobile band after filter paper electrophoresis. When this band was eluted with water, dried, and then extracted with chloroform, the lipid that was extracted contained three amino acids; serine, glutamic acid, and glycine. Fraction S also contained several lipid-free peptides. None of the amino acids in the lipid fraction reacted with fluorodinitrobenzene, indicating that there were no free amino groups.

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