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Studies on the pulmonary surface-active lipoprotein*

I. Isolation and chemical characterization of its constituents

by

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Chemical analyses have shown that the surfactant agent of the mammalian lung is a lipoprotein complex. The first attempt to establish its nature was made by PATTLE and THOMAS in 1961 (38); since then many other investigators have studied the chemical composition of the lung surface-active lipoprotein (SA-lipoprotein), though most of the works have focused their attention on the lipid fraction of the surfactant agent (2, 3, 4, 9, 10, 15, 26, 37, 55).

In 1967 MCCLENEHAN (33) showed for the first time that the surface activity of the SA-lipoprotein can be significantly reduced by an alteration of its peptide moiety alone, suggesting that both the lipid and the peptide moieties are essential to its biological activity.

The present study is concerned with the isolation and establishment of the chemical characteristics of the SA-lipoprotein and its two main components, the lipid and the peptide moieties. It has been undertaken in the hope that new findings may extend present knowledge on the structure of the lung surface active agent and of lipoproteins in general.

Precise information concerning the chemical composition of the SA-peptide moiety, particularly its amino acid composition, and the participation of carbohydrates is included in this paper. Ultracentrifugal studies and some

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of the physicochemical characteristics of the SA-peptide moiety will be presented in a later paper.

MATERIAL AND METHODS

Ordinary laboratory chemicals used in this research were of analytical reagent grade. Unless stated otherwise, optical densities for each test were read with 1 cm quartz cuvettes in the Beckman DB Spectrophotometer.

ISOLATION OF THE SA-LIPOPROTEIN COMPLEX

During the experimental part of this study, eight lots of the SA-lipoprotein were isolated from fresh lung tissue: two lots from guinea pigs (Hartley strain from the Vivarium of the University of Southern California); three lots from human beings (obtained post-mortem from the Los Angeles County-University of Southern California Medical Center); and three lots from cows (from the Los Angeles Meat Company, Vernon, California).

SA-lipoproteins were isolated from lungs according to ABRAMS (2). Fresh lungs were homogenized in a Servall Omnimixer and lipoprotein materials were separated by means of differential centrifugation in salt gradients utilizing a Servall RC-2 Superspeed refrigerated centrifuge (SS-34 rotor). The flotation and pelleting of the SA-lipoprotein materials were repeated twice. The pellicle containing the SA-lipoprotein complex was dialyzed against 0.85 per cent sodium chloride containing 0.01 per cent of the sodium salt of EDTA. The SA-lipoprotein concentrations in these dialysates were estimated by drying aliquots to constant weight. The protein content of dried SA-lipoprotein was estimated on the basis of its nitrogen content.

CHEMICAL CHARACTERIZATION OF THE SA-LIPOPROTEIN COMPLEX

In order to establish the chemical composition of the materials isolated in our laboratory, a series of tests were carried out as follows:

Total nitrogen. This was estimated according to the Technicon Micro-Kjeldahl method (56) which involves complete digestion of organic material in a continuous digestion unit. Determination of ammonia in the resulting digest was achieved with a phenol-hypochlorite system, by which ammonia is converted to a blue-colored compound of indophenol type. A factor of 6.25 was used for calculation of protein content from total nitrogen content.

Lipid analysis. The lipid composition of SA-lipoprotein was studied using lipid extracts obtained at very low temperature with 2:1 chloroform-methanol (v/v) by freezing and thawing the mixture three times in a solution of 2-ethoxyethanol (Matheson) and dry ice. The chloroform-methanol solvents were removed from the extracts by heating the tubes on a hot plate at 80C.

Phospholipids. Phosphorus was estimated according to FISKE and SUBAROW (16). For total phosphorus, the samples were digested at 160C in

a perchloric-sulfuric acid mixture until the solution was clear (29). The color was subsequently developed by the procedure of BARTLETT (8). Phospholipids were calculated by multiplying lipid phosphorus values by 25 (assuming a 4 per cent of total phosphorus per mol of lecithin). Readings of total phosphorus were done in a Klett-Summerson photoelectric colorimeter, utilizing the Nº 66 filter.

Total cholesterol. The method used here was that recommended by SEARCY and BERGQUIST (45). Readings of cholesterol were done in a Klett-Summerson photoelectric colorimeter with filter Nº 50.

Triglycerides. These were determined according to the method of VAN HANDEL and ZILVERSMIT (57). Lipids were extracted with the aid of a zeolite (sodium silico aluminate) and chloroform (18); the glycerol moiety of the triglycerides was determined according to LAMBERT and NEISH (27).

Fatty acids. Determined according to the method proposed by STERN and SHAPIRO (54).

ISOLATION AND PURIFICATION OF THE PEPTIDE MOIETY OF THE SA-LIPOPROTEIN

Although SA-lipoproteins were isolated from three different species of mammals, the physical and chemical characterization of the peptide moiety was carried out only with lipoproteins isolated from bovine lung.

Delipidation Comparative delipidation studies were carried out utilizing the methods proposed by FOLCH *et al.* (17), the modification of SCANU (40) to the technique of SCANU *et al.* (42) and with a new method developed in our laboratory (25) as follows: One volume of the SA-lipoprotein suspension was extracted with 25 volumes of a mixture of 3:2 of ethanol-diethyl ether (v/v) in a flat-bottom, glass-stoppered flask. The mixture was cooled to —70C by immersion for four hours in a container with 2-ethoxyethanol and dry ice, then shaken gently in an automatic shaker operated at very low speed. The cooled lipoprotein-organic solvent mixture was then centrifuged at 15,000 rpm for 30 minutes at 4C in a Serv-all refrigerated centrifuge. The precipitate was suspended in a fresh 3:2 ethanol-ether solution (50 volumes of organic solvent for each volume of precipitate) and allowed to stand for twenty-four hours at —20C in the freezer. The delipidized lipoprotein was recovered by centrifugation at 15,000 rpm for 30 minutes at 4C. The precipitated peptide material was then washed three times with the ethanol-ether solution, and finally three times with diethyl ether in order to remove all traces of alcohol. The precipitate was allowed to dry under nitrogen and stored at —10C until used. Delipidized materials obtained by three different techniques were analyzed for the presence of phospholipids and cholesterol.

Purification of SA-peptide. The dried delipidized lipoprotein material was rendered soluble by stirring at room temperature with 0.1 N sodium hydroxide for 24 hours. These crude peptide solutions were concentrated and ultrafiltered under pressure over a Diaflo Membrane, type UM-1. The ultrafiltered,

concentrated SA-peptide solutions were then successively gel filtered through columns of Sephadex G-20, G-50, and G-100 of medium particle size. Elutions were performed with 0.1 M sodium chloride solutions at pH 10.0. Recovery of the peptide was determined by measuring light absorption at 280 mμ of the collected fractions. The SA-peptide concentrations were estimated by drying aliquots to constant weight and the protein content was estimated by measuring its total nitrogen content, and/or by use of the microbiuret method (5).

Purity of SA-Peptide. Analytical ultracentrifugation, electrophoresis and gel filtration were employed to assess the purity of the isolated SA-peptide. In addition, efforts were made to determine whether the carbohydrates present were an intrinsic part of the peptide or resulted from contamination with a glycoprotein.

Chemical characterization of the SA-peptide moiety. Presence of lipids and carbohydrates on the purified SA-peptide materials was investigated. The content of sialic acid (as N-acethyl-neuraminic acid) was measured according to WARREN (58). The technique of DISCHE and SHETTLES (14), as modified by DISCHE (13), was used in order to measure the content of fucose (as methyl pentose). The orcinol test proposed by LUSTIG and LANGER (30), as modified by FRANCOIS *et al.* (19), was utilized for the estimation of neutral hexoses, and hexosamines (as glucosamine) were determined by the modification of the Elson-Morgan test by RONDLE and MORGAN (39).

Establishment of the peptide-carbohydrate moiety existence. In order to establish that the presence of carbohydrates in the peptide moiety solutions could not arise from protein impurity of high carbohydrate content, the purified SA-peptide was precipitated with 5 per cent trichloroacetic acid or with 0.6 M perchloric acid, to separate it from known glycoproteins (44). Precipitates were collected by centrifugation, washed, suspended in distilled water and measurements of the sialic acid content were then performed.

Amino acid analysis.* One milliliter of concentrated hydrochloric acid (12 N) was added to 1.0 to 4.5 mg of delipidized SA-lipoprotein in 1 ml water solution; the glass tubes were evacuated, sealed under nitrogen, and heated at 110 ± 1C for 22, 46, or 72 hours. Hydrolysates were dried over NaOH pellets *in vacuo*. The dry samples were dissolved in 2.0 ml 0.20 N sodium citrate-HCl buffer, pH 2.2.

Aliquots were analyzed by ion exchange chromatography with the technique of MOORE, SPACKMAN, and STEIN (36), in a Beckman/Spinco model 120B amino acid analyzer (49). The analyzer was calibrated with the type 1 amino acid calibration mixture (provided by Beckman Instruments, Inc., Palo Alto, California) and, in studies of performate-oxidized peptide, with cysteic acid. Chromatography was carried out according to the procedure for "Standard 50º run" (48).

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Half-cystine was determined as cysteic acid after oxidizing the SA-peptide with performic acid according to the methods of SCHRAM *et al.*, (43), MOORE (35), as recommended by BAILEY (7). The oxidized material was hydrolyzed in the usual way with 6 N HCl at 110 ± 1C for twenty-two hours, and aliquots were analyzed by ion exchange chromatography with the Beckman/Spinco model 120B amino acid analyzer.

The tryptophan content of the intact SA-peptide moiety was determined colorimetrically according to procedure L of the method proposed by SPIES and CHAMBERS (52). Reactions were carried out at room temperature and protected from light. Optical densities were read at 590 mμ and transmittancy values were converted to weight of tryptophan from standard curve C of SPIES and CHAMBERS (51).

Calculation of amino acid composition. Data from the Analyzer were corrected for losses of unstable amino acids during acid hydrolysis.

Values of glutamic acid, aspartic acid, lysine, hydroxylysine, threonine and serine were obtained by extrapolation to zero time of hydrolysis to account for apparent loss of these compounds. The amide ammonia content was also estimated by extrapolation to zero time of hydrolysis. Values for the contents of half-cystine and trytophan were obtained by methods already described. The moles of each amino acid residue were converted into grams of amino acid residue per 100 grams of peptide. The number of moles of each amino acid present in 100,000 grams of peptide was also calculated, and from these values minimal molecular weights of the peptide moiety were computed. The nitrogen value for each amino acid residue was also calculated as per cent of total nitrogen.

Biological activity. The possibility that either the SA-lipoprotein complex or the SA-peptide moiety possess biological activity was explored, especially with regard to phosphatase activity. Alkaline phosphatase activity was studied using the Technicon method (56).

RESULTS

ISOLATION AND CHEMICAL ANALYSIS OF SA-LIPOPROTEIN

Normal guinea pig, human and bovine lungs have been found to contain 7.5 ± 2.5 mg of SA-lipoprotein complex per gram of wet tissue.

Table 1 shows the chemical composition of the three species of SA-lipoproteins isolated in our laboratory from guinea pig (n=4), human (n=6), and cow (n=6) lungs. The three species of lipoproteins have a practically identical chemical composition. Peptide moieties represent about 60 per cent of the whole SA-lipoproteins. The lipid fractions consist mainly of about 70 per cent phospholipids, 6 per cent total cholesterol, 16 per cent triglycerides and 10 per cent fatty acids. No attempt was made to estimate the amount of cholesterol esters, or to separate and determine the components of the phos-

TABLE 1

Chemical composition of various lung surface-active lipoproteins[A]

Constituent	Guinea Pig[B]	Human[C]	Cow[D]
Peptide (N × 6.25)	59.2 ± 0.70*	60.0 ± 0.65*	59.7 ± 0.59*
Phospholipid	28.2 ± 0.50	28.0 ± 0.57	28.1 ± 0.48
Total cholesterol	3.3 ± 0.10	2.3 ± 0.16	2.5 ± 0.18
Triglycerides	5.7 ± 0.30	5.6 ± 0.24	5.5 ± 0.25
Fatty acids	4.6 ± 0.20	4.1 ± 0.24	4.2 ± 0.24

A. Content in 100 mg of dry weight.
B. Average of four determinations.
C. Average of six determinations.
D. Average of six determinations.
* Standard deviations.

TABLE 2

Total cholesterol, phospholipid and total nitrogen content in 1 ml of a concentrated suspension of cow lung SA-lipoprotein before and after delipidation by three different techniques

Content In	Cholesterol		Phospholipid		Nitrogen	
	Mg %	%	Mg %	%	Mg %	%
SA-lipoprotein	2.5	100	28.1	100	9.6	100
Extracted once, Folch	1.05	42*	1.97	7*	8.8	91.7*
Extracted twice, Folch	0.47	31*	0.28	1*	8.4	87.5*
Extracted once, Scanu	ND	——	1.0	3*	9.1	95.0*
Extracted once, Jiménez	ND	——	ND	—	9.2	95.8*

* Percent of the original concentration.
ND Not detectable by the analytical methods used.

TABLE 3

Chemical composition of SA-lipoprotein and SA-peptide

	SA-lipoprotein %	SA-peptide %
Protein:	100	100
Total nitrogen	9.55 ± 0.08*	15.73 ± 0.50*
Lipid:		
Phospholipid	28.10 ± 0.48*	
Total cholesterol	2.50 ± 0.18	
Triglycerides	5.50 ± 0.25	
Fatty acids	4.20 ± 0.24	
Carbohydrate:		
Glucosamine		1.40 ± 0.01*
Methyl pentose (as L-fucose)		0.70 ± 0.01
Neutral hexose (as galactose + mannose)		3.00 ± 0.02
Sialic acid (as N-acetyl neuraminic acid)		0.75 ± 0.01

* Standard deviations.

pholipid fractions, since these have been fairly well established and reported in the literature (2, 3, 4, 9, 10, 15, 24, 26, 33, 38).

Delipidation. Amounts of total cholesterol, phospholipids and total nitrogen present before and after delipidation of 1 ml aliquots of a concentrated suspension of bovine lung SA-lipoprotein are given in Table 2. Peptide recoveries were 91.7, and 87.5 per cent after one and two successive extractions, respectively, with the technique of FOLCH (17) at room temperature. Ninety-five per cent peptide recovery was obtained after one extraction at —10C according to the technique proposed by SCANU (40), and finally 95.8 per cent when lipid extraction was carried out according to our technique (25) at —70C. Losses of peptide could be partially attributed to mechanical handling of material during delipidation, or to the presence of a peptide-lipid complex soluble in the organic phase, similar to that reported for other lipoproteins (40). The presence of such a complex was not investigated here.

Lipid analyses conducted on the delipidized materials have shown that those extracted at room temperature by Folch's technique still contain 31 per cent of the original cholesterol and 1 per cent of the phospholipids after two successive extractions. On the other hand, 3 per cent of the original phospholipid was found in the delipidized materials after one extraction according to Scanu's technique, but no cholesterol was detected. Delipidation at —70C removed essentially all cholesterol and phospholipids from the SA-lipoprotein materials.

Chemical analysis of SA-Peptide. The delipidation procedure developed in our laboratory yielded SA-lipoprotein materials apparently free of lipids. The soluble and purified SA-peptide moiety was shown to consist of 15.73± 0.05 per cent total nitrogen, 1.40 ± 0.01 per cent glucosamine, 0.70± 0.01 per cent methyl pentose (as L-fucose), 3.00 ± 0.02 per cent neutral hexose (as galactose + mannose), and 0.75 ± 0.01 per cent sialic acid (as N-acetyl neuraminic acid), and no detectable lipids. Table 3 shows a comparative study of the chemical composition data for both SA-lipoprotein and for its SA-peptide moiety. Among the carbohydrates, neutral hexose is the major component. No quantitative individual determination of D-galactosamine or D-mannose, nor of any hexosamine other than glucosamine was attempted.

Peptide-carbohydrate moiety. As illustrated in Table 3, sialic acid accounted for about 0.75 per cent of the weight of the SA-peptide. Studies with the peptide solutions have shown that the sialic acid content did not change after precipitation with either 5 per cent trichloroacetic acid or 0.6 molar perchloric acid (Table 4).

Amino acid analysis. The amino acids present in the hydrolysates of the SA-peptide were eluted from the columns in the following order: cysteic acid, aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, half-cystine, and valine (by means of a sodium citrate buffer solution of pH 3.28), methionine, allo-isoleucine, isoleucine, leucine (glucosamine), tyrosine, and phenylalanine (by means of a sodium citrate buffer solution of pH 4.25). The order of elution of the basic amino acids was: hydroxylysine, lysine, histidine,

TABLE 4

Effect of various purification procedures on sialic acid content of SA-peptide

Sample	Additional purification procedure	Sialic Acid Content[A] Weight %
1	None	0.74
2	None	0.75
1	Trichloroacetic acid[B]	0.74
2	Trichloroacetic acid[B]	0.76
1	Perchloric acid[C]	0.73
2	Perchloric acid[C]	0.75

A. Determined on sample hydrolysates acording to WARREN (58).
B. Precipitation procedure with 5 % trichloroacetic acid.
C. Precipitation procedure with 0.6 M perchloric acid.

TABLE 5

Amino acid recoveries from SA-peptide hydrolysates[A]

Amino acid	Time of hidrolysis 22 Hrs.	46 Hrs.	70 Hrs.	Average or extrapolated value
Half-cystine[B]	4.09	——	——	4.09 ± 0.01*
Aspartic acid	8.14	7.70	7.30	8.60[C] ± 0.34
Threonine	3.33	3.09	2.71	3.60[C] ± 0.25
Serine	3.26	2.74	2.35	3.60[C] ± 0.37
Glutamic acid	12.52	11.80	11.30	13.15[C] ± 0.50
Proline	4.53	4.60	4.54	4.55 ± 0.01
Glycine	4.66	4.64	4.66	4.65 ± 0.02
Alanine	4.52	4.46	4.47	4.48 ± 0.02
Valine	4.55	4.86	4.87	4.76 ± 0.19
Methionine	2.84	(2.79)	(2.75)	2.84[D] ± 0.03
Allo-isoleucine	0.15	0.12	0.13	0.13 ± 0.01
Isoleucine	4.37	4.67	4.83	4.62 ± 0.18
Leucine	8.40	8.60	8.80	8.60 ± 0.10
Tyrosine	3.10	(3.08)	(3.04)	3.10[D] ± 0.02
Phenylalanine	4.35	4.64	4.63	4.54 ± 0.13
Hydroxylysine	2.02	1.90	1.74	2.20[C] ± 0.11
Lysine	5.78	5.47	5.08	6.20[C] ± 0.28
Histidine	2.45	3.31	2.32	2.36 ± 0.02
Ammonia (NH_2)	0.80	0.88	0.95	0.69[C] ± 0.02
Arginine	6.95	6.76	6.80	6.83 ± 0.08

A. Values expressed as grams of amino acid residue per 100 grams of peptide.
B. Value obtained by averaging the results from two 22-hour hydrolysis periods. Determined on performic acid oxidized peptide.
C. These values were obtained by extrapolating to zero time of hydrolysis in order to correct for apparent destruction or, in case of amide groups (NH_2), to correct for increased liberation of ammonia from the destruction of amino acids.
D. These values are averages of the recoveries after 22 hours of hydrolysis; the parenthetical values are omitted.
* Standard deviations.

ammonia, and arginine (by means of a sodium citrate buffer solution of pH 5.28).

Data of duplicated experiments after hydrolysis of the SA-peptide for periods of 22, 46, and 70 hours at 110 ± 1C are given in Table 5. Most amino acids were completely released after twenty-two hours and were not destroyed by increasing hydrolysis time. Since the recoveries of aspartic acid, threonine, serine, glutamic acid, hydroxylysine, and lysine decreased as the hydrolysis time was lengthened, the initial content of these amino acids was obtained by a linear extrapolation (47) to zero time of hydrolysis (Fig. 1). The initial contents of methionine and tyrosine were calculated on the basis of an average of the recoveries after twenty-two hours of hydrolysis. Ammonia values increased with longer periods of hydrolysis owing to the destruction of amino acids and glucosamine. The initial content of ammonia was also obtained by extrapolation to zero time of hydrolysis. The content of half-cystine was established by averaging duplicate values from cysteic acid recoveries after twenty-two hours of hydrolysis of performic acid oxidized peptide.

Based on the results outlined in Table 5, the following correction factors have been applied to amino acid values obtained after twenty-two hours of hydrolysis of SA-peptide: aspartic acid +5.3%, threonine +8.1%, serine +10.4%, glutamic acid +5.0%, hydroxylysine +8.9%, and ammonia —13.7%.

During each analysis a small ninhydrin-positive peak was eluted at the same buffer volume as that reported for glucosamine (between leucine and tyrosine). Since most of the glucosamine is destroyed during acid hydrolysis, calculation of the glucosamine content of SA-peptide was not possible by this method.

The content of tryptophan in the intact peptide was found to be in the order of 0.58 ± 0.01 g residue per 100 g of peptide, as determined colorimetrically by the method of SPIES and CHAMBERS (52). In performing the Spies and Chambers reaction for tryptophan with intact SA-peptide, we obtained an appropriate absorption spectrum with a true final solution.

Composition of SA-Peptide Moiety. Present estimates of the composition of the SA-peptide moiety are summarized in Table 6. In the second column, mean values for the amino acids and carbohydrates are listed, together with their standard deviations. The nitrogen content of SA-peptide preparations, 15.73 ± 0.05 g per cent, was used to derive the figures in the third column.

Salient features of the SA-peptide composition include the presence of the amino acid constituents, allo-isoleucine and hydroxylysine, with values of 1.1 and 17.2 residues per 10^5 grams of peptide, respectively, and the presence of the carbohydrates glucosamine, neutral hexose, fucose, and sialic acid with values of 8.7, 18.5, 4.8, and 2.6 residues per 10^5 grams of peptide, respectively.

The average molecular weight has been calculated on the basis of 4 half-cystine residues per mole, giving a value of 100,454 for all residues. Also, the average molecular weight was calculated on the basis of 1 allo-isoleucine residue per mole, giving a value of 91,272 for all residues, but this value was not used

in the calculation of the number of residues for average molecular weight stated in the seventh column of Table 6.

TABLE 6

Composition and molecular weight of SA-peptide

Constituent	Gm of residue per 100 gm of peptide	N. as % of total nitrogen	Minimal molecular weight	Calculated molecuuar weight	Assumed number of residues	Calculated No. of residues for average Mol. Wt. of 100,454
Half-cystine[A]	4.09 ± 0.01*	3.57	25,000	100,000	4	4.0
Aspartic acid	8.60 ± 0.34	6.65	1,340	100,500	75	75.0
Threonine	3.60 ± 0.25	3.17	2,810	101,160	36	35.7
Serine	3.60 ± 0.37	3.68	2,410	98,810	41	41.7
Glutamic acid	13.15 ± 0.50	9.10	980	99,960	102	102.5
Proline	4.55 ± 0.01	4.17	2,130	100,110	47	47.2
Glycine	4.65 ± 0.02	7.26	1,220	100,040	82	82.3
Alanine	4.48 ± 0.02	5.61	1,580	99,540	63	63.6
Valine	4.76 ± 0.19	4.28	2,080	99,840	48	48.3
Methionine	2.84 ± 0.03	1.93	4,600	101,200	22	21.8
Allo-isoleucine	0.13 ± 0.01	0.10	90,900	90,900	1	1.1
Isoleucine	4.62 ± 0.18	3.64	2,440	100,040	41	41.2
Leucine	8.60 ± 0.10	6.77	1,300	98,800	76	77.3
Tyrosine	3.10 ± 0.02	1.69	5,260	99.940	19	19.1
Phenylalanine	4.54 ± 0.13	2.75	3,230	100,130	31	31.1
Hydroxylysine	2.20 ± 0.11	2.72	5,810	98,770	17	17.3
Lysine	6.20 ± 0.28	8.62	2,070	99,360	48	48.5
Histidine	2.36 ± 0.02	4.60	5,810	98,770	17	17.3
Ammonia (NH_2)	0.69 ± 0.02[B]	3.61	2,460	100,860	41[B]	40.8[B]
Arginine	6.83 ± 0.08	15.58	2,280	100,320	44	44.1
Tryptophan[C]	0.58 ± 0.01	0.55	32,260	96,780	3	3.1
Glucosamine[D]	1.40 ± 0.01	0.77	11,490	103,410	9	8.7
Neutral hexose[D]	3.00 ± 0.02		5,400	102,600	19	18.6
Methyl pentose[D]	0.70 ± 0.01		20,490	104,150	5	4.8
Sialicic acid[D]	0.75 ± 0.01	0.23	38,460	115,380	3	2.6
Totals	99.33	101.05		100,454[E]	853	856.9

A. Value determined on performic acid oxidized peptide.
B. These values are omitted from the totals.
C. Determined colorimetrically on the intact peptide.
D. Determined colorimetrically.
E. Average molecular weight for all residues.
* Standard deviation.

Accurate determination of the small amount of allo-isoleucine is difficult because of the minor base-line variations when the usual quantity of hydrolysate was used (0.5 ml of sample containing the equivalent of 0.47 mg of peptide). The analysis of the acidic and neutral amino acids was also performed with relatively large amounts of hydrolysate (2.8 mg of peptide) to obtain as precise an estimate of allo-isoleucine as possible.

The weight and nitrogen recoveries given in Table 6 indicate that the composition of this peptide has been satisfactorily estimated.

BIOLOGICAL ACTIVITY OF SA-LIPOPROTEIN AND OF SA-PEPTIDE

Preparations of SA-lipoprotein complex showed traces of alkaline phosphatase activity, which has been estimated as approximately 0.01 King-Armstrong units per milligram of dry weight lipoprotein. The SA-peptide moiety preparations showed no alkaline phosphatase activity.

DISCUSSION

The chemical characteristics of various species of lung surface-active lipoproteins isolated in our laboratory agree with data previously reported by other investigators (2, 3, 4, 9, 15, 24, 26, 33, 38). Thus, it is apparent that this lipoprotein is a non-artifactual entity, consistently isolated from lungs by a variety of techniques.

The results of chemical studies of normal guinea pig, human and bovine lung SA-lipoproteins (Table 1), have revealed a remarkable similarity of composition, which may reflect a similarity of their functions in the lungs of these mammals.

A comparison of the chemical composition of the SA-lipoprotein complex with other high-density lipoproteins, such as plasma lipoproteins and mitochondrial structured proteins (12, 21, 23, 28, 46) shows that the peptide moieties in all of these proteins represented nearly 57-60 per cent of their total dry-weight. Phospholipids represent 20-24 per cent of the dry-weight of plasma proteins, but constitute 28 per cent of the SA-lipoprotein dry-weight. Moreover, whereas the total cholesterol of plasma lipoproteins represents 12-17 per cent, in the SA-lipoprotein, this constitutes only 2.6 per cent of its total dry-weight. It is clear from these findings that although the SA lipoprotein may be classified as a high-density lipoprotein, it differs from other high-density lipoproteins (in particular from plasma lipoproteins) with respect to the proportions of its lipid fractions.

By means of the delipidation technique developed in our laboratory (25), a peptide containing no detectable lipids, 15.73 per cent total nitrogen, and approximately 5.85 per cent carbohydrate was obtained. The yields of SA-peptide are considered satisfactory and greater than those obtained by other techniques.

The presence of carbohydrate moieties in other liproproteins has been reported previously (1, 6, 32, 40, 42, 53) but this is the first time that a detailed carbohydrate anylisis has been performed on the SA-lipoprotein. The complete analysis furnished here (Table 3) indicates that its sugar composition is quantitatively different from that reported for other lipoproteins. Thus, for example, egg yolk lipoprotein contains 0.67 per cent glucosamine, 1.3 per cent neutral hexose, and 0.38 per cent sialic acid (1), and human serum lipoprotein, 1.51 per cent glucosamine, 0.60 per cent fucose, 0.85 per cent neutral hexose, and 0.48 per cent sialic acid (40). It can be observed that in the SA-lipoprotein the neutral hexose content (3.00 per cent) is approximately two and three fold greater than the values for this constituent in egg yolk and human serum lipoproteins, respectively. Moreover, the sialic acid content (0.75 per cent) of the SA-lipoprotein, is approximately three and two times larger than the values for this constituent in egg yolk and human serum lipoproteins, respectively.

Our studies on the purity of the peptide solutions have shown that the sialic acid content did not change after precipitation with two different reagents (Table 4). Although not conclusive, these findings suggest that carbohydrates form an integral part of the SA-peptide molecule and do not result from the presence of glycoprotein impurities. The role of the carbohydrate moiety in the SA-lipoprotein is not yet clear; it may be considered as contributing to the stability of the peptide backbone and perhaps to the antigenic specificity of the molecule, as has been proposed for other lipoproteins (41), or it may play some role in the solubility of the molecule in the lung cells that synthesize it.

The amino acid composition of the SA-lipoprotein is reported for the first time in this paper. The results of amino acid analysis attest the uniformity of our SA-peptide preparations, and the amino acid distribution is consistent with a calculated molecular weight of 100,000 based on a content of four half-cystine residues per mole of peptide. Salient features of the amino acid composition are the presence of hydroxylysine (17.3 residues per mole of peptide), and the geometrical isomer of isoleucine, allo-isoleucine (1.1 residues per mole of peptide). Hydroxylysine is generally found only in collagen and certain connective tissue proteins. The content of this amino acid in the SA-peptide is approximately 2.2 per cent, which is larger than that generally reported for collagen and gelatin (20). It should be emphasized that identification of allo-isoleucine and hydroxylysine was based on chromatographic experiments, where ninhydrin-positive peaks were consistently eluted at the same buffer volumes as those reported for specific substances by other investigators (36, 48). Nevertheless, it is agreed that an absolute identification must be based on independent criteria such as isolation of the compound responsible for the peak (22), followed by chemical and physical characterization, paper chromatography (59), etc. If our findings are correct, the presence of these two amino acids may be of great importance in future investigations as markers in searching for the place of origin of the surfactant lipoprotein.

A comparison of the amino acid composition of SA-lipoprotein with other well known lipoproteins, such as the structural protein from mitochondria

(21) and various serum lipoproteins (28, 31, 46), reveals qualitative and quantitative differences among them. The differences between the amino acid composition of these proteins clearly demonstrate that high-density lipoproteins do not share the same peptide component; their peptide moieties are not identical, and interconversions cannot take place by merely removing or adding lipid constituents. Nevertheless, it should be emphasized that acidic amino acids predominate over basic amino acids in all the proteins studied, including the SA-lipoprotein.

A careful comparison of the amino acid composition of SA-lipoprotein with human and bovine serum mercaptalbumins (50) has been made. Although some similarities were found with regard to the amino acid composition among the SA-lipoprotein and the serum albumins, tremendous discrepancies were also found. Thus, for example, the presence of the amino acids allo-isoleucine and hydroxylysine, as well as of carbohydrate moities in serum albumins, has never been reported. These findings seem to disagree with the conclusions of McCLENEHAN and OLSEN (34) that albumin is closely associated with the pulmonary surfactant molecule and that it may constitute the SA-peptide moiety. Nevertheless, since it has been found that the delipidized SA-poprotein may be electrophoretically separated into two major components (25), one of which is an albumin-like migrating component, and since the amino acid analysis studies were carried out in the whole lipid-free materials (25), a more elaborate study is required to clarify this apparent discrepancy.

The fact that only a low level of phosphatase activity is present in the fairly well purified SA-lipoprotein materials is indicative that the main function of this lipoprotein is not as a phosphatase enzyme. On the other hand, it was expected that the SA-peptide would not exhibit alkaline phosphatase activity, since removal of the lipids is apt to denature the lipoprotein and abolish any biological activity. Since there is the possibility of contamination of SA-lipoprotein with other substances, such as phosphatase enzymes, and with the lung thromboplastic lipoprotein (11), which exhibits phosphatase activity, it remains to be seen whether this biological activity represents a characteristic of the SA-lipoprotein or arises from minor impurities.

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SUMMARY

Studies on the pulmonary surface-active lipoprotein complex were carried out in order to establish, primarily, its biochemical characteristics.

By means of differential centrifugation in salt gradients, surface-active lipoprotein complex was obtained from fresh human, guinea pig and bovine lung tissue.

The three species of SA-lipoproteins proved remarkably similar in chemical composition, in accordance with their homologous functions.

After removal of lipids with an extraction technique developed by the author, the peptide of the cow lung SA-lipoprotein was subjected to an exhaustive characterization study. The delipidized peptide contained 15.73 per cent total nitrogen, approximately 5.85 per cent carbohydrate, and no detectable lipids. The sialic acid content of the delipidized peptide did not change after precipitation with trichloroacetic acid and perchloric acid reagents, which suggests that carbohydrates form an integral part of the SA-peptide molecule and do not result from the presence of impurities.

Amino acid analysis of SA-peptide revealed the presence of all the amino acids normally found in protein hydrolysates. In addition, two unusual amino acid constituents were found, allo-isoleucine and hydroxylysine. These relatively uncommon amino acid constituents may serve as useful markers for identifying the peptide moiety of SA-lipoprotein in future studies. The amino acid composition of SA-lipoprotein differs qualitatively and quantitatively from that of other well-known lipoproteins and of serum albumin as well.

A low level of alkaline phosphatase activity was detected in SA-lipoprotein. No such enzymatic activity was found in the SA-peptide moiety. This biological activity may be characteristic of the SA-lipoprotein but could also result from contamination with discrete lung alkaline phosphatase enzymes.

RESUMEN

Se llevaron a cabo estudios de la lipoproteína tensoactiva pulmonar con la finalidad de establecer, principalmente, sus características bioquímicas.

Mediante la utilización de técnicas de centrifugación diferencial, en gradientes de concentración salina, el complejo lipoproteico tensoactivo fue aislado de tejidos pulmonares frescos provenientes de cobayos, humanos y bovinos.

Los análisis bioquímicos de las tres especies de lipoproteínas estudiadas, demostraron que éstas son extremadamente similares en cuanto a su composición química, lo que está de acuerdo con sus funciones homólogas.

La remoción total de los lípidos del complejo lipoproteico fue lograda mediante una nueva técnica desarrollada por el autor. El constituyente péptido de la lipoproteína tensoactiva pulmonar, libre de lípidos, fue sometido a intenso estudio de caracterización. Dicho estudio mostró que el péptido no posee lípidos y que está constituído por 15.73 por ciento de nitrógeno total y aproximadamente 5.85 por ciento de carbohidratos. No se observó cambios en el contenido del ácido siálico del constituyente péptido cuando éste fue precipitado repetidas veces con reactivos de ácido tricloroacético o de ácido perclórico, esto sugiere que los carbohidratos forman parte integral de la molécula de la lipoproteína tensoactiva pulmonar y que su presencia no se debe a impurezas.

El análisis de los amino ácidos del constituyente péptido de la lipoproteína reveló la presencia de todos los amino ácidos normalmente encontrados en hidrolizados proteicos. Además se encontraron dos constituyentes poco comunes: los amino ácidos alo-isoleucina e hidroxilisina. Este hallazgo es considerado de gran utilidad, ya que estos amino ácidos poco comunes podrían servir en el futuro como "marcadores" en la identificación del péptido *in vivo*. La composición en amino ácidos de la lipoproteína tensoactiva pulmonar ha mostrado diferencias, tanto cualitativas como cuantitativas, con respecto a la composición en amino ácidos de otras lipoproteínas, así como de la sero-albúmina.

La lipoproteína tensoactiva pulmonar mostró un bajo nivel de actividad enzimática relacionada con la fosfatasa alcalina Dicha actividad biológica podría ser una característica del complejo lipoproteico pulmonar, pero también podría atribuírse a la contaminación con enzimas fosfatásicas presentes en el tejido pulmonar.

El constituyente péptico no mostró ninguna actividad biológica.

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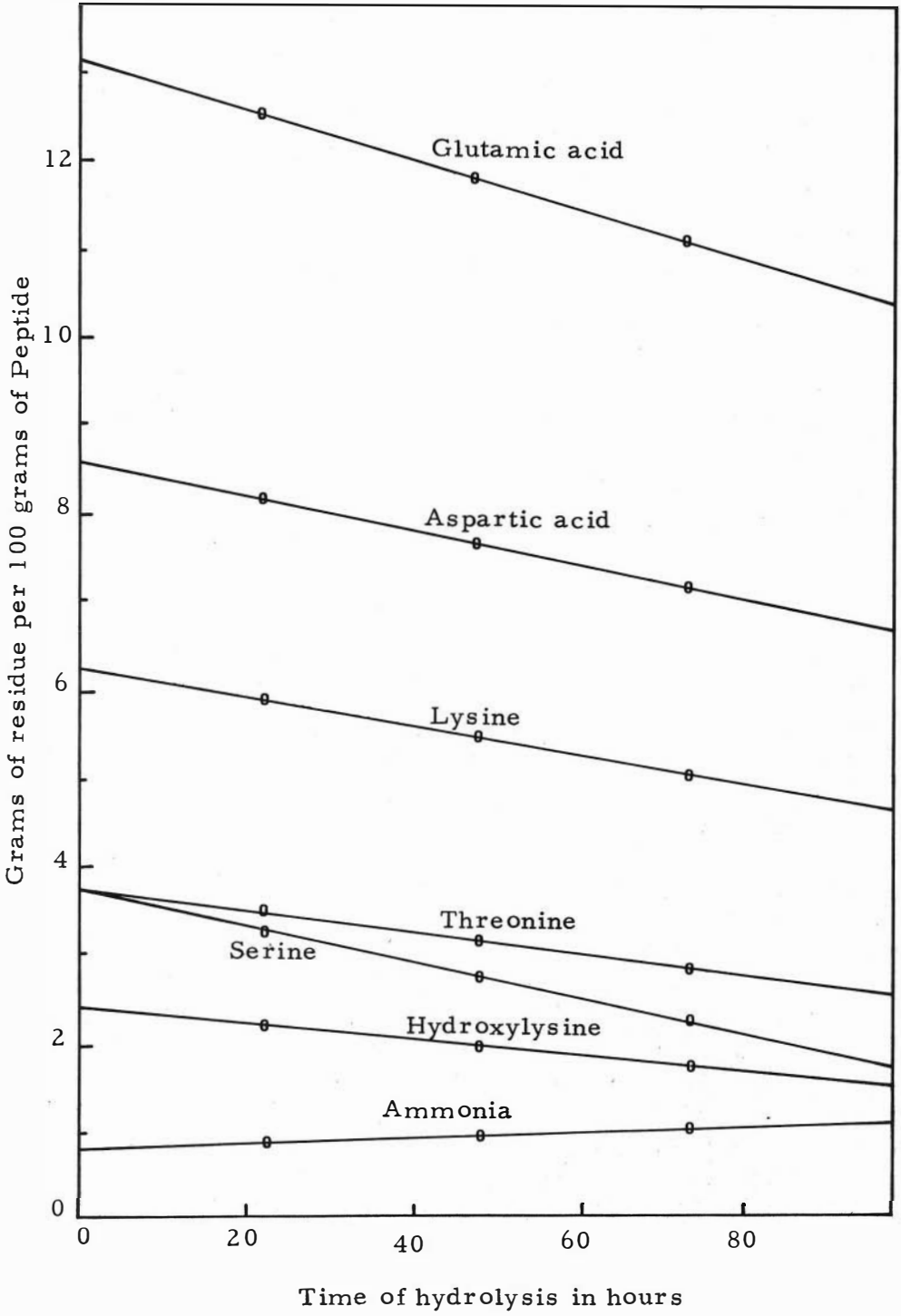
Fig. 1. Recoveries as a function of time of hydrolysis for various amino acids and ammonia, given as grams of residue per 100 grams of SA-peptide.


Glutamic acid
Aspartic acid
Lysine
Threonine
Serine
Hydroxylysine
Ammonia
Grams of residue per 100 grams of Peptide
0
2
4
6
8
10
12
0
20
40
60
80
Time of hydrolysis in hours