Rev. Biol. Trop., 19(1, 2): 73-95, 1971

Studies on the pulmonary surface-active lipoprotein* II. Biophysical characterization of its peptide moiety

by

José Miguel Jiménez**

(Received for publication, March 27, 1969)

It has been proven that the lung alveoli of mammals, birds, reptiles, and amphibians, possess a surface-active lining film which is highly insoluble in water and freely permeable to gases (6, 22, 23, 24, 25, 26, 35).

In mammals, the surfactant material has been identified as a lipoprotein complex (the SA-lipoprotein), and its composition is fairly well known (1, 2, 10, 14, 15, 16, 17, 18, 27). Recently, a new technique for separating a saline-soluble, essentially lipid-free peptide from the SA-lipoprotein of mammals has been developed by the author (14). In the light of these important developments, further studies on the lung surface-active material were undertaken in an attempt to define in detail the chemical and physical characteristics of the SA-peptide moiety (15).

The present paper is concerned with the establishment of the biophysical features of the peptide component of cow lung SA-lipoprotein. Information concerning solubility, electrophoresis, and sedimentation behavior of SA-peptide is presented. An extensive account of the biochemical findings has been given elsewhere (15).

MATERIAL AND METHODS

Isolation and characterization of the lung SA-lipoprotein

The preparation and characterization of the cow lung SA-lipoprotein utilized in this study is described fully in a separate report dealing with the

* Part of a Dissertation presented to the Faculty of the Graduate School of the University of Southern California in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

** Present address: Departamento de Biología, Universidad de Costa Rica, San José, Costa Rica.

chemistry of this substance (15). In summary, highly purified materials of cow lung SA-lipoprotein complex were obtained from fresh lungs according to the procedure of ABRAMS (1).

ISOLATION OF THE PEPTIDE MOIETY OF THE SA-LIPOPROTEIN

Since the most important topic to be studied in the present research problem is the isolation and characterization of the peptide moiety of the pulmonary surface-active lipoprotein complex, a major emphasis has been given to the aspect of separating lipids from the lipoprotein without denaturing the peptide residue. This was accomplished by utilizing the delipidation technique developed by the author (14) and fully described in an associated paper (15).

SOLUBILITY OF THE DELIPIDIZED SA-LIPOPROTEIN

Solubility studies of the lipid-free SA-lipoprotein materials were conduced at 4 C and at 24 C (room temperature) in 0.1 M sodium chloride solutions at pH 5.0, 7.0, and 10.0; in 0.1 N sodium hydroxide solution, and in distilled water. Solute and solvents were stirred for twenty-four hours in an automatic magnetic stirrer. Samples were centrifuged at 15,000 rpm for thirty minutes at 4 C in a Servall super-speed refrigerated centrifuge (rotor SA-34), and nitrogen content of the supernates was determined (38).

To determine whether the solubilized SA-peptide was dialyzable or not, a 0.5 per cent solution was prepared and dialyzed against 0.1 M sodium chloride pH 10.0, in a cold room at 4 C. Nitrogen contents of the delipidized sample, before and after dialysis, and of the dialysates, were determined.

PURIFICATION OF SA-PEPTIDE

The crude dried delipidized SA-liproprotein materials were rendered soluble by stirring them at room temperature with 0.1 N sodium hydroxide for 24 hours. These crude peptide solutions were concentrated and ultrafiltered under pressure over Diaflo Membranes, Type UM-1 (Diaflo Ultrafiltration, Amicon Corp.). This membrane retains solutes larger than 10,000 molecular weight. The ultrafiltered, concentrated, SA-peptide solutions were then successively filtered through columns of Sephadex G-20, G-50, and G-100 (Pharmacia Fine Chemicals Inc.), of medium particle size. Gel filtrations were carried out with columns of bed dimensions: 2.5 × 45 cm, and the Sephadex gels were equilibrated with 0.1 M sodium chloride, pH 10.0. The SA-peptide solutions were placed in the columns and elutions were performed with the same saline solutions. Recovery of the peptide was determined by measuring the light absorption at 280 $m\mu$ of the collected fractions and/or by determining the presence of protein by the microbiuret method. All the eluted fractions containing protein were pooled, lyophilized, and redissolved with distilled water. The final peptide solution collected after gel filtration through Sephadex G-100 was extensively dyalized against 0.1 M sodium chloride,

pH 10.0, for four days in the cold room, with changes of dialysate every 24 hours.

The dialyzed SA-peptide solutions were analyzed spectrophotometrically over the range of visible light and ultraviolet light (from 800 to 200 mμ) in a Beckman DB Spectrophotometer, to detect absorption peaks other than those of pure proteins.

The dry weight of the materials to be used in the physical experiments was determined in the same way as described elsewhere (15). Aliquots of 50.0 ml each of the dialyzed SA-peptide solutions were used in these experiments.

PHYSICAL CHARACTERIZATION OF THE SA-PEPTIDE MOIETY

Calculation of the partial specific volume: Since it has been found that the volume of macromolecules is closely equal to the sum of the individual residue contributions (19, 20, 32), the partial specific volume of a protein can be estimated quite reliably from its chemical composition.

In the calculation of the partial specific volume of the SA-peptide this method of adding the individual specific volumes of the residues that constitute it was used. The values for the specific volumes of the amino acid residues are those given by COHN and EDSALL (7), and the values for the specific volumes of the carbohydrate residues are those given by GIBBONS (11). The specific volume of hidroxylysine was calculated from lysine Vi value (7), assuming the value of 2.3 cc/mole for the extra atom of oxygen present in hydroxylysine.

Electrophoresis: The cellulose acetate electrophoresis technique of BREIER and MULL (5), with a commercial apparatus (Gelman Instrument Co.), was used. A Beckman model RB Analytrol was adapted to scan the cellulose acetate strips by means of the addition of an external synchronous motor (Scan-A-tron, Gelman) which drew the strips through at the same rate of two inches per minute. The narrowest slit in the Analytrol was shortened to 1 cm in length with black masking tape. The B-2 optical density cam was used with the green 500 mμ filter.

A 1 percent solution of the SA-peptide was prepared and dialyzed for three days against a barbital-barbituric acid buffer, pH 8.6, ionic strength 0.05, with daily changes of dialysate. Electrophoresis was performed on these dialysate materials; it was carried out at 1.5 ma per strip for thirty minutes.

Analytical ultracentrifugation: Ultracentrifugation runs were conducted in a Model E Spinco Analytical Ultracentrifuge, operated at room temperature (36). The two-hole AN-D rotor was used and its temperature was regulated to within ± 0.1 C during the experiments. Cells were tightened 2½ divisions on a torque wrench. The schlieren optical system was used throughout these experiments and the patterns photographed on Kodak metallographic plates.

A Nikon Shadograph Model 6 C optical comparator (Nikon Co., Japan), was used to read the photographic plates.

Determination of sedimentation coefficients of SA-peptide

Approximately 1 gm/dl solution of SA-peptide was dialyzed for four days at 4 C against 0.1 ionic strength, pH 8.0, 0.1 M sodium chloride solution. The protein solution was diluted with the final dialysate to give a solution of concentration 0.76 gm/dl, determined by the microbiuret and/or the micro-Kjeldahl methods (14, 38). Sedimentation velocity runs were performed with peptide solution at 20.85 C, according to Schachman (32). Sedimentation coefficients were calculated and corrected to the standard state ($s_{20,w}$). The values for the viscosity of water at t° relative to that at 20 C, the relative viscosity of the solvent to that of water, and the densities of water at 20 C and the solvent at t°, were calculated from data in appendices I, II, and III of Svedberg and Pedersen (37). It was implicitly assumed that $\bar{V}$ in the reference solvent was the same as in the actual solvent used in the experiments.

Determination of the molecular weight

In the determination of the molecular weight of the SA-peptide moiety, two different ultracentrifugation experiments were carried out: a) sedimentation equilibrium runs, and b) synthetic boundary runs.

Sedimentation equilibrium experiments: A technique with short columns of solutions (39) as recommended by Yphantis (40) was used. A standard 1 mm aluminum centerpiece cell was used with the dummy counterbalance centerpiece in the two-hole AN-D rotor. Four-tenths ml of "FC-43" fluorocarbon (Beckman) was placed in the cell and 0.050 ml of SA-peptide solution (0.76 gm/dl in 0.1 ionic strength, pH 8.0, 0.1 M sodium chloride solution) was layered over the oil. The rotor was run at a speed of about 20,0000 rpm to develop concentration gradients at the meniscus and base (31) and was then slowed to 6,166 rpm. The distribution was allowed to reach equilibrium overnight at 6,166 rpm. All schlieren observations were made at a constant phase-plate angle of 70° to the vertical, and exposures were taken on photographic plates after equilibrium was reached. Experiments were done at 20.85 C. A similar run, of 30 to 60 min duration, was performed with the saline dialysate instead of the peptide solution.

Synthetic boundary experiments: A 4° sector, aluminium centerpice cell (valve-type synthetic boundary cell) was used with the dummy counterbalance in the two-hole AN-D rotor. Four-tenths ml of the SA-peptide solution were placed in the sector compartment and 0.30 ml of dialysate in the cup of the cell. The procedure for accelerating the rotor and taking photographs was that recommended by Schachman (32). The schlieren optical system was used with the phase-plate at an angle of 70° to the vertical. Experiments were performed at 20.85 C, and the final rotor speed was 12,590 rpm.

Reading of the photographic plates and calculations: In general, reading and measuring the photographic records was done in the same way as recommended by ALLERTON (3). Vertical distances of the schlieren patterns were measured for both the peptide run (distances = Ys) and the dialysate run (distance = Yb). The value of the concentration gradient (dc/dx) at the mid-point (Xm) of the cell is proportional to the difference between the peptide solution and the solvent traces (Ys—Yb = ΔY). The mid-point Xm is equal to (a + b)/2, where a and b are the distances from the center of rotation to the meniscus and base of the solution (40).

Synthetic boundary records were aligned by having the horizontal cross-hair bisect the flat sections of the patterns on either side of, and adjacent to, the boundaries. The bisectors were taken as baselines in evaluating boundary areas. Vertical distances (Y coordinates) of the schlieren pattern from these baselines were measured for points (X ordinates) located at equal intervals throughout the boundary.

For the computation of the initial uniform protein concentration (Co), the area beneath the synthetic boundary peak was evaluated. This could be done either by means of a planimeter giving results in square centimeters or by trapezoid summation, with data from large numbers of equally spaced points. The value obtained by either of these two methods is then multiplied by 1/F to correct for magnification in the x direction, and the resultant value is proportional to Co (32).

The weight-average molecular weight of the SA-peptide moiety was calculated according to standard procedures.

RESULTS

Analytical ultracentrifugation, electrophoresis and gel filtration were employed to assess the purity of the isolated SA-peptide.

Ultracentrifugal analyses have shown the presence of at least two main distinguishable components. In addition, two small very rapidly moving peaks could be observed at the beginning of the sedimentation experiments. These two high molecular weight components were observed in the schlieren patterns when the rotor speed was relatively low, approximately 20,000 rpm (Fig. 1).

By cellulose acetate electrophoresis two main electrophoretic boundaries were found; a large symmetrical peak (fast moving), and a small, more slowly moving component, close to the origin, which was estimated by densitometry to represent less than 5 per cent of the total peptide (Fig. 4).

Gel filtration on Sephadex G-100 showed that all the protein material emerged from the column as a single peak (Fig. 5); no protein was detected in later fractions, either by the biuret reagent, or by absorbence at 280 mμ wavelength in the spectrophotometer. Protein recoveries from Sephadex G-100 were of the order of 90-95 per cent.

When the isolated SA-peptide was examined spectrophotometrically over the range of visible and ultraviolet light, it showed only one absorption peak at 280 mμ wavelength (Fig. 6).

Solubility studies of the delipidized cow lung SA-lipoprotein materials are summarized in Table 1. It can be seen that the lipid-free materials are insoluble in distilled water, and in acid or neutral sodium chloride solutions. They may be slightly soluble in 0.1 M sodium chloride solution at pH 10, and fairly soluble in 0.1 normal sodium hydroxide solutions. Solubility values were low at 4 C but considerably increased at 24 C (room temperature). It was observed that the insoluble residues or gels dissolved on addition of 6 M urea. It was also found that the lipid free SA-peptide is not dialyzable.

TABLE 1

*Solubility values of the delipidized cow lung SA-lipoprotein**

	4 C gm%	24 C gm%
Sodium chloride, 0.1 M, pH 5.0	——	——
Sodium chloride, 0.1 M, pH 7.0	——	——
Sodium chloride, 0.1 M, pH 10.0	0.10	0.15
Sodium hydroxyde, 0.1 N	0.50	0.85
Distilled water	——	——

* Content of peptide in solution was estimated from its total nitrogen content.

Knowledge of the chemical composition of the SA-peptide moiety (15) has permitted the calculation of various physical features of this substance. Thus, for example in Table 2, the number of ionic groups of SA-peptide is given on the assumption that a single terminal alpha-carboxyl group and also a single free alpha-amino group are present in this peptide. The slightly greater number of anionic over cationic groups may be in accordance with the acidic properties of this peptide, which should have an isoelectric point of less than pH 7.

TABLE 2

*Ionic groups of the SA-peptide**

Constituent	N° of groups	Constituent	N° of groups
Aspartic acid	75	Hydroxylysine	17
Glutamic acid	102	Lysine	48
Sialic acid	3	Histidine	17
Terminal alpha-carboxyl[A]	1	Arginine	44
	181	Glucosamine[B]	9
Amide groups	— 41	Terminal alpha-amino[A]	1
Total anionic groups (—)	140	Total cationic groups (+)	136

* The number of each residue per molecule of SA-peptide was obtained from data of Jiménez (15).

A. It was assumed that a single alpha-carboxyl group and a single alpha-amino group are present in this peptide.

B. Assumed to be glucosamine, rather than N-acethylglucosamine, residue for this tabulation.

Partial specific volume: The relatively complete accounting of the weight of SA-peptide (15) has also permitted the calculation of the partial specific volume $\bar{V}$ of this peptide. To take into account the forty amide groups per molecule of SA-peptide it was assumed that these were approximately equally distributed between the aspartic and glutamic residues. The assignment of the amide groups has no significant effect on calculation of $\bar{V}$. The data are given in Table 3 and lead to a value for $\bar{V}$ of 0.7263 cc/gm.

TABLE 3

Calculation of the partial specific volume of the SA-peptide from its constituents

Residue	Grams of residue per 100 gm peptide Wi [A]	Specific volume of residue Vi [B]	% by volume of residue WiVi
Half-cystine	4.09	0.63	2.577
Aspartic acid	6.31	0.60	3.786
Asparagine [C]	2.29	0.62	1.419
Threonine	3.60	0.70	2.520
Serine	3.60	0.63	2.268
Glutamic acid	10.51	0.66	6.937
Glutamine [C]	2.64	0.67	1.769
Proline	4.55	0.76	3.458
Glycine	4.65	0.64	2.976
Alanine	4.48	0.74	3.315
Valine	4.76	0.86	4.094
Methionine	2.84	0.75	2.130
Allo-isoleucine	0.13	0.90	0.117
Isoleucine	4.62	0.90	4.158
Leucine	8.60	0.90	7.740
Tyrosine	3.10	0.71	2.201
Phenylalanine	4.54	0.77	3.496
Hydroxylysine	2.20	0.74 [D]	1.628
Lysine	6.20	0.82	5.084
Histidine	2.36	0.67	1.581
Arginine	6.83	0.70	4.781
Tryptophan	0.58	0.74	0.429
Glucosamine	1.40	0.666 [E]	0.932
Neutral hexose	3.00	0.613 [E]	1.839
Methyl pentose	0.70	0.678 [E]	0.475
Sialic acid	0.75	0.584 [E]	0.438
Totals	99.33		72.148

$$\bar{V} = \sum WiVi \,/\, \sum Wi = 72.148 \,/\, 99.33 = 0.7263 \text{ cc/gm}$$

A. The values of Wi for the residues are those given by Jiménez (15).
B. The values of Vi are those given by Cohn and Edsall (7).
C. The assignment of the amide groups as asparaginyl and glutaminyl is arbitrary in this calculation. For convenience these were approximately equally distributed between aspartic and glutamic residues.
D. Calculated from Lysine Vi value acording to Cohn and Edsall (7).
E. These values of Vi for the carbobydrates are those given by Gibbons (11).

Sedimentation constants of SA-peptide: Data from a typical sedimentation velocity experiment have been plotted in Fig. 7 for the two main components of SA-peptide (fast and slow moving components only). The plot shows essentially two straight lines over a broad range of the abscissa (time), and sedimentation coefficients can be calculated from the slopes of these lines. The results of these experiments are summarized in Table 4. In the calculation of the corrected sedimentation coefficient values, it was assumed that $\bar{V} = 0.726$ cc/gm, in the reference solvent, is the same as in the solvent actually used in the experiments. For the experimental data presented in Table 4, calculations of $s_{20,w}$, for the slow and fast moving major components of SA-peptide, lead to the results.

Slow moving component, $s_{20,w} = 1.53 \times 10^{-13}$ sec. or 1.53S

Fast moving component, $s_{20,w} = 3.10 \times 10^{-13}$ sec. or 3.10S

TABLE 4

Sedimentation velocity experiment of SA-peptide. Calculation of sedimentation coefficients

F = 2.114; T = 20.85 C; rotor speed = 56,100 rpm; Cp = 0.76 gm/dl

Time [A]	Component [B]	ΔX cm	$\frac{\Delta X}{F}$ ΔX_{cm}	$(\Delta X + 5.700)$ X_{cm}	log x
37	Slow	0.6800	0.3217	6.0217	0.77972
37	Fast	0.8450	0.3997	6.0997	0.78531
53	Slow	0.7450	0.3524	6.0524	0.78193
53	Fast	0.9750	0.4612	6.1612	0.78966
69	Slow	0.8100	0.3832	6.0832	0.78413
69	Fast	1.1050	0.5227	6.2227	0.79397
85	Slow	0.8750	0.4139	6.1139	0.78632
85	Fast	1.2350	0.5842	6.2842	0.79825

$$s = \frac{1}{w^2 x} \frac{dx}{dt} = \frac{2.303\ d \log x/dt}{(60)\ (w^2)} = \frac{(\text{slope})\ (2.303/60)}{(2\pi)\ (\frac{56{,}100}{60})^2}$$

$S_{slow} = 1.50 \times 10^{-13} = 1.50S$

$S_{fast} = 3.04 \times 10^{-13} = 3.04S$

Sedimentation coefficients were corrected to the standard state:

$s_{20,w}$, slow = 1.53S

$s_{20,w}$, fast = 3.10S

A. Time in minutes after the rotor has attained the final speed of 56,100 rpm.

B. Refers to the two main components of the SA-peptide material.

Molecular weight of SA-peptide: A representative set of data from one of the synthetic boundary experiments performed with SA-peptide materials (Fig. 2) is presented in Table 5. Horizontal and vertical coordinates of the schlieren pattern are given in the first two colums. Differences (Z) between Y values for the pattern and baseline are given in the third column. Calculation of the area under the peak was done by the trapezoid summation method, by adding the ordinates, Z, at the x intervals and then multiplying the sum by the respective x interval value to obtain the area contribution in the fourth column. The value obtained by adding all the areas was then multiplied by 1/F to correct for magnification in the x direction, and the resultant value is Co.

An average of three determinations gave the value Co of 0.174 square centimeters.

Figure 3 shows ultracentrifuge patterns for a sedimentation equilibrium experiment performed with SA-peptide. Table 6 shows the data obtained from sedimentation equilibrium experiments with SA-peptide. The location of the reading points on the schlieren patterns is given in the first column, and the horizontal and vertical coordinates and reference marks in the second and third columns. The net vertical displacement, at the mid-point Xm, (Ys - Yb), is equal to ΔY.

Calculations presented in Table 6 lead to the results of

$$\Delta Y = 1.201 \text{ centimeters}$$
$$Xm = 6.469 \text{ centimeters}$$

Based upon the experimental data collected, the weight-average molecular weight for the SA-peptide was calculated according to standard methods:

$$M_w = 2.3 \times 10^5 \text{ gm/mole}$$

DISCUSSION

By means of a new delipidation method (14) it is possible to obtain SA-peptide containing no detectable lipids, 15.73 per cent total nitrogen, and aproximately 5.85 per cent carbohydrate, which could be utilized in physical and chemical characterization studies. Chemical characteristics of the SA-peptide have been presented in an associated paper (15).

Knowledge of the entire composition of the SA-lipoprotein has permitted the calculation of various physical constants of this substance.

Assuming that a single terminal alpha-carboxyl group and a single alpha-amino group are present in the SA-peptide, calculations have shown that there is a slightly greater number (1.4 per cent of total charged groups) of negative

than positive groups in this peptide. This result would be anticiped from the acidic properties of this peptide.

TABLE 5

*Synthetic boundary experiment of SA-peptide. Calculation of area under the peak (Co)**

F = 2.114; T = 20.85 C; rotor speed = 12,590 rpm; Cp = 0.76 gm/dl

1	2	3	4	1	2	3	4
Xcm	Ycm	Zcm	$\sum Z \cdot dx$ trap.	Xcm	Ycm	Zcm	$\sum Z \cdot dx$ trap.
1.45	3.400	—		2.12	2.390	1.010	
1.50	3.400	0.000	(0.020) (0.05)	2.14	2.430	0.970	
1.55	3.390	0.010	= 0.00100	2.16	2.460	0.940	
1.60	3.380	0.020		2.18	2.500	0.910	
1.62	2.375	0.025		2.20	2.565	0.835	
1.64	3.365	0.035		2.22	2.635	0.765	
1.66	3.355	0.045		2.24	2.725	0.675	
1.68	3.345	0.055		2.26	2.810	0.590	
1.70	3.340	0.060		2.28	2.895	0.505	
1.72	3.330	0.070		2.30	2.980	0.420	
1.74	3.315	0.085		2.32	3.055	0.345	
1.76	3.300	0.100		2.34	3.125	0.275	
1.78	3.285	0.115		2.36	3.175	0.225	
1.80	3.265	0.135		2.38	3.215	0.185	
1.82	3.245	0.155		2.40	3.250	0.150	
1.84	3.215	0.185		2.42	3.275	0.125	
1.86	3.180	0.220		2.44	3.295	0.105	
1.88	3.140	0.260		2.46	3.310	0.090	
1.90	3.100	0.300		2.48	3.320	0.080	
1.92	3.055	0.345		2.50	3.330	0.070	
1.94	3.000	0.400		2.52	3.340	0.060	
1.96	2.935	0.465		2.54	3.350	0.050	
1.98	2.860	0.540		2.56	3.360	0.040	(18.2825) (0.02)
2.00	2.770	0.630		2.58	3.370	0.030	= 0.36565
2.02	2.675	0.725		2.60	3.375	0.025	
2.04	2.555	0.845		2.65	3.390	0.010	(0.0275) (0.05)
2.06	2.425	0.975		2.70	3.395	0.005	= 0.001375
2.08	2.400	1.000		2.75	3.400	—	
2.10	2.360	1.040					
						$\sum (\sum Z \cdot dx)$ =	0.368250

$\sum (\sum Z \cdot dx)_{true} = 0.368250 / 2.114 = 0.1740 \text{ cm}^2$

$C_O = 0.1740 \text{ cm}^2$

* Area under the peak was calculated by the trapezoid summation method.

TABLE 6

Sedimentation equilibrium of SA-peptide
Calculation of ΔY at the Mid-Point Xm in short columns

F = 2.114; T = 20.85 C; rotor speed= 6,166 rpm; Cp = 0.76 gm/dl

Location*	X_{cm}	Y_{cm}	ΔX_{cm}	Δx_{cm}	x_{cm}	ΔY_{cm}
Sample run:						
Left reference bar	1.000					
Baseline reference mark		3.357				
Air-sample line	1.630					
Sample-oil line	1.816					
Mid-point Xm	1.723	2.102	1.623	0.7677	6.468	1.255
Solvent run:						
Left reference bar	1.000					
Baseline reference mark		3.344				
Air-sample line	2.524					
Sample-oil line	2.728					
Mid-point Xm	2.626	3.290	1.626	0.7691	6.469	0.054

$\Delta Y = (1.255) - (0.054) = 1.201$ cm

$Xm_{true} = (6.468 + 6.469) / 2 = 6.469$

* These correspond to prints in the original ultracentrifuge pattern.

The partial specific volume, 0.726 cc/gm, of the SA-peptide moiety has been calculated. Moreover, it has been possible to estimate the partial specific volume of the anhydrous cow lung SA-lipoprotein (Table 7). This value, 0.852 cc/gm, is calculated in the same way as the partial specific volume of the peptide. The Wi values for the cow lung SA-lipoprotein constituents used here are those given by JIMENEZ (15). The Vi value for the peptide constituent (0.726) is that shown in Table 3, and the Vi values for the lipid fractions (phospholipid 1.031, free cholesterol 0.943, cholesterol esters 1.01, triglycerides 1.087, and fatty acids 1.087) are obtained from density data given by ONCLEY (21), as the reciprocal of the density value of each lipid constituent. From data provided by ABRAMS (1), it is possible to calculate the values for free cholesterol and cholesterol esters of the SA-lipoprotein, these are estimated as 2.3 and 0.2 per cent, respectively. In calculating the $\bar{V}$ value for the SA-lipoprotein it is also assumed that the specific volume value for the fatty acids is equal to that of the triglycerides.

From the calculated $\bar{V}$ value for cow lung SA-lipoprotein the anhydrous density of this lipoprotein can be estimated (Table 7). This calculated density value of 1.17 gm/cc is in the range regarded as characteristic of high density lipoproteins: 1.06-1.21 gm/cc (9, 13).*

TABLE 7

Calculation of the partial specific volume of the cow lung SA-lipoprotein complex from its constituents

Constituent	Grams of constituent per 100 gm of lipoprotein A Wi	Specific volume of constituent B Vi	Per cent by volume of constituent WiVi
Peptide	59.7	0.726[C]	43.342
Phospholipids	28.1	1.031	28.971
Free Cholesterol[D]	2.3	0.943	2.169
Cholesterol Esters[D]	0.2	1.010	0.202
Triglycerides	5.5	1.087	5.979
Fatty Acids	4.2	1.087[E]	4.565
Totals	100.0		85.228

$$\bar{V} = \Sigma \text{ WiVi} / \Sigma \text{ Wi} = 85.228 / 100 = 0.852 \text{ cc/gm}$$

$$\therefore \text{ of SA-lipoprotein} = 1 / 0.852 = 1.17 \text{ gm/cc}$$

A. Values of Wi for constituents according to Jiménez (15).
B. The Vi values were calculated from data given by Oncley (21).
C. V value from Table 3.
D. Calculated as 6 per cent of free cholesterol and 0.5 per cent cholesterol esters based on data given by Abrams (1).
E. Assumed to be equal to the Vi value of the triglycerides.

A weight-average molecular weight of SA-peptide was determined from sedimentation equilibrium data with short columns of solutions, and a value of 230,000 was obtained. The calculated minimal molecular weight of SA-peptide on the basis of its chemical compositon, on the other hand, was 100,000 (15). The discrepancy between these two values may be explained by assuming that polymerization occurs in solution, leading to a high value for the weight-average molecular weight but does not affect the molecular weight value derived from the chemical composition.

ABRAMS (1) has reported a value of 190,000-290,000 for the molecular

* A lower density value would be expected for the hydrated bind lipoprotein. For example, if the lipoprotein were to bind 0.3 gm water per gram of lipoprotein, a density near 1.13 would be calculated for the hydrated species.

weight of SA-lipoprotein, calculated from surface tension measurements, which would give a molecular weight of 110,000-170,000 for the SA-peptide. The molecular weight figure we obtained for the SA-peptide on the basis of its amino acid and carbohydrate composition (15), may be in close agreement with the lower value reported by Abrams.

With regard to homogeneity of the isolated lipid-free SA-peptide ultracentrifugal and electrophoretic data have shown that the material is heterogeneous. The SA-peptide was resolved into two components by cellulose acetate electrophoresis, and two main peaks were also found in sedimentation velocity experiments with the analytical ultracentrifuge. Furthermore, sedimentation runs have also revealed at least two minor fast-sedimenting components, presumably of very high molecular weight.

The properties of the SA-peptide were markedly influenced by solvent pH. Solubility and ultracentrifugal data indicated that changes in pH in the medium influence the solubility of SA-peptide. Aggregation occurred at pH 4-6, as evidenced by the formation of a gel which dissolved on addition of 6 molar urea. At pH values higher than 7.0 or less than 4.0 the SA-peptide became more soluble. However, ultracentrifugal experiments performed at pH 8.0 have shown the presence, in the analyzed peptide solutions, of materials with different velocity constants, which suggest that aggregation is still occurring at this pH value. At higher pH values solubility of the peptide was greatly increased, and it appeared to be more nearly monodisperse, as judged by its elution from Sephadex G-100 columns as a single peak when chromatographed at pH 10.0 with 0.1 molar sodium chloride solutions.

The observed dependence of solubility and the tendency to aggregate of SA-peptide on pH of the medium suggest that the forces involved are electrostatic. That is, the solution behavior of SA-peptide is in part controlled by its net electrical charge. The tendency of SA-lipoprotein to aggregate when lipids are removed recalls comparable behavior of other lipoproteins such as human serum lipoproteins (29, 30, 34), mitochondrial structural proteins (12), and structural proteins of cell membrane systems (4, 8, 28).

This polymerization tendency of the SA-peptide, which seems to be a general physicochemical characteristic of lipoprotein-peptides, probably accounts for the high weight-average molecular weight of SA-peptide determined by sedimentation equilibrium experiments. It is obvious that the calculated molecular weight value obtained on the basis of the chemical composition of SA-peptide is unaffected by the aggregation phenomena, since the primary chemical analyses are based on gram masses, rather than moles, of material examined.

If it is assumed that the SA-peptide consists of a number of sub-units, then the maximal number of these may be calculated from the amino acid composition. Since it has been found that half-cystine occurs to the extent of approximately four residues per 100,000 grams of peptide (15), the minimal chain molecular weight may be estimated as approximately 25,000. The presence of one residue of allo-isoleucine and three residues of tryptophan per 100,000 grams of peptide (15) does not seriously challenge this assumption, since the proposed subunits may not have identical amino acid composition. A model

of subunit structure in lipoproteins has been reported previously (30, 33, 34).

The calculated values of the sedimentation constants ($s_{20,w}$) 1.53S and 3.10S, for the two major components of SA-peptide (Table 5) permitted the calculation of their minimal anhydrous molecular weights. The calculated minimal molecular weight values are:

Slow moving component, M = 8,600 gm/mole
Fast moving component, M = 24,800 gm/mole

We are thus led to the following considerations:

1. If we assume that the anhydrous SA-peptide molecule is a sphere, the observed sedimentary species ($s_{20,w}$, 1.53S, and 3.10S) should have molecular weights of not less than 8,600 and 24,800, respectively.
2. If, under the experimental conditions in which the ultracentrifugal analysis was made at pH 8.0, polymerization had ocurred, the fast-moving component would represent a trimer of the slow moving component. The consistency of the calculated molecular weight of 100,000 with the amino acid composition of this peptide seems to support the view that these two sedimenting species share the same chemical composition and do not constitute different substances.
3. We assume that the true molecular weight values of these subunits are much higher than the lower limits given above, depending on the degree of hydration of the peptide molecules, and their asymmetry of shape.

SUMMARY

Studies on the lung surface-active lipoprotein complex were carried out in order to establish, primarily, the physicochemical characteristics of its peptide moiety. It has been undertaken in the hope that new findings may extend present knowledge on the nature of the surfactant agent and of lipoproteins in general.

After removal of lipids from the SA-lipoprotein, the peptide moiety of the cow lung SA-lipoprotein was subjected to an exhaustive characterization study.

It was observed that the lipid-free materials are insoluble in distilled water, in acid, and in neutral physiological saline solutions. They are slightly soluble in 0.1 molar sodium chloride solutions at pH 10.0, and soluble in 0.1 normal sodium hydroxide solutions. The delipidized SA-peptide is not dialyzable.

The soluble lipid-free materials were concentrated by ultrafiltration through Diaflo Membranes and purified by gel filtration in columns of Sephadex.

It was observed that the properties of the purified SA-peptide material were markedly influenced by solvent pH. In the pH range 4-6, where the peptide was nearly isoelectric, it formed a gel, which could be dispersed by 6 molar

urea. At pH values higher than 7.0 or less than 4.0, traces of insoluble, perhaps aggregated material were observed in the solutions. It is apparent that the solution properties of this peptide material are controlled, at least in part, by its net charge.

Ultracentrifugal and electrophoretical data indicate that the SA-peptide preparations are heterogeneous. Two bands appeared after electrophoresis of SA-peptide on cellulose acetate strips: one major fast-moving component; and a minor one, close to the origin, which constituted about 5 per cent of the total peptide. Sedimentation patterns in the analytical ultracentrifuge showed two main components, with sedimentation constants ($s_{20,w}$) of 1.53S and 3.10S, with minimal anhydrous molecular weights of 8,600 and 24,800 (assuming spherical shape), respectively.

Calculations have shown that the SA-peptide contains more negative than positive groups, in accord with its acidic properties.

Molecular weight determinations with SA-peptide materials have led to values of 100,000 and 230,000, on the basis of chemical composition and sedimentation equlibrium data, respectively. The discrepancy between these two values may be explained by assuming that polymerization of SA-peptide occurs in solution, leading to a high value for the weight-average molecular weight determined by sedimentation equilibrium.

RESUMEN

Estudios para establecer las características fisicoquímicas de la liproteína tensoactiva pulmonar fueron llevados a cabo. Se espera que los resultados de esta investigación pueden servir para compender mejor la naturaleza del agente tensoactivo pulmonar así como para extender nuestro conocimiento general de las lipoproteínas.

Un estudio exhaustivo del constituyente péptido de la lipoproteína tensoactiva pulmonar fue realizado después de que los lípidos fueron removidos en su totalidad en material proveniente de pulmones bovinos.

Se observó que los materiales lipoprotéicos pulmonares libres de lípidos son insolubles en agua destilada, así como en soluciones salinas fisiológicas con pH ácido o neutro. Son ligeramente solubles en cloruro de sodio 0.1 molar pH 10.0, y completamente solubles en hidróxido de sodio 0.1 normal. El componente péptido de esta lipoproteína no es dializable.

Los materiales libres de lípidos fueron concentrados mediante ultrafiltración en membranas Diaflo y purificados en columnas de Sephadex.

Se observó que las propiedades del componente peptídico purificado son marcadamente influenciadas por el pH de los solventes, por ejemplo, el péptido en su estado isoeléctrico (pH 4-6) forma un gel que se dispersa con urea 6 molar. A pH mayores de 7.0 o menores de 4.0, se pudo observar trazas de material insoluble, quizás agregados. Se asume que las propiedades de solubilidad de este péptido están controladas, al menos parcialmente, por su carga neta.

La ultracentrifugación y la electroforesis indican que las preparaciones del componente péptido estudiado son heterogéneas. Las electroforesis del péptido en tiras de acetato de celulosa mostraron dos bandas: un componente mayor de rápida migración electroforética, y un componente menor más lento, localizado cerca del punto de origen, que se considera constituye aproximadamente el 5 % del total del péptido. Los patrones de sedimentación en la ultracentrífuga analítica mostraron dos componentes principales con constantes de sedimentación ($s_{20,w}$) de 1.53S y 3.10S, cuyos pesos moleculares anhidros mínimos fueron estimados en 8,600 y 24,800 respectivamente (suponiendo una forma esférica).

Los cálculos mostraron que el componente péptido de la lipoproteína tensoactiva pulmonar posee más grupos negativos que positivos en su constitución, lo que está de acuerdo con sus propiedades ácidas.

Las determinaciones del peso molecular de este péptido arrojaron valores de 100,000 y 230,000, con base en su composición química y con base en datos derivados de experimentos de sedimentación-equilibrio en la ultracentrífuga. Tal discrepancia entre estos dos valores puede ser explicada asumiendo que una polimerización del componente péptido ocurre en solución, determinando así un valor más alto para el peso molecular medio establecido mediante sedimentación-equilibrio.

LITERATURE CITED

1. ABRAMS, M. E.
1966. Isolation and quantitative estimation of pulmonary Surface-Active Lipoprotein. *J. Appl. Physiol.*, 21: 718-720.

2. ABRAMS, M. E., & F. B. TAYLOR
1964. Isolation and quantitative estimation of pulmonary Surface-Active Lipoprotein and its interaction with fibrinogen. *Physiologist*, 7: 78.

3. ALLERTON, S. E.
1961. *Ultracentrifugal studies on proteins.* Ph.D. Theis, Harvard University, 211 pp.

4. BAKERMAN, S., W. GOURLEY, G. WASEMILLER, & J. PETERSON
1967. Characterization of human erithrocyte membrane. *In Abstracts of Amer. Chem. Soc., Div. Biol. Chem.,154th Meeting* (Chicago; Sept. 11-15, 1967). Nº 294.

5. BREIER, R. O., & J. D. MULL
1964. Electrophoresis of serum protein with cellulose acetate. A method for quantitation. *Amer. J. Clin. Path.*, 42: 547-551.

6. CLEMENTS, J. A.
1962. Surface tension in the lungs. *Sci. Amer.*, 207: 121-130.

7. COHN, E. J., & J. T. EDSALL
1943. *Proteins, amino acids and peptides.* Reinhold Publishing Corp., New York, 686 pp.

8. COOK, R. T., W. E. NEELEY, & S. BAKERMAN
1967. Characterization of a human liver cell membrane preparation. *In Abstracts of Amer. Chem. Soc., Div. Biol. Chem., 154th Meeting* (Chicago: Sept. 11-15, 1967). N° 297.

9. CORNWELL, D. G., & F. A. KRUGER
1961. Molecular complexes in the isolation and characterization of plasma lipoproteins. *J. Lipid Res.*, 2: 110-134.

10. FINLEY, T. N., T. E. MORGAN, H. C. FLALKOW, & G. L. HUBER
1962. Surface activity of phospholipid components of dog pulmonary surfactant collected *in vivo*. *Fed. Proc.*, 23: 156.

11. GIBBONS, R. A.
1966. Physicochemical methods for the determination of purity, molecular size and shape of glycoproteins. *In* A. Gottschalk, ed. *Glycoproteins*, vol. 5 B. B. A. Library. Elsevier Publishing Co., New York. 628 pp.

12. GREEN, D. E., & S. FLEISCHER
1963. The Role of lipids in mitochondrial electron transfer and oxidative phosphorylation. *Biochim. Biophys. Acta*, 70: 554-582.

13. HANAHAN, D. J.
1960. *Lipid Chemistry*. John Wiley & Sons, Inc. New York. 330 pp.

14. JIMÉNEZ, J. M.
1968. *Studies on the pulmonary surface-active lipoprotein: isolation and characterization of its peptide moiety.* Ph. D. Dissertation. University of Southern California. 148 pp.

15. JIMÉNEZ, J. M.
1968. Studies on the pulmonary surface-active lipoprotein. I. Isolation and chemical characterizations of its constituents. *Rev. Biol. Trop.*, 17: 125-145.

16. KLAUS, M. H., J. A. CLEMENTS, & R. J. HAVEL
1961. Composition of surface-active materials isolated from beef lung. *Proc. Nat. Acad. Sci. (Wash.)*, 47: 1858-1859.

17. MCCLENEHAN, J. B.
1967. Abnormal "surfactant" activity secondary to protein component alteration. *Ann. Intern. Med.*, 66: 1055.

18. MCCLENEHAN, J. B., & J. D. OHLSEN
1968. Protein component of human surfactant. *Clin Res.*, 15: 134.

19. MCMEEKING, T. L., M. L. GROVES, & N. J. HIPP
1949. Apparent specific volume of alpha-casein and beta-casein and the relationship of specific volume to amino acid composition. *J. Amer. Chem. Soc.*, 71: 3298-3300.

20. MCMEEKING, T. L., & K. MARSHALL
1952. Specific volumes of proteins and the relationship to their amino acid content. *Science*, 116: 142-143.

21. ONCLEY, J. L.
1958. The lipoproteins of human plasma. *In* F. Homburger and P. Bernfeld, eds., *The Lipoproteins. Methods and clinical significance.* S. Karger, Basel.

22. PATTLE, R. E.
1955. Properties, function and origin of the alveolar lining layer. *Nature, (Lond.)*, 175: 1125-1126.

23. PATTLE, R. E.
1958. Properties, function and origin of the alveolar lining layer. *Proc. Roy. Soc. (Lond.), Ser. B,* 148: 217-240.

24. PATTLE, R. E.
1961. The formation of a lining film by foetal lungs. *J. Pathol. Bacteriol.,* 82: 333-343.

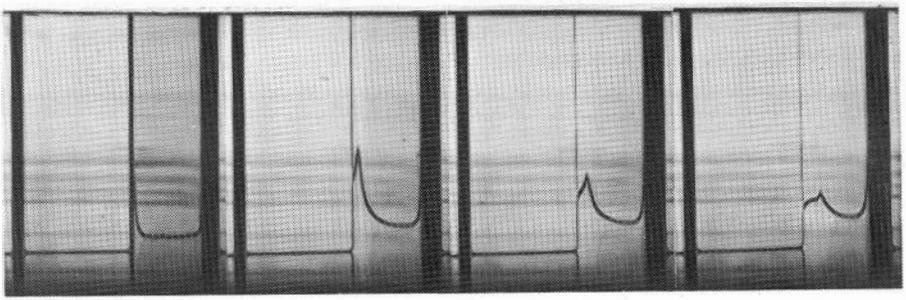
25. PATTLE, R. E.
1965. Surface lining of lung alveoli. *Physiol. Rev.,* 45: 48-79.

26. PATTLE, R. E., & D. A. W. HOPKINSON
1963. Lung lining in bird, reptile and amphibian. *Nature (Lond.),* 200: 894.

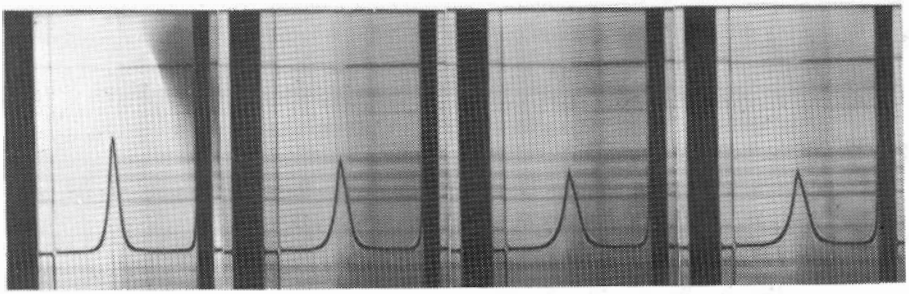
Fig. 1. Ultracentrifuge patterns from a typical sedimentation velocity run of SA-peptide. SA-peptide solution containing 0.76 gm/dl peptide in 0.1 M sodium chloride pH 8.0, at 20.85 c. The schlieren optical system was used with the phaseplate at an angle of 70° to the vertical. Photographs were taken automatically 4, 20, 36, and 52 minutas after the rotor had attained a final speed of 56,000 rpm.

Fig. 2. Ultracentrifuge patterns from a run in the synthetic boundary cell for the determination of the concentration of SA-peptide, required for a study during the approach to equilibrium. SA-peptide solution containing 0.76 gm/dl peptide in 0.1 M sodium chloride pH 8.0, at 20.85°. Photographs were taken with a diaphragm angle of 70°, and the final rotor speed was 12,590 rpm.

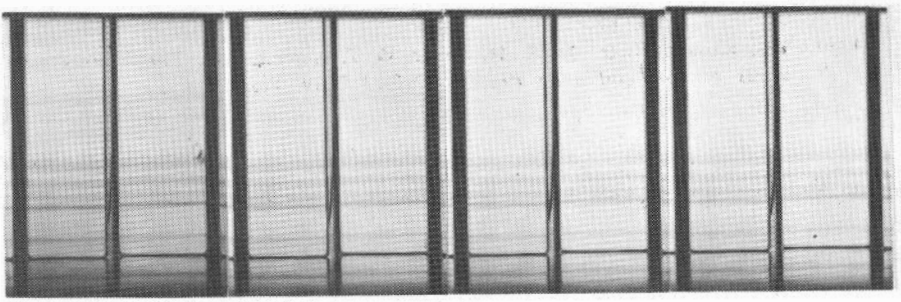
Fig. 3. Representative ultracentrifuge patterns from a study of SA-peptide during the approach to sedimentation equilibrium in short columns. SA-peptide solution contains 0.76 gm/dl peptide in 0.1 M sodium chloride pH 8.0, at 20.85°. Photographs were taken with a phase plate angle at 70° to the vertical after equilibrium was reached at a rotor speed of 6,166 rpm.



1



2



3

27. PATTLE, R. E., & L. G. THOMAS
1961. Lipoprotein composition of the film lining the lung. *Nature (Lond.)*, 189: 844.

28. RICHARDSON, S. H., H. O. HULTIN, & D. E. GREEN
1963. Structural proteins of membrane systems. *Proc. Nat. Acad. Sci. (Wash.)*, 50: 821-827.

29. SANBAR, S. S., & P. ALAUPOVIC
1963. Effect of urea on behavior of the protein moiety of human-serum alpha-lipoprotein in solution. *Biochim. Biophys. Acta*, 71: 235-236.

30. SCANU, A.
1966. Forms of human serum high density lipoprotein protein. *J. Lipid Res.*, 7: 295-306.

31. SCHACHMAN, H. K.
1959. *Ultracentrifugation in biochemistry.* Academic Press, New York. 272 pp.

32. SCHACHMAN, H. K.
1965. Ultracentrifugation, diffusion and viscometry. *In* S. P. Colowick and N. O. Kaplan, eds., *Methods in Enzymology.* Vol. 4. Academic Press, New York. 979 pp.

33. SHORE, B.
1957. C— and N— terminal amino acids of human serum lipoproteins. *Arch. Biochem. Biophys.*, 71: 1-10.

34. SHORE, V., & B. SHORE
1962. The protein subunit of human serum lipoproteins of density 1.125-1.200 gram/ml. *Biochem. Biophys. Res. Commun.*, 9: 455-460.

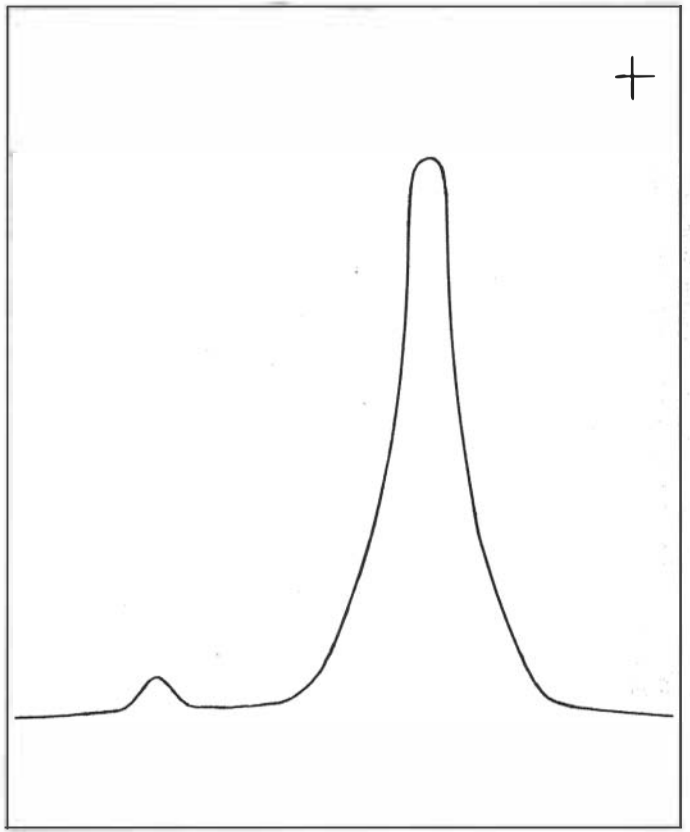
35. SHORT, R. H. D.
1950. Alveolar epithelium in relation to growth of the lung. *Phil. Trans. Roy. Soc. (Lond.), Ser. B.*, 235: 35-86.

36. SPINCO DIVISION, BECKMAN INSTRUMENTS, INC.
1961. *Instruction manual. Spinco Model E., analytical centrifuge.* Beckman Industries, Inc., Stanford Industrial Park, Palo Alto, California.

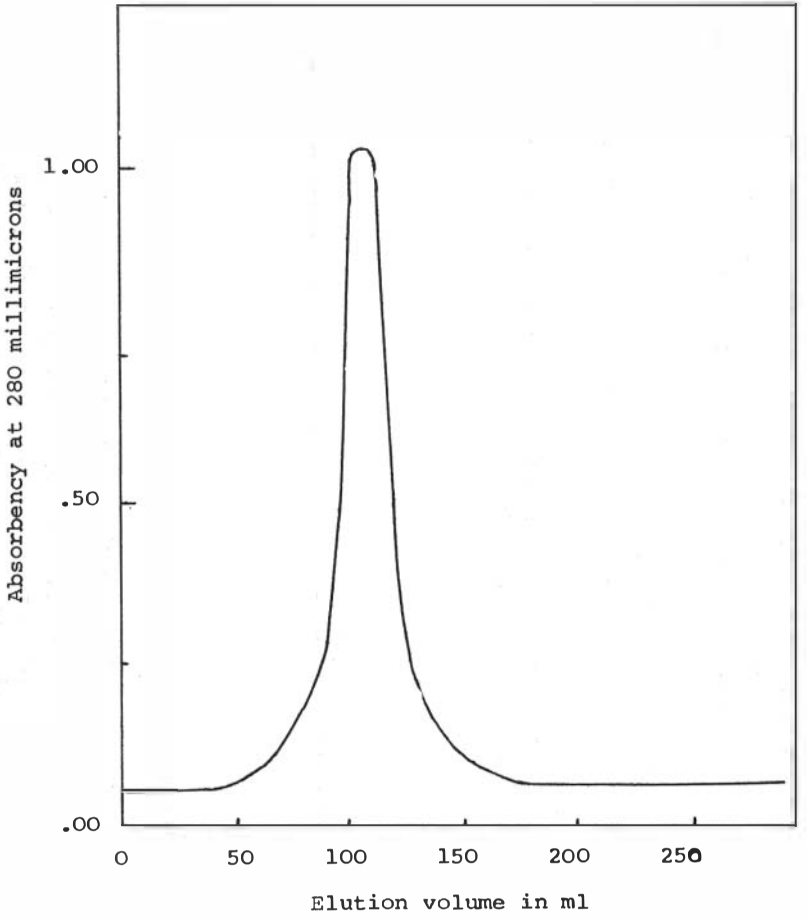
37. SVEDBERG, T., & K. O. PEDERSEN
1940. The ultracentrifuge. *In* R. H. Fowler & T. Kapitza, eds., *The international series of monographs on Physics.* Oxford Univ. Press. 478 pp.

Fig. 4. Cellulose acetate electrophoretic pattern of SA-peptide.

Fig. 5. Behavior of SA-peptide when gel filtered on a Sephadex G-100 column of medium grain.



4


Absorbency at 280 millimicrons
1.00
.50
.00
0
50
100
150
200
250
Elution volume in ml

5

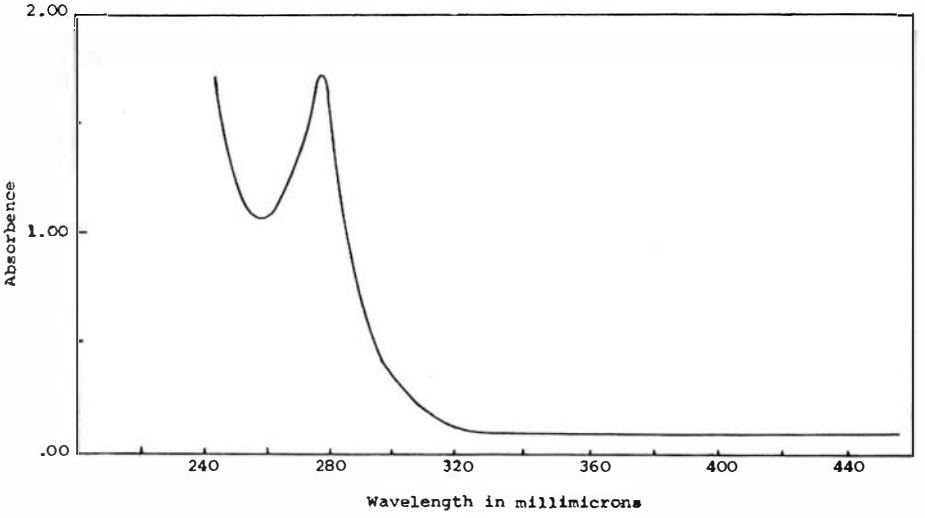
38. TECHNICON AUTO-ANALYZER SYSTEM
1965. Technicon Symposia. Micro-Kjeldahl nitrogen method. *In* L. T. Skeggs, Jr., ed., *Automation in analytical chemistry.* Mediad Incorporated, New York. 722 pp.

39. VAN HOLD, K. E., & R. L. BALDWIN
1958. Rapid attainment of sedimentation equilibrium. *J. Phys. Chem.,* 62: 734-743.

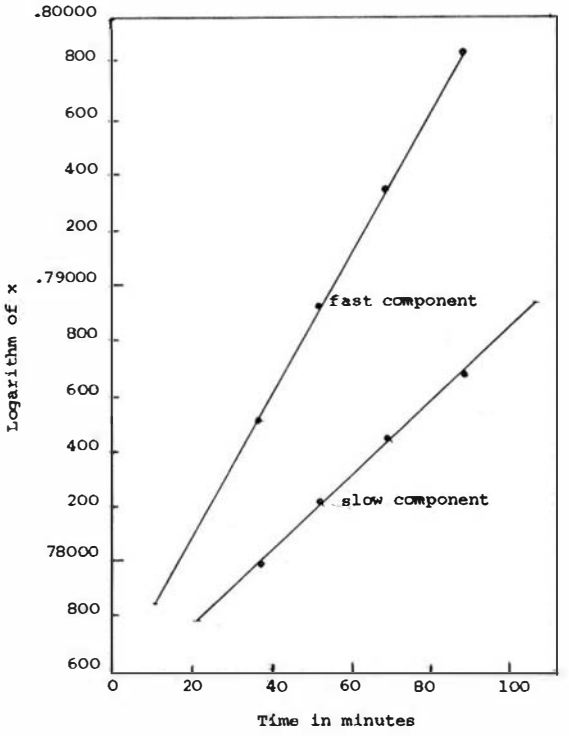
40. YPHANTIS, D. A.
1960. Rapid determination of molecular weights of peptides and proteins. *Ann. N. Y. Acad. Sci.,* 88: 586-601.

Fig. 6. Absorption spectra of SA-peptide.

Fig. 7. Plot of data from a typical sedimentation velocity experiment of SA-peptide, for the calculation of the slopes of the lines for each one of the two main components (slow and fast moving components). Data from Table 4.


2.00
1.00
.00
Absorbence
240
280
320
360
400
440
Wavelength in millimicrons

6


.80000
800
600
400
200
.79000
800
600
400
200
78000
800
600
Logarithm of x
fast component
slow component
0
20
40
60
80
100
Time in minutes

7