[CONTRIBUTION FROM THE RHEUMATIC FEVER RESEARCH INSTITUTE]

The Separation and Properties of a High Molecular Weight Simple Protein from a Lymphatic Organ[1,2]

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A third component has been isolated from the cytoplasmic extracts of bovine palatine tonsils. This component appears to be a simple protein of high molecular weight. Chemical and physical data, including diffusion behavior, light scattering studies, intrinsic viscosity, partial specific volume, isoelectric point, electrophoretic behavior, ultraviolet absorption characteristics, specific refractive increment, nitrogen, lipid, nucleic acid and hexose contents are reported.

Introduction

The isolation of a mucoprotein[3] and an albumin[4] from cytoplasmic extracts of bovine palatine tonsils has been reported previously. The conditions of separation, the yield and some chemical and physical properties of a third component isolated from extracts of bovine palatine tonsils will be discussed in this report. This fraction appears to be a simple protein[2] and possesses, at least to some extent, the solubility properties of the class of proteins called glutelins. We have hesitated to call it a glutelin since this term has hitherto been ascribed only to proteins of vegetable origin. Since it is the third component separated from a lymphatic organ in this Laboratory it has been designated as L3.

Experimental

The earlier steps in the fractionation procedure as well as most of the experimental techniques employed have been described previously.[3-5] For the light scattering studies an American Instrument Company microphotometer was employed. The instrument was calibrated using a 3% solution of Ludox[6] according to the method of Oster.[7] The validity of the calibration constants was verified by determining the molecular weight of crystalline bovine serum albumin[8] for which an average value of 66,400 was obtained from a series of measurements at three ionic strengths at *p*H 4.7. This value is in good agreement with the generally accepted value for the molecular weight of this protein.[9,10] Both the dissymmetry method[11] and the Zimm extrapolation method[12] were used in calculation of the molecular weight of L3 from light scatter measurements.

The refractive index increment $(n_s - n_0)/C = k$ was obtained simultaneously with the diffusion coefficient. An electrophoresis assembly was used for this purpose with T 0.4° and λ = 5780 Å. The necessary apparatus constant was determined indirectly using 0.1000 N KCl solution as a standard. The refractive index of 0.1000 N KCl at 0.4° at λ = 5780 Å. was taken as 0.001109.[13] The correctness of this constant was verified by determining the specific refractive increment of crystalline bovine serum albumin.[8] This was found to agree within 3% with that given by Perlmann and Longsworth.[14]

The values k_{4360} and k_{5460} were calculated from the relationship of Perlmann and Longsworth[14] $k_\lambda = k_{5780}(0.940 + 2.00 \times 10^6/\lambda^2)$. A temperature correction[14] was applied as well, in order to adjust the value of $k_{4360}^{0.4°} = 0.00191$ to $k_{4360}^{25°} = 0.00189$ used in the light scatter calculations.

The specific refractive increment was determined far less accurately than is desirable, especially since it occurs as a square term in the light scatter equation. At the present time, however, the above method is the only one available to us. Because of the inherent errors in the method the reported value for the refractive index increment of L3 cannot be considered reliable beyond the fourth decimal.

Most of the physical studies have been performed at *p*H 3.0. This *p*H has been chosen for reasons of stability and solubility. The protein is insoluble at the isoelectric point and the solubility away from the isoelectric point is an inverse function of ionic strength. In order to obtain solutions of 1% concentration at an ionic strength (μ) 0.15 it is necessary to approach *p*H 3.0. The protein is unstable in the *p*H range 6.5–8.5 and spontaneously oxidizes, even at cold room temperatures, due to dissolved oxygen in the solvent. This substance is also unusually sensitive to ultraviolet radiation in the *p*H range 6.5–8.5.[15] At *p*H 3.0 component L3 appears to be quite stable even at room temperature.

Dry samples of L3, prepared by freeze drying, although initially soluble in water at *p*H 3–4, become insoluble upon standing a few weeks at cold room temperatures. For this reason, all physical measurements have been made upon freshly prepared materials kept in solution at *p*H 3.0.

The electron microscope studies were performed on an RCA instrument equipped with Em U microscope.[16]

The sedimentation studies were performed by Dr. Virgil Koenig,[17] employing a model E Spinco ultracentrifuge. The average centrifugal field was 260,000 *g* and the average temperature 25°. Analyses were performed in sodium phosphate buffers at *p*H 3.0, at the ionic strength specified.

All electrophoretic patterns are photographs of the descending limb of the cell after 120 minutes under the potential gradient indicated.

Fractionation

The fractionation steps (to include fraction 6.2P) have been discussed previously.[5,15] Fraction 6.2P (Fig. 1A) contained from 0.5 to 1.5% nucleic acid which may represent from 5 to 15% nucleoprotein[15] admixed with component L3. It was found that at *p*H 9 and at μ 0.05 component L3 precipitated and the contaminant remained in the supernatant solution.

The precipitate called 6.2P which amounted to 750 mg., was dissolved in 50 ml. of water by lowering the *p*H to 3.0. The ionic strength of this solution was made 0.05 with NaCl and the *p*H of the solution was raised to 9.0. The solution was centrifuged and the precipitate dissolved in 40 ml. of water by the addition of 0.05 N HCl to *p*H 3.0. This solution after being dialyzed against water and lyophilized

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(2) Presented in part before the Division of Biological Chemistry (Paper 69) at the 124th National Meeting of the American Chemical Society at Chicago, September 9, 1953. At that time L3 was thought to be a phosphoprotein. Subsequent work disclosed that this conclusion was incorrect. The phosphorus found in the earlier preparations was due to small amounts of nucleic acid in these preparations.

(3) E. L. Hess, W. Ayala and A. Herranen, THIS JOURNAL, **74**, 5410 (1952).

(4) E. L. Hess, M. Campbell and A. Herranen, *ibid.*, **76**, 4035 (1954).

(5) E. L. Hess and S. E. Lagg, submitted for publication.

(6) We wish to thank Mr. R. J. Kepfer of E. I. du Pont de Nemours & Co., Grasselli Chemicals Department, Chicago, Illinois, who kindly supplied us with this material.

(7) G. Oster, *J. Polymer Sci.*, **9**, 525 (1952).

(8) Armour & Co. Control #51.

(9) H. Neurath and K. Bailey, "The Proteins," Academic Press, Inc., New York, N. Y., 1953, p. 721.

(10) J. T. Edsall, *J. Polymer Sci.*, **12**, 270 (1954).

(11) Ref. 9, p. 610.

(12) B. H. Zimm, *J. Chem. Phys.*, **16**, 1099 (1948).

(13) L. G. Longsworth, THIS JOURNAL, **69**, 2510 (1947).

(14) G. E. Perlmann and L. G. Longsworth, *ibid.*, **70**, 2719 (1948).

(15) E. L. Hess, D. Yasnoff and S. Lagg, *Arch. Biochem. Biophys.*, **57**, 323 (1955).

(16) In the School of Dentistry of Northwestern University.

(17) Veterans Research Hospital, Chicago, Illinois.

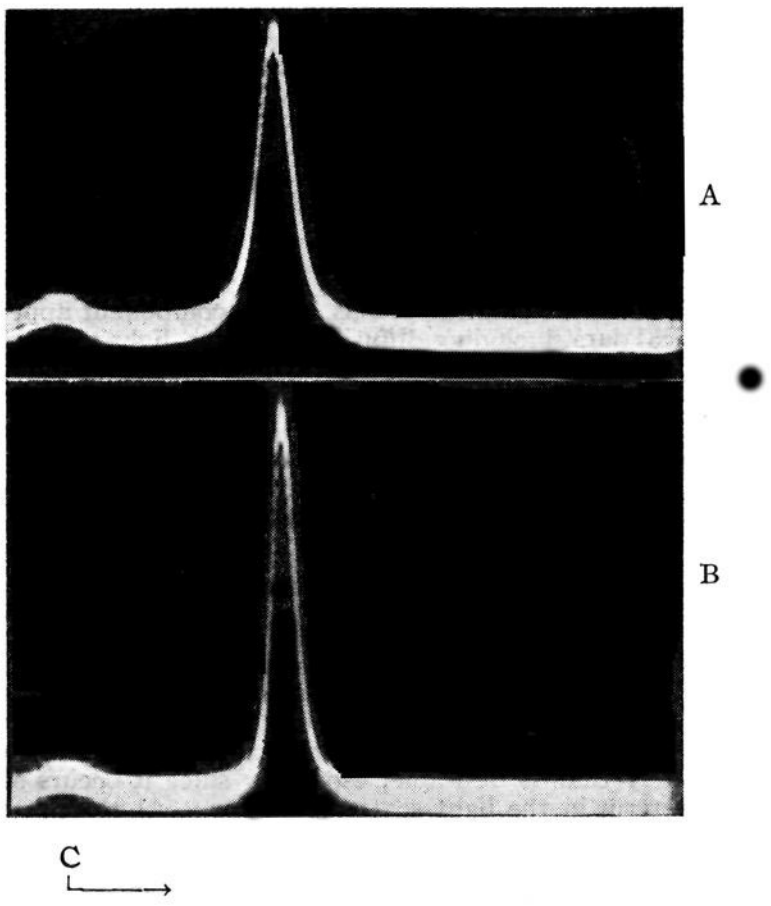

Fig. 1.—Bovine palatine tonsil fractions. A photograph of the descending limb cell after 120 min. under a potential gradient of 5.4 volt cm.$^{-1}$ in μ 0.10 sodium phosphate *p*H 3.0. Protein concentration 1%, diagonal slit angle 40 degrees: A, fraction 6.2P; B, fraction L3.

yielded 600 mg. of solids. The dried material could be dissolved in water at *p*H 3.0 or in μ 0.10 phosphate at *p*H 3.0, and in the latter solvent gave the electrophoretic pattern seen in Fig. 1B. This material has been called L3.

Data and Discussion

Characterization data are listed in Table I.

TABLE I

CHARACTERIZATION DATA ON BOVINE TONSIL COMPONENT L3

V_{20w}	0.752	*p*H 3.0
pI_e	6.2	Solubility $\mu < 0.005$
H, dl./g.	0.17	μ0.15 NaCl *p*H 3.0
$(n_p - n_0)/c$, 5780 Å., dl./g.	0.00183	μ(0.10 phosphate) (0.05*N* NaCl *p*H 3.0)
u, cm.^{2}/sec., v.	6.2×10^{-5}	μ0.10 PO_4 *p*H 2.85
	3.9×10^{-5}	μ0.10 NaOAc *p*H 4.2
$E_{280}^{1\%}$	10.0	
Nitrogen, %	15.5	
Lipid, %	1.1	Alcohol–ether ext.
Nucleic acid, %	<0.1	Schneider[20]
Phosphorus, %	<0.01	Fiske-SubbaRow[21]
Hexoses, %	0.5	Anthrone[22]
Z	1.5	Light scattering
M	$2.3 \pm 0.4 \times 10^6$	Light scattering

Light Scattering Studies.—The reduced intensities $(K_c)/R_{90}$ obtained in buffers of various ionic strengths were plotted against concentration as shown in Fig. 2. Although the agreement between the intercepts at zero concentration is not as good as desired it is apparent that the weight average molecular weight for L3 is approximately 2.3×10^6 as determined from the equation[18] $K_c/R_{90} = 1/M + 2Bc$. This is an unusually high molecular weight for an apparently simple protein.

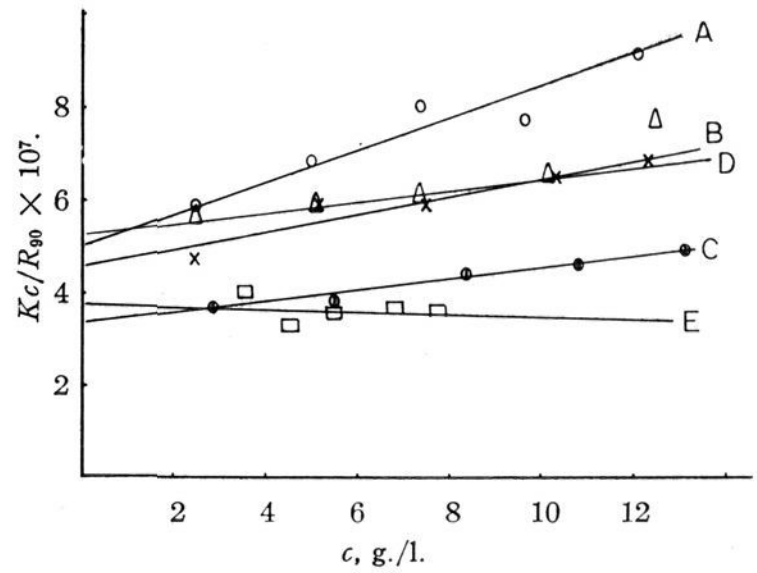

Fig. 2.—A plot of reduced intensities against concentration of component L3 made at λ 4360 Å.: A, μ 0.00 *p*H 3.0; B, μ 0.02 NaH_2PO_4 *p*H 3.0; C, μ 0.05 NaH_2PO_4 *p*H 3.0; D, μ 0.10 NaH_2PO_4 *p*H 3.0; E, μ 0.15 (0.05 NaCl) (0.10 H_2PO_4) *p*H 3.0.

It is of interest that with a dissymmetry coefficient (Z) of about 1.5 an assumption concerning particle shape does not affect the calculated molecular weight greatly. However, using the extrapolation method, which does not require an assumption about shape in order to obtain a molecular weight, a value of $M = 2.5 \times 10^6$ was obtained. This value agrees well with that calculated by means of the dissymmetry method.

As would be expected,[18] where the determinations were carried out with a rather large positive charge on the molecule,[19] the interaction constant B was greater at the lower ionic strengths and decreased progressively with an increase in ionic strength.

There was some evidence that the value of reduced intensity extrapolated to zero concentration of protein $(K_c/R_{90})_0$ was a function of ionic strength. When samples of L3, taken from the same preparation, were studied in solutions of different ionic strengths, and this was done on three occasions, $(K_c/R_{90})_0$ was always found to be lower at the higher ionic strength. A marked increase in opalescence occurred when inorganic ions were added to an aqueous solution of L3. There was no evidence that Z was a function of ionic strength.

Electrophoretic Studies.—Electrophoretic studies have been limited by both solubility and stability factors, nevertheless the electrophoretic patterns obtained in several buffer systems and at several *p*H values indicated a high degree of electrokinetic homogeneity. L3 has been examined at *p*H 3 in 0.10 ionic strength sodium phosphate and sodium monochloroacetate buffers; at *p*H 4.2 in 0.10 ionic strength sodium acetate; and at *p*H 9.2 in 0.02 ionic strength sodium phosphate buffer. In each instance a single symmetrical peak was observed. It is of interest that at *p*H 3 the ascending and descending boundaries were not enantiographic. In the phosphate system the ascending pattern

(18) P. Doty and J. T. Edsall, *Advances in Prot. Chem.*, **6**, 43 (1951).

(19) From titration data, the isoelectric point, and the molecular weight of 2.3×10^6 it was calculated that the molecule must have an average of 2500 protonic charges at *p*H 3.

(20) W. C. Schneider, *J. Biol. Chem.*, **161**, 293 (1945).

(21) C. H. Fiske and Y. SubbaRow, *ibid.*, **66**, 375 (1925).

(22) S. Seifter, S. Dayton, B. Novic and E. Muntwyler, *Arch. Biochem.*, **25**, 191 (1950).

was much sharper than the descending patterns shown in Fig. 1; in monochloroacetate the reverse obtained. At *p*H 4.2 in μ 0.10 sodium acetate the two patterns were enantiographic.

Diffusion Studies.—The diffusion coefficient was determined at one concentration; 0.4% in 0.15 *M* NaCl at *p*H 3.1. A value of D_{20w} of 1.0×10^{-7} was obtained from the height and area method of calculation. The value calculated using the inflection point method was 0.8×10^{-7} while the method of moments gave $D_{20w} = 3.1 \times 10^{-7}$ cm.^{2} sec.$^{-1}$. The normalized refractive index gradient distance curve did not show skewness but did differ from the ideal curve with respect to the height of the maximum ordinate. This can be taken as evidence of polydispersity.[23] The diffusion results are subject to question, however, for the same reasons as are the sedimentation studies.

Other Studies.—The only criteria of molecular kinetic homogeneity available to us at this time are electrophoresis and turbidity[24] studies. The results of the turbidity measurements made on L3 are consistent with the viewpoint that we are dealing with a single substance.

Sedimentation velocity experiments were run at several ionic strengths at *p*H 3.0. Because the solubility of the material decreased with an increasing concentration of inorganic ions the greatest ionic strength employed was 0.15. Since we were unable to reduce the primary charge effect[25-27] to a negligible amount it was concluded that these experiments did not provide worthwhile information concerning the sedimentation properties of L3.

The intrinsic viscosity (*H*) is indicative of pronounced molecular asymmetry or marked hydration or both. The ultraviolet absorption behavior of L3 is that of a typical protein with a maximum at 278 mμ and a minimum of 250 mμ.

The electron microscope studies were difficult to interpret. Several possible artifacts must be considered. In preparing the sample for examination both drying and shadowing may produce alterations in size and shape. Exposure to high intensity radiation may likewise alter the molecule; L3 has been shown to be unusually sensitive to ultraviolet radiation.[15] Both rods and spheres were observed in the electron micrographs at a magnification of 23,000. The rods were calculated to be approximately 1800 Å. in one dimension and 400 Å. in the other. Such a particle would have a volume about six times that of tobacco mosaic virus obviously inconsistent with the observed molecular weight. If one assumes a rod-shaped molecule and uses the value of $Z = 1.5$ a particle with a dimension of 1400 Å. may be calculated. The diameters of the spheroids were approximately 450 Å. Other particles of various shapes were also observed. These particles appeared to be aggregates of the spheroids. Spheres arranged like beads on a string or in the form of T's, X's and V's were prevalent.

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(23) H. Neurath, *Chem. Revs.*, **30**, 374 (1942).
(24) E. L. Hess and D. S. Yasnoff, THIS JOURNAL, **76**, 931 (1954).
(25) T. Svedberg and K. Pedersen, "The Ultracentrifuge," Oxford, Press, New York, N. Y., 1940, p. 23.
(26) L. Varga, *J. Biol. Chem.*, **217**, 651 (1955).
(27) We have estimated from the electrophoretic mobility and the molar frictional constant that at *p*H 3.0 and $\mu = 0.15$ the effective charge of the molecule is still about 6% of the total indicated in reference 19 or approximately 150 protonic charges. A charge of this magnitude would certainly affect the sedimentation behavior of L3. In fact the sedimentation patterns bore a striking resemblance to the pattern obtained by Varga[26] with hyaluronic acid at μ 0.02 where the estimated effective charge was 173.

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The Amino Acid Sequence of Polymyxin B_1

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Totally substituted DNP-polymyxin B_1 was partially hydrolyzed in acid and the resulting peptide mixture was fractionated by means of multiple dialysis and countercurrent distribution in various systems. Fourteen DNP-peptides were isolated in a state of purity indicated by the criteria of countercurrent distribution, paper chromatography and paper electrophoresis, where the latter technique was applicable. Resubstitution of these peptides with fluorodinitrobenzene and subsequent study by countercurrent distribution and paper chromatography before and after hydrolysis permitted determination of the positions of all the amino acids save one, for which two possibilities still exist. Di-DNP-α,γ-diaminobutyric acid was found to be destroyed to the extent of 60–65% under the hydrolysis conditions. By the same treatment DNP-threonine was 95% converted to dinitroaniline.

Introduction

In an earlier communication[1] it was shown that upon total hydrolysis of polymyxin B_1 (PMB_1) six moles of predominantly L-α,γ-diaminobutyric acid (DAB), two moles of L-threonine (Thr), one mole of L-Leucine (Leu), one mole of D-phenylalanine (Phe) and one mole of 6-methyl-octan-1-oic acid, an isopelargonic acid (Ipel), were liberated. An empirical formula $C_{56}H_{99}O_{14}N_{16}$ for the free base was found and a molecular weight of 1220, assuming the fatty acid residue to be attached to one of the amino groups and the presence of one free carboxyl group in the peptide molecule. The present paper reports experiments designed to determine the amino acid sequence and additional fine

(1) W. Hausmann and L. C. Craig, THIS JOURNAL, **76**, 4892 (1954).