may be explained on the basis of one of two structures for normal methylene: (a) a free radical structure, (b) a molecular (unpromoted electron) structure with heat of formation but slightly above that of the free radical.

NEW YORK, N. Y. RECEIVED APRIL 24, 1941

[CONTRIBUTION FROM THE LABORATORY OF PHYSICAL CHEMISTRY, UNIVERSITY OF WISCONSIN]

The Physical-chemical Investigation of Certain Nucleoproteins. III. Molecular Kinetic Studies with Calf Thymus Nucleohistone

BY R. OWEN CARTER[1,2]

In an earlier report certain striking physical and chemical properties of the nucleohistone of the calf thymus gland have been presented.[3] The electrophoretic behavior of this nucleohistone also has been considered.[4] Further characterization of the material has been carried out by making observations of its sedimentation and diffusion behavior in solutions containing relatively large amounts of sodium chloride or calcium chloride.

Experimental Procedures

Sedimentation Velocity Measurements.—Measurements of sedimentation velocity were made, using the refractive index method,[5] in a standard Svedberg oil-turbine ultracentrifuge at a speed of 50,000 r. p. m., corresponding to a centrifugal force of about 200,000 times gravity at the center of the sector-shaped solution cell. A 6-mm. cell was used, with a scale distance which varied between 5 and 10 cm., depending upon the concentration of the solution in the cell. The temperature in the cell varied somewhat from experiment to experiment, sometimes reaching a low value near 22°, and at other times rising as high as 27°. During any individual experiment the variation in temperature from the time of attainment of full speed did not exceed 2°. Solvent experiments were made under identical centrifuging conditions and the scale photographs obtained in this way were used as standard reference scales from which the deviations, caused by the sedimentation of the nucleohistone, were measured.

The line positions in the scale photographs were measured to the nearest micron with a Gaertner micro-comparator. The sedimentation curves, obtained by plotting the line displacements as a function of distance from the center of rotation, were for the most part regular and normal in character.

The sedimentation constants were calculated from the equation

$$s = \frac{\Delta x}{\Delta t} \times \frac{1}{\omega^2 x_m}$$

and, for purposes of comparison, reduced to the basis of sedimentation in water at 20° by the expression

$$s_{20} = s \left(\frac{\eta_t}{\eta_{20}}\right)_{H_2O} \left(\frac{(1 - V\rho_{20})}{(1 - V\rho_t)}\right)_{H_2O} \cdot \frac{\eta}{\eta_0} \frac{(1 - V\rho_0)}{(1 - V\rho)}$$

The symbols used have now been standardized. The partial specific volume, V, of sodium nucleohistone is 0.658.

Sedimentation Equilibrium Measurements.—The low-speed ultracentrifuge with direct motor drive developed by Svedberg was used in the sedimentation equilibrium studies. In this form of instrument the rotor rests upon the conical top of the vertical shaft of the motor and is surrounded by a brass rotor casing so that it can be thermostated.

In this case the shape of the cell was of no concern, but a centrifugal force giving optimal concentration distribution had to be found in order to have a reasonably accurate result. The cell and counter cell were fitted into the rotor and the rotation was continued at constant temperature until test measurements showed that equilibrium between sedimentation and diffusion was attained.

Provisions are made in the instrument for passing light through a uniform linear scale and the cell contained in the rotor, in order, during the operation. The method of observation of the concentration gradient in the cell was again that of Lamm.

The molecular weights of the nucleohistone were computed from the equation

$$M = \frac{2RT \ln z_2x_1/z_1x_2}{(1 - V\rho)\omega^2 (x_2^2 - x_1^2)} = 4.02 \times 10^6 \frac{\log z_2x_1/z_1x_2}{(x_2^2 - x_1^2)}$$

where z_2 and z_1 are the refractive-index gradients at the distances x_2 and x_1 from the center of rotation. The calculations followed closely the procedures recently described in detail by Svedberg and Pedersen.[5c]

Diffusion Measurements.—The method used for measuring the diffusion constants was that of Lamm and Polson.[6] In this method a uniform transparent scale is photographed through the glass diffusion cell. The gradient in refractive index at the diffusion boundary produces a distorted image of the scale, with scale line displacement being proportional to the concentration gradient when the refractive index is a linear function of the concentration.

---

(1) More complete details of this work may be found in a thesis by R. Owen Carter presented in June, 1939, to the Faculty of the University of Wisconsin in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

(2) Present address: Research Laboratory, The Procter and Gamble Co., Cincinnati, Ohio.

(3) Carter and Hall, THIS JOURNAL, **62**, 1194 (1940).

(4) Hall, *ibid.*, **63**, 794 (1941).

(5) (a) Lamm, *Z. physik. Chem.*, **A138**, 313 (1928); **A143**, 177 (1929); Dissertation, Upsala (1937); (b) McFarlane, *Biochem. J.*, **29**, 407 (1935); (c) Svedberg and Pedersen, "The Ultracentrifuge," Clarendon Press, Oxford, 1940.

(6) Lamm and Polson, *Biochem. J.*, **30**, 528 (1936); **31**, 1903 (1937).

The diffusion cell was very much like that described by Svedberg.[7] The internal diameter of the cylindrically ground and polished diffusion arm of the apparatus was 1 cm. The linear scale was a photographic reduction of a large scale of 200 divisions to a length of 53 mm. The light source was a sodium vapor lamp. The final line displacement caused by the blurring of the boundary was read by means of the Gaertner micro-comparator. The normal undeviated scale, photographed through the solvent, was computed and used as a reference scale.

The differences between corresponding scale divisions of the undeviated and deviated scales were plotted against distance as measured by the deviated scale. It is this curve which approaches the normal probability curve for a homogeneous dispersed substance. The diffusion constant is computed from formulas derived from the classical equation of Wiener

$$\frac{dn}{dz} = \frac{n_1 - n_0}{2\sqrt{\pi Dt}} e^{-z^2/4Dt}$$

where n_1 and n_0 are the refractive indices of solution and solvent, and z is the distance from the original boundary.

Experimental Results

The sedimentation constant of calf thymus nucleohistone was determined between *p*H 5 and 12, at various protein concentrations and in the presence of either sodium chloride or calcium chloride. The solutions were made up immediately before the start of the experiment. The results of a number of representative experiments have been tabulated in Table I.

TABLE I

SEDIMENTATION VELOCITY DETERMINATIONS: CALF THYMUS NUCLEOHISTONE, CENTRIFUGAL FORCE 200,000 × GRAVITY

Expt.	Protein concn.	Solvent	*p*H	s_{20}
7	0.56	5% NaCl, buffer	5.3	13.9
8	.56		6.4	14.2
9	.56		7.3	14.0
10	.56		9.0	13.0
11	.56		10.4	11.3
12	1.25		11.9	3.1
13	0.82		6.4	12.5
14	.62		6.4	14.0
15	.41		6.4	17.3
16	.27		6.4	20.3
17	.21		6.4	23.0
18	.10		6.4	24.7
21	.29		10.4	12.8
22	.20		10.4	14.3
23	.13		10.4	16.0
24	.67		11.9	4.5
25	.72	2% $CaCl_2$, unbuffered		32.0
26	.41	2% $CaCl_2$, unbuffered		32.0

Experiments 7 to 12 were carried out in order to study the *p*H stability of the protein. Since the sedimentation constant for the nucleohistone in solutions containing 5% sodium chloride is a function of protein concentration, this factor had to be carefully controlled. The data of Table I show the sedimentation constants of 0.56% protein solutions to be independent of *p*H over the interval from 5.3 to 9.0. A definite (and irreversible) break in stability occurs at *p*H 10.4 and the sedimentation constant has fallen to almost one-fifth of its apparent value at *p*H 11.9. At this high *p*H a precipitate which has the characteristics of a simple histone is formed. It is the clear liquid remaining after the removal of the precipitate which was studied in the ultracentrifuge. This solution (expt. 12) now contains a homogeneous main component, and, in addition, a quantity of much lighter material.

The sedimentation constant for solutions with protein concentration 0.56% has been referred to as an apparent value. The reason for this description will be evident from a study of the results of expts. 13 to 18, in which the protein concentration has been varied continuously from 0.82% down to 0.10%, while the solvent has remained unchanged. In seeking to find a function of the sedimentation constant that could be readily extrapolated to give the limiting value for zero protein concentration it was found that all the experimental values lie on a straight line when the reciprocal of s_{20} is plotted as a function of protein concentration. This is equivalent to plotting molar frictional coefficient against concentration. Signer and Gross[8] observed a similar behavior when studying the sedimentation behavior of fractionated polystyrenes. The true value for s_{20} is 31×10^{-13} cm./sec./dyne.

This series of experiments illustrates the importance of studying the variation of sedimentation constant with protein concentration when new substances are considered. If, as is sometimes done, we had reported only the sedimentation constant datum for a solution which is one (or some other) per cent. in protein concentration, the figure given could not be described as a characteristic molecular constant. The extreme variation found in this case is due to the elongated nature of the molecule.

A similar study of the effect of protein concentration change on the sedimentation constant of the nucleohistone unit in buffered solution at *p*H 10.4 has been made. The data of experiments 21

(7) Svedberg, "Colloid Chemistry," 2nd edition, The Chemical Catalog Co., New York, N. Y., 1928

(8) Signer and Gross, *Helv. Chim. Acta*, **17**, 59 (1934).

to 23 show again a large variation of the kind just described, and a plot of $1/s_{20}$ *versus* protein concentration gives as a limiting value $s_{20} = 20 \times 10^{-13}$ cm./sec./dyne. The scale line displacement–distance curves for this species show that the new component is quite monodisperse and that no other material is present in appreciable quantity.

Experiment 24 forms a second experiment in a concentration series with expt. 12. In both experiments the "molecular unit mass spectrum" curve indicates that the principal component is homogeneous. These solutions form gels on standing in an ordinary refrigerator over a two or three week period.

Another series of sedimentation velocity experiments was performed in unbuffered medium in the presence of 2% calcium chloride. These solutions are much less viscous than the solutions of corresponding protein concentration in 5% sodium chloride. In this type of solution it was observed that the sedimentation constant is essentially independent of protein concentration. However, the maxima in the line displacement–distance curves are broad enough to indicate a limited, but nevertheless definite range of sizes. The relatively high calcium ion concentration appears to have rendered the nucleohistone polydisperse without appreciable change in the average sedimentation constant. The average value, $s_{20} = 32 \times 10^{-13}$, differs but slightly from the limiting value, $s_{20} = 31 \times 10^{-13}$, obtained for the sodium nucleohistone. A more extensive characterization and study of the protein in calcium chloride solution is planned.

Several sedimentation equilibrium experiments were made with the nucleohistone. Anomalous behavior was observed when the protein concentration exceeded 0.1%. For a material of high molecular weight like nucleohistone low rotor speeds are required. With the instrument available for the work the slowest speed that can be used is 2840 r. p. m., a speed which gives relatively high protein concentration toward the bottom of the cell at equilibrium. Under such conditions viscosity and inter-molecular hindrance may build up to make the attainment of the true equilibrium a very slow process. Also when the material is present at such high concentrations it may behave in an abnormal osmotic manner and give a "swelling effect."

In Table II there have been collected the data and results for two successful experiments at high dilution of protein. In the table x is the distance from the center of rotation, z is the displacement of the scale line corresponding to the distance x, and M is the molecular weight of the nucleohistone calculated between the position x and the one 0.5 mm. nearer to the center of rotation. Since no appreciable variation of molecular weight with distance is observed it is concluded the protein is nearly monodisperse.

TABLE II

SEDIMENTATION EQUILIBRIUM DETERMINATIONS AT 25°

Expt. 1: Concentration of thymus nucleohistone at start: 0.08 g. protein/100 cc.; buffer solutions, 0.855 (M) NaCl + 0.005 (M) KH_2PO_4 + 0.005 (M) Na_2HPO_4; pH 6.4; rotor speed, 2840 r. p. m. Computations from two sets of exposures, 124 and 144 hours after starting. Expt. 2: Concentration at start 0.06 g. protein/100 cc. Duration of experiment, 144 hours; other conditions same as Expt. 1.

x (mm.)	z (mm.)	M
	Experiment 1	
51.0	0.191	
		1,700,000
50.5	.114	
		2,100,000
50.0	.059	
		2,100,000
49.5	.032	
		2,100,000
49.0	.017	
		2,500,000
48.5	.008	
		Av. 2,100,000
51.0	.201	
		1,900,000
50.5	.116	
		2,300,000
50.0	.059	
		2,000,000
49.5	.033	
		1,900,000
49.0	.019	
		2,200,000
48.5	.010	
		Av. 2,070,000
	Experiment 2	
51.0	.134	
		1,500,000
50.5	.085	
		1,200,000
50.0	.060	
		1,200,000
49.5	.042	
		1,400,000
49.0	.028	
		1,900,000
48.5	.016	
		2,400,000
48.0	.008	
		2,500,000
47.5	.004	
		Av. 1,700,000

The experimental error in expt. 2 is relatively large because the protein concentration was only 0.06%. Solutions which are more dilute cannot be studied in this way.

The results of three typical diffusion experiments are collected to form Table III. Diffusion experiments were usually abnormal in that skewed line displacement-distance curves were obtained. Two methods were used in the computation of the diffusion constants; the method of area and maximum height, and the method of moments. This second method probably gives the better value.

TABLE III

DIFFUSION EXPERIMENTS AT 25°

Expt. 1: Protein concn. 0.51 g./100 cc. (0.855 (*M*) NaCl, 0.005 (*M*) KH_2PO_4, 0.005 (*M*) Na_2HPO_4: *p*H 6.4). Expt. 2: protein concn. 0.20 g./100 cc. (0.855 (*M*) NaCl, 0.005 (*M*) KH_2PO_4, 0.005 (*M*) Na_2HPO_4: *p*H 6.4). Expt. 3: protein concn. 0.46 g./100 cc. (0.01 (*M*) KH_2PO_4, 0.01 (*M*) Na_2HPO_4: *p*H 7.0).

$A \times 10^3$, sq. cm.	$H_m \times 10^3$, cm.	*t*, sec.	μ, cm.	$D_A \times 10^7$, sq. cm./sec.	$D_\mu \times 10^7$, sq. cm./sec.
		Experiment 1			
9.52	25.74	128,080	0.1953	0.76	1.33
9.96	23.20	169,720	.2159	.77	1.22
9.90	20.59	218,155	.2522	.75	1.30
			Average	.76	1.28
$D_{A_{25}} = 0.76 \times 1.087 = 0.83 \times 10^{-7}$ cm.²/sec.					
$D_{\mu_{25}} = 1.28 \times 1.087 = 1.39 \times 10^{-7}$ cm.²/sec.					
		Experiment 2			
3.79	10.32	93,720	0.1915	1.01	(1.73)
3.50	9.86	136,200	.1850	0.65	1.11
3.47	9.01	180,150	.1981	.57	0.96
3.49	7.89	246,600		.56	
			Average	.59	1.03
$D_{A_{25}} = 0.59 \times 1.087 = 0.64 \times 10^{-7}$ cm.²/sec.					
$D_{\mu_{25}} = 1.03 \times 1.087 = 1.12 \times 10^{-7}$ cm.²/sec.					
		Experiment 3			
9.60	24.76	85,710	0.1739	1.25	(1.57)
9.25	19.45	133,845	.1860	1.20	1.15
8.89	17.68	175,080	.2101	1.02	1.12
9.04	15.90	223,380		1.03	
9.77	15.09	262,760		1.14	
9.10	13.88	313,785		1.01	
9.16	13.40	349,370	0.2888	0.95	0.95
			Average	1.03	1.07
$D_{A_{25}} = 1.03 \times 1.01 = 1.04 \times 10^{-7}$ cm.²/sec.					
$D_{\mu_{25}} = 1.07 \times 1.01 = 1.08 \times 10^{-7}$ cm.²/sec.					

In expt. 3 conditions were nearly ideal and curves were obtained after the various intervals of time which correspond closely to the normal curves having the same area and the same standard deviation. Reference to the data for this experiment shows that the diffusion constants calculated by the two methods are in good agreement. The experience with this experiment shows that under proper experimental conditions the material diffuses normally and is essentially monodisperse.

The data which have been obtained do not indicate that the diffusion constant changes rapidly with concentration.

Calculations

The molecular weight of the calf thymus nucleohistone may be calculated by combining sedimentation velocity and diffusion data in the well-known formula

$$M = RTs/D(1 - V\rho)$$

The limiting value of s_{20} is 31×10^{-13} cm./sec./dyne. The corresponding diffusion constant is $D_{20} = 0.93 \times 10^{-7}$ sq. cm./sec. Thus

$$M = \frac{8.31 \times 10^7 \times 293 \times 31 \times 10^{-13}}{0.93 \times 10^{-7} \times 0.344} = 2{,}300{,}000$$

It will be seen this value is in reasonably good agreement with the figure $M = 2{,}000{,}000$ obtained in the equilibrium ultracentrifuge.

From the molecular weight the molar frictional coefficient may be computed to be $f = 2.09 \times 10^{17}$. The molar frictional coefficient for a spherical particle of the same size is $f_0 = 0.83 \times 10^{17}$. The ratio f/f_0, the molar dissymmetry constant, is 2.5.

This molar dissymmetry constant also may be obtained by combining viscosity and diffusion data. The Kuhn equation[9] for the viscosity of a very dilute solution of elongated units is

$$\left(\frac{\eta}{\eta_0} - 1\right)\frac{1}{G} = 2.5 + \frac{1}{16}\left(\frac{a}{b}\right)^2$$

In this equation η is the viscosity of a solution which contains G volume fraction of solute. The particles have a ratio of long to short axis of a/b. The left-hand number of this equation is the viscosity increment. The data of a previous article[3] give for the most dilute and practically isoelectric solutions a viscosity increment of 84, so that a/b has the value 36.

If the kinetic unit is assumed to have the shape of an elongated ellipsoid the Perrin equation can be applied. Then

$$\frac{D}{D_0} = \frac{\sqrt[3]{(b/a)^2}}{\sqrt{1 - (b/a)^2}} \ln \frac{1 + \sqrt{1 - (b/a)^2}}{b/a}$$

in which D is the observed diffusion constant and D_0 is the diffusion constant of the equivalent sphere. Solving for D_0/D, the result is 2.6 for the molar dissymmetry constant, so that sedimentation, diffusion and viscosity data are in agreement as regards a highly elongated shape for the nucleohistone molecule.

Acknowledgment.—The writer wishes to thank J. W. Williams for assistance, advice and encouragement. Grateful acknowledgment is also made to the Wisconsin Alumni Research Foundation for the research appointment which made this work possible. Assistance in the construction and installation of the apparatus for diffusion was furnished by the personnel of the Work Projects Adm. Off. Project No. 65-1-53-2349.

(9) Kuhn, *Z. physik. Chem.*, **A161**, 1 (1932).

Summary

Measurements of sedimentation velocity, sedimentation equilibrium and diffusion have been made with solutions of the nucleohistone found in the calf thymus gland. The protein is found to be essentially monodisperse and to show the same molecular kinetic behavior between *p*H 5.3 and 9.0.

The following data are obtained for the nucleohistone dissolved in buffer solutions

Sedimentation constant s_{20}	31×10^{-13} cm./sec./dyne.
Sedimentation constant s_{25}	35×10^{-13} cm./sec./dyne.
Diffusion constant D_{20}	0.93×10^{-7} sq. cm./sec.
Diffusion constant D_{25}	1.06×10^{-7} sq. cm./sec.
Frictional coefficient f/f_0	2.5
Axial ratio a/b	35
Molecular weight	2,150,000

MADISON, WISCONSIN RECEIVED APRIL 11, 1941

[CONTRIBUTION FROM THE BURROUGHS WELLCOME & CO. U. S. A. EXPERIMENTAL RESEARCH LABORATORIES]

Preparation of Secondary Amines

BY JOHANNES S. BUCK AND RICHARD BALTZLY

Most of the methods for preparing secondary aliphatic amines leave much to be desired in the way of yield and homogeneity. The authors, by the use of reductive debenzylation of benzyldialkylamines, have devised a method which gives good yields of dialkylamines, and in which, moreover, purification is automatically effected at several points.

Benzaldehyde is condensed with a primary aliphatic amine to give a Schiff base II, and this is catalytically reduced to the corresponding benzylalkylamine III. Addition of alkyl halide (or, in the case of methylamines, reductive methylation[1]) gives the hydrohalide of the benzyldialkylamine IV. On catalytically reducing this, or the corresponding base V, the benzyl group is smoothly removed, giving the dialkylamine VI and toluene and, sometimes, methylcyclohexane.

$$\underset{\text{I}}{C_6H_5CHO} + NH_2R \longrightarrow \underset{\text{II}}{C_6H_5CH{=}NR} \longrightarrow$$

$$\underset{\text{III}}{C_6H_5CH_2NHR} \longrightarrow \underset{\text{IV}}{C_6H_5CH_2NRR'HX} \longrightarrow$$

$$\underset{\text{V}}{C_6H_5CH_2NRR'} \longrightarrow \underset{\text{VI}}{NHRR'} + C_6H_5CH_3$$

Stage I to II is virtually quantitative. The Schiff base II may be purified (*e. g.*, by distillation) but ordinarily this is unnecessary as any unchanged primary amine is eliminated on distilling the benzylalkylamine III, while unchanged benzaldehyde is eliminated with the neutral fraction.

Stage II to III may be regarded as quantitative. In stage III to IV any unchanged benzylalkylamine not separated by distillation of the tertiary base V is eliminated on reduction, as it does not undergo fission. Any quaternary compounds which might have been formed are separated by their non-volatility and water solubility. Alternatively, the tertiary amine hydrohalide IV may be purified by crystallization when the solubility permits.

After reduction it is only necessary to separate the secondary amine from the non-basic toluene or methylcyclohexane and any unchanged benzyldialkylamine which might have escaped reduction. This offers no difficulty as the tertiary amine is relatively non-volatile. Alternatively, the separation may be done by acetylation or other simple chemical methods.

In the experimental section the general method of preparation is given and a number of examples recorded. The process is based on the work of Baltzly and Buck.[2] The authors hope to extend the work to other than purely aliphatic amines.

Experimental

Schiff Bases.—One mol of primary amine, either in aqueous or benzene solution, was mixed with one mol of benzaldehyde. Some heat was evolved, and, after standing overnight, the Schiff base was separated from the water by extraction with ether. If benzene had been used as solvent, the water formed was separated in a separatory funnel. The ether solution was dried (anhydrous potassium carbonate); the benzene solution usually not. After evaporation of the solvent, the residual base was used, without characterization, for the next stage. In some cases the Schiff base was distilled, but this appears to be unnecessary. Some of the Schiff bases are recorded in the literature.[3]

Benzylalkylamines.—The Schiff bases obtained as above were reduced in glacial acetic acid solution, with a

(1) Clarke, Gillespie and Weisshaus, THIS JOURNAL, **55**, 4571 (1933).

(2) Baltzly and Buck, *ibid.*, **62**, 164 (1940).

(3) Zaunschirm, *Ann.*, **245**, 279 (1888); Einhorn and Pfeiffer, *ibid.*, **310**, 225 (1900).