propenyl grouping is accompanied by a shift of the absorption maxima in the ultraviolet. The absorption maxima of 3,3'-propenyldiethylstilbestrol and of 3,3'-propenylhexestrol are at identical wave lengths.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, NORTHWESTERN UNIVERSITY MEDICAL SCHOOL]

Osmotic Pressure of β-Lactoglobulin Solutions

BY HENRY B. BULL AND BYRON T. CURRIE[1]

A number of values for the molecular weight of β-lactoglobulin from cow's milk have been reported. There is, however, a disconcerting lack of agreement between these values as is shown in Table I.

TABLE I

MOLECULAR WEIGHT OF β-LACTOGLOBULIN AS REPORTED BY VARIOUS WORKERS

Method	Molecular weight	Workers
Ultracentrifuge (equilibrium) and diffusion	38,000	Pedersen[a]
Ultracentrifuge (rate sedimentation)	41,500	Pedersen[a]
X-Ray (wet crystals)	33,000	Crowfoot[b]
		McMeekin and Warner[c]
X-Ray (air dried crystals)	35,000	Crowfoot[b]
		McMeekin and Warner[c]
Chemical analysis } mini-	42,000	Brand and Kassel[d]
Chemical analysis } mum	42,000	Chibnall[e]
Chemical analysis } (m. w.)	42,020	Brand *et al.*[f]
Osmotic pressure	38,000	Gutfreund[g]
Film pressure	2 × 17,100	Bull[h]
Light scattering	35,000	Heller and Klevens[i]
Osmotic pressure	35,020	Bull and Currie

[a] Pedersen, *Biochem. J.*, **30**, 961 (1936). [b] Crowfoot, *Chem. Rev.*, **28**, 215 (1941). [c] McMeekin and Warner, THIS JOURNAL, **64**, 2393 (1942). [d] Brand and Kassel, *J. Biol. Chem.*, **145**, 365 (1942). [e] Chibnall, *Proc. Roy. Soc. London*, B131, 136 (1942). [f] Brand, Saidel, Goldwater, Kassell and Ryan, THIS JOURNAL, **67**, 1524 (1945). [g] Gutfreund, *Nature*, **155**, 237 (1945). [h] Bull, THIS JOURNAL, **68**, 745 (1946); [i] Heller and Klevens, private communication.

The present paper reports the result of 20 osmotic pressure measurements on solutions of β-lactoglobulin along with the calculation of the molecular weight of this protein.

Experimental

The osmotic pressure apparatus was a modification of that described by Bull.[2] This modification is diagrammed in Fig. 1. The capillary tube had an inner diameter of 0.2 mm. and was furnished by Corning Glass Works. About 5 cc. of the outside solution (solution whose composition was identical with that of the protein solution except for the absence of protein) was placed in the bottom of the apparatus. Toluene was added from the top of the clean, dry capillary and forced down the capillary with gentle air pressure. Suction was then applied to the capillary to remove the trapped air. The apparatus was then filled with outside solution. The sack which contained the protein solution was made from Visking sausage casing whose flat width was 1.5 cm. and was supplied through the courtesy of Dr. C. J. B. Thor. The moist casing was securely knotted at one end and the other end was slipped over a rubber stopper which had been trimmed to size, and attached to the end of the tube which was to contain the protein. The stopper had been previously coated with stopcock grease. The end of the sack which had been slipped over the rubber stopper was wrapped tightly with a rubber band to complete the attachment to the rubber stopper. The sack was then filled with protein solution through the larger glass tube to which was attached a second, larger rubber stopper which was coated with stopcock grease. The apparatus was then assembled as shown in Fig. 1. The larger rubber stopper was held in place by a metal clamp which is not shown in Fig. 1. Small one-hole rubber stoppers were placed in the tubes containing the outside solution and the protein solution. Their purpose was to decrease the evaporation of these solutions during an experiment.

The whole apparatus was clamped in a fixed position in a constant temperature bath at 25°. The stopcock was allowed to remain open until the apparatus had come to temperature. The position of the toluene level in the capillary was marked by appropriate means and the stop cock was closed. The toluene meniscus immediately began to rise or fall depending on whether the hydrostatic pressure was greater or less than the osmotic pressure. Protein solution was added to or removed from the protein solution column to bring the toluene meniscus to its original level. Adjustment of the protein solution level was made from time to time as needed. The toluene column thus acted essentially as an indicator and in the ideal case there is no net motion of liquid across the sausage casing membrane. Usually, no further adjustment of the protein solution column was necessary after about two hours. In all cases, however, the experiment was allowed to continue overnight. At the end of this time, the position of the toluene column had usually shifted one or two mm. The drift of the toluene level was multiplied by the density of toluene and applied as a correction to the osmotic pressure. The difference in level between the outside solution and the protein solution was measured with a cathetometer. This difference in level when multiplied by the density of the protein solution gave, after applying the correction due to the small excursion of the toluene column, the osmotic pressure of the protein solution in centimeters of water.

The β-lactoglobulin was prepared by a modification of a method suggested in a private communication by Dr. A. H. Palmer. Raw whole milk was brought to 50% saturation with ammonium sulfate. The solution was filtered and the filtrate brought to 80% saturation with ammonium sulfate. A small amount of water along with some toluene was added to the precipitate. The precipitate was transferred to sausage casing and dialyzed against frequent changes of distilled water at 5° for four or five days. The protein solution was then removed from the sausage casing and any extraneous solid material filtered off. The filtrate was adjusted to *p*H 5.1 by the cautious addition of dilute hydrochloric acid. The turbid solution was then dialyzed against distilled water and the dialysis continued until no further separation of the "oily" phase took place. The protein was then crystallized as described by Palmer.[3]

(1) On leave of absence from the Corn Products Refining Company.

(2) Bull, *J. Biol. Chem.*, **137**, 143 (1941)

(3) Palmer, *ibid.*, **104**, 359 (1934).

As will be pointed out later, the recrystallization of β-lactoglobulin by the addition of dilute sodium hydroxide to produce a *p*H of 5.8 followed by subsequent neutralization with dilute hydrochloric acid does not, in our hands, yield a product satisfactory for osmotic pressure measurements. After recrystallizing twice according to Palmer's method, the following additional crystallization was made. The crystals of β-lactoglobulin were dissolved in 0.07 *M* sodium chloride. The turbid solution was centrifuged and the clear solution dialyzed against distilled water with frequent agitation so that many small plate-like crystals were produced. This type of recrystallization was repeated several times. The final crystals, when dissolved in sodium chloride solutions, gave water-clear solutions up to a protein concentration of 6%.

For the osmotic pressure measurements, the β-lactoglobulin solutions were made up in such a way that the final concentration of sodium chloride was 0.5 *M*. The concentrations of β-lactoglobulin solutions were determined with a dipping refractometer (Zeiss). The refractive increment was measured on eleven different protein solutions whose concentration extended from 0.745 g. per 100 cc. to 6.175 g. per 100 cc. of solution. The concentrations of these solutions were obtained by drying the solutions to constant weight in a vacuum oven at 105° and subtracting from these weights the known weight of sodium chloride present. The concentration of protein in grams per 100 cc. of solution was then calculated by the empirically found relation

$$C = 0.2121\Delta - 0.00024\Delta^2$$

where Δ is the dipping refractive increment of the protein solution above that of 0.5 *M* sodium chloride. Fresh solutions from the protein crystals were prepared for each osmotic pressure measurement. Two separate lots of β-lactoglobulin were used; the data from these two lots were indistinguishable. Osmotic pressure measurements were also made on successive recrystallizations.

The *p*H of the protein solutions were between 5.1 and 5.2 and the protein acted as its own buffer to maintain the *p*H constant.

The osmotic pressure of β-lactoglobulin was also measured in the presence of a series of concentrations of urea. These solutions were prepared from a mother solution of β-lactoglobulin and maintained at room temperature for several days prior to the osmotic pressure determination. The sodium chloride concentration in these solutions was 0.5 *M*.

Results

Twenty-three osmotic pressure measurements were made on β-lactoglobulin recrystallized by adding dilute sodium hydroxide to produce a *p*H of 5.8 and then neutralizing the alkali with dilute hydrochloric acid followed by subsequent dialysis. These measurements were very unsatisfactory as there was a wide spread of values and osmotic equilibrium appeared to be approached very slowly. Indeed it is doubtful in a number of cases that equilibrium was ever attained. It was noted that solutions of the protein recrystallized by the sodium hydroxide–hydrochloric acid procedure were never entirely clear. Repeated recrystallizations by this technique failed to improve the situation. The average of the twenty-three determinations gave a molecular weight of 40,500, with a standard deviation of the mean of 2,000. It appears from the ultracentrifugal studies of Pedersen[4] that β-lactoglobulin is peculiarly sensitive to alkali. Even at a *p*H as low as 7.5 the sedimentation constant had begun

(4) Reference [a] of Table I

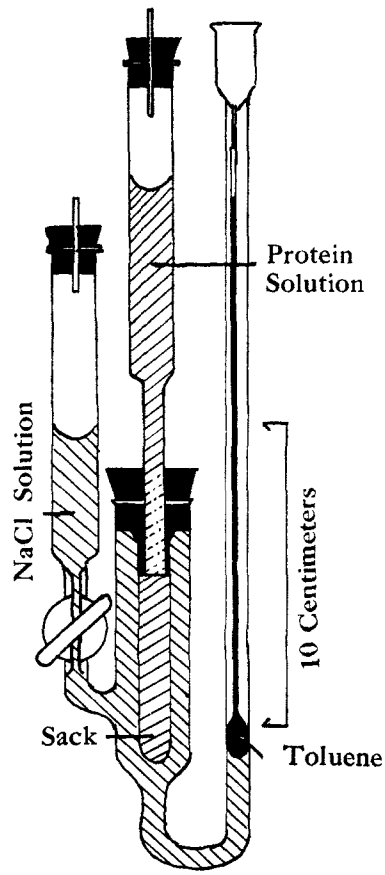

Fig. 1.—Osmotic pressure apparatus.

to decrease. It was concluded that the sodium hydroxide–hydrochloric acid technique tends to produce a heterogeneous product by virtue of local accumulation of alkali as the sodium hydroxide is added to the protein. All data obtained on β-lactoglobulin recrystallized by the sodium hydroxide–hydrochloric acid technique were rejected as unacceptable.

Table II gives the osmotic pressure and the calculated molecular weight of β-lactoglobulin. These measurements were made on water clear solutions of the protein which were prepared from protein recrystallized by dialyzing sodium chloride solutions of the protein against distilled water. Table II includes all data obtained on these solutions and no results have been rejected. The osmotic pressures of most of the solutions were measured in duplicate. The concentration of protein is given in grams of protein per 100 grams of 0.5 *M* sodium chloride solution.

The average molecular weight is 35,050 with a standard deviation of the mean of 144.

The results for the osmotic pressure of β-lactoglobulin in the presence of urea are given in Table III.

Solutions containing 7 and 8 *M* urea were distinctly turbid.

TABLE II

OSMOTIC PRESSURE OF β-LACTOGLOBULIN SOLUTIONS AT 25°

Protein concn.	Osmotic press., cm. of H_2O	Mol. wt.
1.485	10.95	34,860
1.485	11.03	34,600
1.931	14.35	34,600
1.931	14.28	34,760
1.622	12.31	33,890
2.328	17.33	34,530
1.418	10.46	34,860
1.418	10.53	34,630
2.331	17.35	34,550
2.331	17.30	34,630
1.346	9.78	35,380
1.346	9.91	34,900
1.747	12.79	35,120
1.747	12.71	35,350
1.848	12.91	36,790
1.848	13.40	35,450
1.281	9.15	35,990
1.281	9.32	35,320
1.972	14.42	35,170
1.972	14.26	35,560

TABLE III

OSMOTIC PRESSURE OF β-LACTOGLOBULIN IN THE PRESENCE OF UREA

Molar urea concn.	Protein concn., g. per 100 g. of solvent	Osmotic press., cm. of H_2O	Mol. wt.
0	...	..	35,000
2	0.977	8.46	30,500
3	.965	9.04	28,600
4	.953	9.06	28,500
5	.468	5.06	25,400
6	.464	5.95	21,700
7	.457	4.93	26,100
8	.452	<4.0	>30,000

In all cases, the molecular weight has been calculated by the equation

$$M = 2.528 \times 10^5 \rho C/P$$

where ρ is the density of the solvent, C is the concentration of the protein solution in grams of protein per 100 g. of sodium chloride solution and P is the osmotic pressure in centimeters of water.

Discussion

As can be seen from Table I, the value of 35,050 for the molecular weight of β-lactoglobulin is in good agreement with the molecular weight as determined by X-ray analysis of Crowfoot[5] and the density measurements of McMeekin and Warner.[6] It is also in good agreement with the value found by light scattering and by film pressure studies. There is, however, considerable divergence between this value and those calculated by other methods.

(5) Reference [b] in Table I.

(6) Reference [e] in Table I.

The reason for this disagreement is to some extent obscure. Our work indicates that β-lactoglobulin has considerable tendency to undergo reactions which lead to a heterogeneous product. The recent paper by Briggs and Hull[7] substantiates this conclusion. It is our belief that much of the difficulty associated with an accurate determination of the molecular weight of β-lactoglobulin can be explained on this basis.

It had been reported[8] that the spread film molecular weight was about 44,000. This value was obtained on a sample of protein which had been recrystallized by the sodium hydroxide–hydrochloric acid technique and was probably aggregated. The spread film molecular weight of β-lactoglobulin has been reinvestigated and is the subject of a separate paper.[9] As noted in Table I the molecular weight of spread films of this protein prepared according to the present procedure is 17,100 which indicates that β-lactoglobulin dissociates into 2 fragments on the surface and twice this weight is 34,200 in good agreement with the osmotic pressure studies. In the presence of Cu^{++} ions β-lactoglobulin does not dissociate and its film molecular weight is 34,300.

Heller and Klevens[10] measured the molecular weight of β-lactoglobulin by the light-scatter method using a sample of protein which we supplied. They state that their value is probably accurate to plus or minus 1,000.

It can be seen from Table II that there is a tendency for the molecular weights in the first part of the table to be lower than those in the last part of the table. The sequence of the values in the table is the sequence in which the osmotic pressure determinations were made. The actual work extended over a period of about a month which means that the last determinations were made on solutions which were prepared from aged crystals. It is possible that there is slight and progressive aggregation of the protein in the crystalline state when stored at 2°. On the other hand, the slight variation noted in the molecular weights may be due to experimental error.

The concentration intrinsic viscosity of our protein was 4.01 which compares favorably with the values reported by Neurath, Cooper and Erickson[11] and by Briggs and Hanig.[12]

The osmotic pressure of β-lactoglobulin in the urea solutions seems to indicate that in the presence of urea this protein splits into two fragments although this conclusion is not entirely certain. The apparent decrease in the molecular weight in urea could conceivably be due to a greatly increased osmotic coefficient resulting from a departure from the ideal gas laws in concentrated

(7) Briggs and Hull, THIS JOURNAL, **67**, 2009 (1945).

(8) Bull, *ibid.*, **67**, 8 (1945).

(9) Reference [h] in Table I.

(10) Reference [i] in Table I.

(11) Neurath, Cooper and Erickson, *J. Biol. Chem.*, **138**, 411 (1941).

(12) Briggs and Hanig, *J. Phys. Chem.*, **48**, 1 (1944).

urea solutions. In view of the small concentration of protein used, we regard this interpretation as unlikely. The apparent increase of the molecular weights in 7 and in 8 *M* urea is undoubtedly due to progressive aggregation as both solutions showed a degree of turbidity which was not present in the other urea concentrations used.

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We also wish to thank Drs. W. Heller and H. B. Klevens for their kind permission to use their unpublished results on light scattering.

Summary

1. Twenty osmotic pressure measurements on solutions of β-lactoglobulin in 0.5 *M* sodium chloride have been reported. A molecular weight of 35,050 with a standard deviation of the mean of 144 has been calculated.

2. The osmotic pressure of β-lactoglobulin solutions in several concentrations of urea have been measured. It is concluded that urea probably dissociates β-lactoglobulin into two fragments.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, NORTHWESTERN UNIVERSITY MEDICAL SCHOOL]

Monolayers of β-Lactoglobulin. II. Film Molecular Weight

BY HENRY B. BULL

In a previous paper[1] it was shown that β-lactoglobulin prepared from fresh, raw whole milk forms a gaseous monolayer on the surface of concentrated ammonium sulfate solutions and from the application of the gas laws in two dimensions, the molecular weight along with the area of the gaseous molecules was calculated. The film molecular weight reported for β-lactoglobulin was about 44,000 and the area of the gaseous film was 1.21 sq. meters per milligram of protein. Since that time, the molecular weight of β-lactoglobulin has been determined by osmotic pressure measurements[2] and found to be 35,050 with a standard deviation of the mean of 144. In view of this discrepancy between the results of these two techniques, it was decided to reinvestigate in greater detail gaseous spread films of β-lactoglobulin on concentrated ammonium sulfate solutions. It has now been found possible to reconcile the results from surface film technique and from osmotic pressure measurements. These studies are reported in this paper.

Experimental

The β-lactoglobulin was prepared from fresh, raw whole milk by the technique described by Bull and Currie.[2] The protein was recrystallized several times by dialyzing a solution of the protein in 0.07 *M* sodium chloride against water. The protein crystals were dissolved in 1 *M* sodium chloride and enough water added to make the solution 0.5 *M* in respect to sodium chloride. The protein concentration was determined with a dipping refractometer (Zeiss). The mother solutions were diluted with water so that one Blodgett pipet would deliver between 0.02 and 0.03 mg. of β-lactoglobulin to the surface of the ammonium sulfate solutions.

A Wilhelmy balance has been used to measure the surface pressures. Two thin microscope cover glasses each 6 cm. wide were suspended from one arm of the analytical balance. This gives a total length of surface of 24 cm. and, accordingly, one milligram of weight was equivalent to 0.0409 dyne per centimeter film pressure. It was noted that "tears" of ammonium sulfate solution tended to form on the Wilhelmy slides when the slides were placed in the clean ammonium sulfate solution surface. This led to confusion in regard to the initial base line weight in the absence of protein since as soon as protein was added to the surface these "tears" drained off the slide and, accordingly, the weight of the suspended glass slides changed. The technique adopted to avoid this difficulty was to spread protein on the surface of the ammonium sulfate solution with the Wilhelmy slides in place and then to sweep the surface of the ammonium sulfate solutions repeatedly with a movable barrier. This gave an unambiguous base line for the weight of the slides in the absence of protein.

Previous to use, the ammonium sulfate solutions were treated with activated charcoal to remove surface active impurities.

In all cases, the film pressures in dynes per centimeter have been multiplied by the corresponding film areas in sq. meters per milligram and those values plotted against the film pressures. These plots included eight or nine points extending from about 0.1 dyne per centimeter up to about 0.5 dyne per centimeter. The best straight line was drawn through these points and the slope and intercept on the *y*-axis determined. The slope of the line is equal to the area occupied by the gaseous molecules in sq. meters per milligram. For one mole of substance in the surface film, the intercept at 25° should equal 24.6×10^3 ergs when the pressure is expressed in dynes per centimeter and the area in sq. meters per milligram. Accordingly the film molecular weight is equal to 24.6×10^3 divided by the value of the intercept.

Results

β-Lactoglobulin was spread on 20% ammonium sulfate and the influence of time on the properties of the gaseous surface film studied. The film was compressed within one minute after spreading and the pressure–area curve measured. The film was then expanded and recompressed at the end of fifteen minutes. It was re-expanded and recompressed and re-expanded again at the end of thirty minutes, at the end of forty-five minutes and at the end of four hours. During this time the base line for the weight of the slide had

(1) Bull, THIS JOURNAL, **67**, 8 (1945).

(2) Bull and Currie, *ibid.*, **68**, 742 (1946).