TABLE I

Pyrene–T. N. B. Log ε			1,2-Benzopyrene–T. N. B. Log ε		
mμ	Calcd.	Exp.	mμ	Calcd.	Exp.
231	4.84	4.84	226	4.73	4.73
240	5.01	5.02	255	4.70	4.73
252	4.38	4.39	264	4.72	4.74
262	4.49	4.51	284	4.64	4.66
273	4.68	4.69	296	4.73	4.74
295	3.70	3.70	332	3.70	3.71
306	4.08	4.09	348	4.09	4.08
320	4.44	4.46	366	4.37	4.36
335	4.69	4.70	380	4.39	4.39
353	2.90	2.90	386	4.42	4.41
358	2.74	2.75	406	3.51	3.52
364	2.67	2.66			
374	2.57	2.58			

1,2,5,6-Dibenzanthracene–T. N. B. Log ε		
mμ	Calcd.	Exp.
221	5.05	5.03
278	4.71	4.68
288	4.98	4.95
297	5.17	5.16
321	4.27	4.25
334	4.21	4.19
350	4.16	4.13
365	2.92	2.91
375	3.08	3.05
385	2.73	2.71
396	3.06	3.03

Anthracene–T. N. B. Log ε		
mμ	Calcd.	Exp.
252	5.29	5.30
298	3.07	3.08
311	3.23	3.23
325	3.49	3.49
340	3.92	3.90
358	3.90	3.87
378	3.76	3.74

Chrysene–T. N. B. Log ε		
mμ	Calcd.	Exp.
242	4.59	4.60
258	4.91	4.91
267	5.11	5.11
295	4.07	4.09
307	4.10	4.11
320	4.09	4.10
345	2.93	2.94
354	2.75	2.77
363	2.87	2.88

Phenanthrene–T. N. B. Log ε		
mμ	Calcd.	Exp.
244	4.85	4.86
251	4.91	4.91
274	4.21	4.22
281	4.08	4.10
293	4.14	4.11
309	2.83	2.82
316	2.77	2.76
324	2.76	2.74
331	2.79	2.78
339	2.72	2.71
347	2.71	2.72

the hydrocarbon recovered by passing a solution of the complex in petroleum ether–benzene through a column packed with activated alumina-Super-Cel. The recovered hydrocarbon was then crystallized from a suitable solvent. The naphthalene was recrystallized from methanol–water (Bureau of Standards sample for calorimetric work). The trinitrobenzene was decolorized with charcoal and recrystallized twice from methanol–water (m. p. 124.3–124.8° cor.). In Table II the melting points of the hydrocarbons and the trinitrobenzene complexes are listed.

TABLE II

Hydrocarbon	m. p. °C. (cor.)	Trinitrobenzene complex, m. p. °C. (cor.)
Naphthalene	81–81.8	155.2–155.8
Phenanthrene	100.8–101.3	165.8–166.4
Anthracene	216.5–217.2	163–164
Pyrene	152.2–152.9	252.5–253.3
Chrysene	255.8–256.3	188.5–189.5
1,2-Benzopyrene	179.9–180.3	227–228.5
1,2,5,6-Dibenzanthracene	269–270	237.0–238.5[7]

Acknowledgment.—The authors wish to acknowledge the assistance of Miss F. A. Lawrence and C. L. McCabe in carrying out the spectra determinations.

Summary

Ultraviolet absorption spectra of dilute methanol solutions of aromatic hydrocarbon–trinitrobenzene complexes have demonstrated the complete dissociation of the complex. The concept of complete dissociation and the additivity of spectra has been applied to representative two, three, four, and five ring hydrocarbons. The application of this concept as an identification tool has been pointed out.

(7) Calculated for $C_{24}H_{14}\cdot 2C_6H_3N_3O_6$: C, 57.96; H, 2.86. Found: C, 57.50; H, 2.74.

PITTSBURGH, PENNSYLVANIA RECEIVED JUNE 12, 1944

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

Adsorption of Water Vapor by Proteins[1]

BY HENRY B. BULL

Many workers have concerned themselves with the combination between water and proteins and the present author cannot lay claim to originality in either his belief in the importance of the problem or in his approach to the subject. He has, however, been somewhat more persistent than most and has arrived at certain conclusions which seem to warrant publication at this time.

As pointed out by Sponsler, Bath and Ellis,[1a] proteins contain two types of hydrophilic groups which are capable of binding water through hydrogen bond formation. These are the polar side chains such as those from lysine, glutamic acid, tyrosine, etc., and, second, the oxygen and nitrogen associated with the peptide bonds in the peptide chains. The amount of water held by a protein should be primarily determined by the number and availability of these two types of groups.

Previous work on the adsorption of water vapor by proteins such as that reported by Briggs,[2] shows quite clearly that a typical S-shaped curve is obtained when the amount of water vapor adsorbed is plotted against the relative humidity. This characteristic adsorption curve can be

(1) This paper was presented at the Cleveland, Ohio, meeting of the American Chemical Society, April 5, 1944.

(1a) Sponsler, Bath and Ellis, *J. Phys. Chem.*, **44**, 996 (1940).

(2) Briggs, *J. Phys. Chem.*, **35**, 2914 (1931); **36**, 367 (1932).

roughly separated into three segments. The first part, at low vapor tensions, has the earmarks of a Langmuir adsorption, the amount adsorbed increasing rapidly with an increase in the vapor pressure. This section of the curve is followed by a more or less linear relation between the amount adsorbed and the vapor pressure. This relation extends up to about 65% relative humidity, and has a much more gentle slope. The third segment of the curve is characterized by a sharp upswing of the amount adsorbed as the relative water vapor pressure is increased.

It is the purpose of this paper to report the results of the study of the adsorption of water vapor by a series of purified proteins and to attempt a tentative interpretation of these results.

Experimental

The protein samples, contained in weighing bottles, were placed above sulfuric acid of the desired concentration in closed containers (fruit jars). The containers were submerged in a constant temperature water-bath at 40° for eighteen days. At the end of this time the weighing bottles were weighed and replaced above the sulfuric acid. The water-bath was then adjusted to 25° for twelve days, at the end of which time the weighing bottles were reweighed and placed in a vacuum oven at 105° for twenty-four hours and again weighed. This last weight gave the dry weight of the protein samples. The sulfuric acid in the fruit-jars was, after appropriate dilution, titrated with standard alkali. From the concentration of the acid samples the relative aqueous vapor pressure for each sample was obtained from a smooth plot of the water vapor pressure against acid concentration.[3] These data allow us to calculate the weight of water taken up by a given weight of protein as a function of the aqueous vapor pressure both at 25° and at 40°.

The question arises as to whether or not equilibrium has been attained in the allotted time. In order to answer this, three weighing bottles containing respectively gelatin, heat coagulated egg albumin and wool were placed over pure water for twenty-four hours and then over 74% sulfuric acid and set in the water-bath at 40° and weighed on successive days. Likewise samples of the above three proteins were dried in a vacuum oven at 80° for two hours and then placed over 10% sulfuric acid at 40°. The gain in weight of these samples was followed on successive days. The results of these studies are shown in Table I.

The results shown in Table I, which represent the extreme adjustment which samples would have to make to the relative humidity, indicate that the time allowed for the attainment of equilibrium is sufficient.

The troublesome problem arises as to whether or not twenty-four hours at 105° in a vacuum oven is sufficient to drive off all the moisture from the protein samples. This question is not easily answered. If the temperature is raised too high, the protein will decompose and the result will be that a higher moisture content will be calculated than is actually present. The following experiment was performed to throw some light on this subject: samples of coagulated egg albumin, gelatin and wool were placed over pure water for twenty-four hours. The samples were then transferred to a vacuum oven and allowed to remain for two hours at a selected temperature and the weight of the protein sample found after each two-hour period in the oven. These results are shown in Table II.

(3) "International Critical Tables."

TABLE I

RATE OF LOSS AND GAIN OF WEIGHT OVER SULFURIC ACID AT 40°

Time in days	Moisture in sample, % — Over 10% H_2SO_4			Over 74% H_2SO_4		
	Gelatin	Egg albumin	Wool	Gelatin	Egg albumin	Wool
0	0.62	0.19	0.21	61.5	31.1	31.7
1	18.4	17.8	22.5	5.56	3.00	2.71
2	41.4	21.0	24.1	3.51	1.75	1.92
3	45.0	22.3	24.4	3.30	1.47	1.76
4	47.8	23.3	24.7	3.20	1.44	1.78
5	49.4	23.7	24.8	3.04	1.45	1.74
6	50.8	24.1	24.9	2.99	1.43	1.76
7	51.4	24.1	24.8	3.04	1.46	1.76
8	51.6	24.1	24.7			
10	52.1	24.1	24.8			
12	52.2					

TABLE II

INFLUENCE OF THE TEMPERATURE OF THE VACUUM OVEN ON THE MOISTURE CONTENT OF PROTEIN SAMPLES

Temp., °C.	Weight of protein samples, g. — Gelatin	Egg albumin	Wool
80	0.4992	1.2012	0.3708
93	.4965	1.1972	.3698
101	.4941	1.1956	.3695
119	.4940	1.1947	.3687
137	.4927	1.1934	.3680
137 (overnight)	.4921	1.1902	.3664

Drying at 137° badly discolored the egg albumin and the wool, indicating a certain amount of decomposition of these proteins at this temperature. There is no temperature at which a constant weight is obtained. In short, the process of the removal of water grades directly into protein decomposition and it is clear that the arbitrary selection of the drying conditions as 105° for twenty-four hours in a vacuum oven is not without ambiguity. It is felt, however, that these conditions do represent as satisfactory compromise as could be selected.

The proteins used were the purest obtainable. Nylon was supplied by Dr. Malcolm Dole in the form of fibers. One lot had been stretched to orient the peptide chains while the other was in an unstretched condition. Both lots were exhaustively washed with water at 60° and then electrodialyzed. Both samples apparently contained some metallic cation because there was a black deposit on the platinum cathode. The nylon is, of course, not a protein

but it is one of the simplest compounds containing the peptide linkage. It is a polymer of hexamethyldiamine and adipic acid. The silk was obtained from Dr. Milton Harris. It was the Bombyxmori type and had been degummed. Before use it was electrodialyzed. The wool also was supplied by Dr. Milton Harris. It was exhaustively extracted with ether and alcohol and then electrodialyzed. The zein was given by Dr. D. R. Briggs already fractionated and electrodialyzed. The B-zein is that fraction which separated out of solution when the per cent. of water in the ethyl cellosolve solution was increased from 39 to 44. C-Zein is that fraction which remained in solution. Salmin sulfate was supplied by Dr. Carl Dragsted. It was electrodialyzed and lyophilized and used as the base. Elastin was prepared from the *ligamenta nuchae* of beef. The ligament was ground in a meat chopper, extracted with 0.9% sodium chloride and then with alcohol and ether. It was then electrodialyzed. The elastin is undoubtedly the most impure of the proteins which are being used for this report. The collagen was a hide collagen and was supplied by Dr. R. A. Gortner. It was extracted with ether and alcohol and washed with dilute hydrochloric acid. It was then electrodialyzed and lyophilized. The gelatin also was supplied by Dr. R. A. Gortner and was made by acid extraction from the same lot of collagen as reported on in this paper. The gelatin was washed with dilute hydrochloric acid, electrodialyzed and lyophilized. The egg albumin was prepared from fresh hen's eggs by the method of Kekwick and Cannan.[4] It was recrystallized three times and dialyzed against distilled water. A part was lyophilized and a part was air dried in a sausage casing at room temperature. Another part was coagulated by heating for five minutes on a boiling water-bath. The coagulated protein was lyophilized. Dr. A. H. Palmer supplied one lot of platy crystals of β-lactoglobulin. This lot was lyophilized. A second lot was prepared according to a private communication from Dr. Palmer. This was also platy crystals but was used in this condition and not lyophilized. The serum proteins were prepared from healthy horse blood by the author while he was at California Institute of Technology and he is grateful for the facilities supplied him. The γ-globulin was assumed to be that part of the serum which was precipitated by 34.0% saturated ammonium sulfate, while the mixture of α- and β-globulin was the fraction which precipitated between 34 and 45% saturated ammonium sulfate. The method of preparation was adopted from that of Cohn, *et al.*[5] The serum albumin was prepared according to the method of McMeekin[6] and belonged to the carbohydrate-free fraction. The serum proteins were dialyzed against pure water and then air dried in a sausage casing.

In the cases of silk, wool, collagen, gelatin and lyophilized egg albumin equilibrium was approached from both the wet and dry side of the equilibrium point. This was accomplished by allowing a sample of protein to remain above water for twenty-four hours; this is the "wet" sample. For the "dry" samples, silk and wool were placed in a vacuum oven at 80° for two hours while the collagen, gelatin and egg albumin were in the lyophilized condition. In general, the agreement between the two adsorption curves was good. There is some hysteresis between a relative vapor pressure of 0.15 and 0.65 while above and below these humidities, the agreement is very good. In reporting the adsorption curves for the above five proteins an average of the adsorption and desorption curves has been given.

The moisture content was measured at, at least, 12 relative humidities for each protein. These relative humidities were selected to cover the range from 0.02 to 0.95.

Results.—Table III shows the percentage water associated with the proteins on a dry weight basis. In view of the extensiveness of the data it was necessary to compare the proteins at the same relative humidities. The function a/x was plotted against x where a is the amount of water adsorbed per 100 g. of dry protein and x is the relative humidity (see Fig. 2). The curves were then interpolated at the relative humidities shown in Table III and the amount of water calculated. This manipulation allows the data to be expressed in a compact form. Very little accuracy is lost in this process.

It is difficult if not impossible to evaluate the experimental errors involved in the measurement of the data reported. The weighing of the protein was done on a good analytical balance and should be accurate to 2 to 3 tenths of a milligram. The protein samples usually weighed between 0.5 and 1.0 g. and, accordingly, except for the samples over the most concentrated acid (containing the smallest amount of water) the error involved in the moisture determination is certainly very small. The error due to the calculation of the acid concentration should likewise be small. Probably the largest errors are involved in the estimation of the aqueous vapor pressures from the acid concentrations.

Discussion

There is an inherent vagueness in any definition of protein hydration. This vagueness arises from the impossibility of defining in a clear manner the standard state of the protein in its dry as well as in its wet condition. In this paper we define protein hydration as that water which is released when moist protein in equilibrium with saturated water vapor at 25° or at 40° is dried in a vacuum oven for twenty-four hours at 105°. This definition neglects entirely the interaction between protein molecules and is, accordingly, very incomplete. It is, however, not possible at the present time to formulate a more meaningful definition. It is not feasible to present adsorption curves for each of the proteins studied. In order to make the discussion of the results somewhat clearer, water adsorption curves for unstretched nylon, for wet and dry silk, for unlyophilized egg albumin and for wet and dry collagen are shown in Fig. 1.

The interpretation of the adsorption isotherms has been approached in two ways. First, the thermodynamic functions of free energy and heat changes have been calculated and secondly an effort has been made to formulate the mechanism of the adsorption reaction.

The free energy required to transfer water molecules from the vapor state to the solid surface is a quantitative measure of the affinity of the solid for the vapor. In an isothermal process involving the transfer of one gram of water the free energy change is clearly

$$\Delta F = \frac{RT}{M}\int_0^1 \frac{a\,dx}{x}$$

where a is the weight of water adsorbed per given

(4) Kekwick and Cannan, *J. Biochem.*, **30**, 227 (1936).

(5) Cohn, McMeekin, Oncley, Newell and Hughes, THIS JOURNAL, **62**, 3386 (1940).

(6) McMeekin, *ibid.*, **62**, 3393 (1940).

TABLE

GRAMS OF WATER ADSORBED PER 100 G. OF DRY PROTEIN AS A

Relative vapor pressures	0.05		0.10		0.20		0.30		0.40	
temperature, °C.	25	40	25	40	25	40	25	40	25	40
Unstretched nylon	0.56	0.40	0.83	0.66	1.26	1.16	1.86	1.74	2.48	2.36
Stretched nylon	.58	.49	.83	.68	1.36	1.16	1.92	1.71	2.48	2.24
Silk	1.96	1.64	2.81	2.46	3.90	3.66	5.19	4.79	6.28	5.90
Wool	2.97	2.50	4.25	3.88	6.25	5.65	8.22	7.50	9.90	9.36
B-Zein	1.78	1.54	2.59	2.46	3.82	3.62	4.98	4.47	5.92	5.28
C-(Zein)	1.70	1.50	2.52	2.37	3.74	3.64	4.65	4.56	5.56	5.44
Salmin	4.50	4.25	5.35	5.00	6.50	6.02	8.16	7.62	11.32	10.80
Elastin	4.18	3.30	4.35	3.73	5.74	5.40	7.56	7.29	10.68	9.28
Collagen	5.45	4.18	7.39	6.33	10.06	8.89	12.44	11.64	15.14	14.46
Gelatin	5.30	3.99	6.75	5.95	8.71	8.40	11.25	10.89	13.82	13.34
Egg albumin (lyophilized)	2.54	2.20	3.70	3.36	5.25	4.97	6.96	6.53	8.70	8.18
Egg albumin (unlyophilized)	2.65	2.53	3.91	3.63	5.86	5.50	7.56	7.14	9.28	8.84
Egg albumin (coagulated)	2.30	2.10	3.36	3.09	4.94	4.54	6.33	6.00	7.68	7.36
β-Lactoglobulin (lyophilized)	2.29	2.05	3.54	3.05	5.38	4.72	6.96	6.51	8.80	8.40
β-Lactoglobulin (crystals)	2.50	2.20	3.62	3.33	5.82	5.30	7.68	7.08	9.52	8.92
Serum albumin	3.08	2.63	4.35	3.90	6.24	6.00	8.10	7.95	9.92	9.60
α- and β-pseudoglobulin	3.34	2.93	4.63	4.34	6.90	6.58	8.88	8.34	10.80	10.16
γ-Pseudoglobulin	3.34	2.95	4.52	4.25	6.86	6.36	8.85	8.34	10.64	10.20

weight of dry protein (taken as 100 g. in this study), x is the relative aqueous vapor pressure and M is the molecular weight of water. The gas laws are assumed to apply. In order to integrate equation 1, a/x is plotted against x and the area under the curve determined. When this area is multiplied by RT/M the free energy change is obtained. Unfortunately, it is difficult to measure the area under the curve directly because at small values of x, the curve swings upward too steeply. To circumvent this difficulty the method used by Boyd and Livingston[7] has been employed.

Figure 2 shows the plot of a/x against x for four selected proteins.

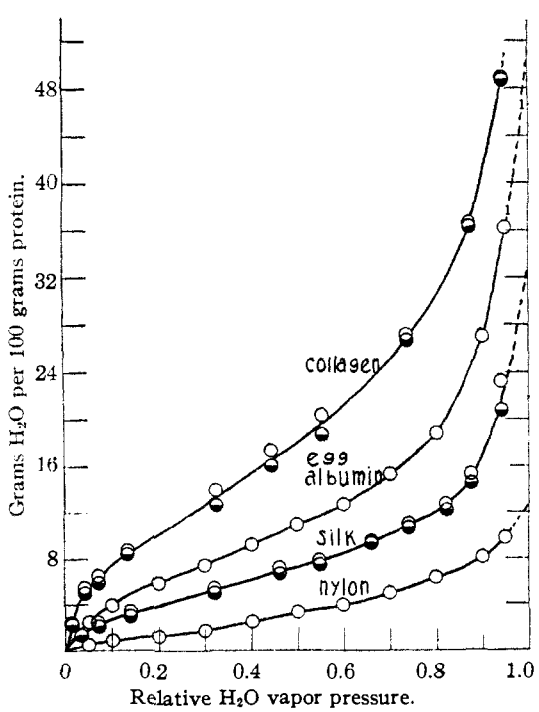

Fig. 1.—Water adsorption curves for unstretched nylon, for wet (open circles) and dry (half circles) silk, for unlyophilized egg albumin, and for wet (open circles) and dry (half circles) collagen at 25°.

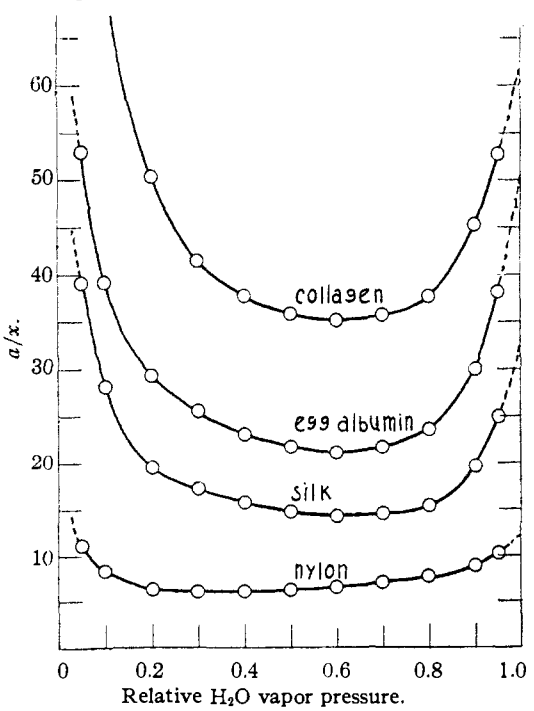

Fig. 2.—Plot of a/x against x for unstretched nylon, for silk (average of wet and dry), for unlyophilized egg albumin, and for collagen (average of wet and dry) at 25°.

(7) Boyd and Livingston, THIS JOURNAL, **64**, 2383 (1942).

III

FUNCTION OF THE RELATIVE WATER VAPOR PRESSURE AT 25° AND AT 40°

0.50		0.60		0.70		0.80		0.90		0.95	
25	40	25	40	25	40	25	40	25	40	25	40
3.20	3.15	3.96	3.90	5.04	4.68	6.24	6.00	8.01	7.65	9.77	9.03
3.10	2.80	3.72	3.36	4.48	4.06	5.28	4.88	6.57	6.03	7.70	6.65
7.43	7.00	8.58	8.10	10.19	9.56	12.36	11.53	17.64	15.93	23.75	20.88
11.43	10.90	13.47	12.66	15.61	14.70	18.05	17.16	22.54	20.97	29.15	25.75
6.50	6.20	8.04	7.38	9.80	9.10	11,76	10.95	15.85	14.22	21.10	18.35
6.75	6.30	8.28	7.62	10.23	9.10	12.16	11.60	16.38	15.12	22.05	19.70
15.40	15.00	21.00	20.52	27.60	27.20	39.40	38.80	66.70	68.00	113.00	100.00
11.60	11.25	13.62	13.14	15.90	15.40	18.57	17.75	25.20	22.90	34.20	29.80
17.95	17.23	21.06	20.16	25.05	23.78	30.20	28.71	40.70	37.35	50.01	44.75
16.30	15.70	18.48	17.64	21.60	20.05	26.73	25.35	40.20	36.45	57.60	49.05
10.30	9.73	12.24	11.61	14.53	13.52	17.76	16.78	25.50	23.25	34.20	29.40
10.95	10.40	12.72	12.12	15.26	14.35	18.97	18.00	27.00	24.90	36.20	32.00
9.30	8.80	11.04	10.20	13.23	11.97	15.77	14.32	20.45	18.90	27.60	24.30
10.90	10.35	12.96	12.30	15.47	14.70	18.80	18.33	28.25	25.75	39.20	33.20
11.40	10.80	13.50	13.02	16.30	15.60	20.80	19.44	31.30	28.80	49.50	42.00
10.80	11.25	13.80	13.08	16.24	14.90	20.65	18.65	28.70	26.60	35.70	33.50
12.65	11.85	14.76	14.02	17.50	16.80	21.60	20.80	31.30	28.80	41.70	36.50
12.40	12.20	14.64	14.16	17.42	16.80	21.42	20.15	31.00	28.90	43.60	38.90

Since the adsorption of water has been studied at two temperatures (25° and 40°), the heats of adsorption can be calculated by well-known methods provided we assume the heat change to be independent of temperature over the temperature range employed. Such calculations have been made and are reported in Table IV. The accuracy of these values is undoubtedly of a low order. The lack of precision arises from two sources. First, the heat changes are calculated from the difference between the free energy changes at 25° and at 40°. This difference is not large and such errors as exist in the values for the free energy changes are magnified greatly in the heat changes and, second, it is entirely possible that the proteins do not exhibit a constant heat change even over the 15° range employed. The heat changes as shown in Table IV are, therefore, to be used with caution.

The plot of a/x against x provides a convenient way of extrapolating the amount of water adsorbed to saturation as the curve obtained is smooth and the change of the slope with x even at high relative humidities is moderate. By this method reasonably unambiguous values are obtained for the amounts of water held by the proteins at saturation. These saturation values along with the thermodynamic functions are given in Table IV. No values for salmin are included because this protein adsorbed so much water (it went into solution at x equal to about 0.7), that it was not possible to extrapolate to saturation. It is to be noted that the per cent. water held by β-lactoglobulin at saturation is 78 at 25°. It will be recalled that McMeekin and Warner[8] in a careful study found β-lactoglobulin crystals to contain 83% water of hydration. Considering the unusual steepness of the slope of the a/x *vs.* x curve as saturation is approached and accordingly the uncertainty of extrapolation to saturation for this protein, the agreement is regarded as good.

The free energy and heat changes were plotted as functions of the water content for each protein from a dry to a saturated condition but the calculations and results are so extensive that they have not been included in this paper. All the proteins showed the same qualitative changes of the thermodynamic functions with the amount of water adsorbed and such changes as were observed can be summarized as follows: the free energy change per gram of water added is a constant from zero water up to 1 to 4% water depending upon the protein. This portion of the curve of constant slope is followed by a steep upswing of the curve. In short, there is a gain in affinity of the protein for water as water is adsorbed. After the steep up-swing there is a gradual decrease in the slope of the curve until at saturation the slope is zero.

The ΔH-curves are somewhat more complex than are the ΔF-curves. The ΔH-curves follow very closely their corresponding ΔF-curves from zero water through the steep rise of the ΔF-curves. The ΔH-curves break more sharply and the point at which they break coincides rather well with the values of a_1 calculated from the Brunauer, Emmett and Teller[9] plot which will be discussed in detail later. The sharpness of the break in the curves varies from protein to protein. In the case of nylon it is very sharp, while the egg albumin and β-lactoglobulin curves are lacking in sharpness and amount to little more than an inflection. The slopes of the ΔH-curves are substantially constant following the above-mentioned break up to a

(8) McMeekin and Warner, THIS JOURNAL, **64**, 2393 (1942).

(9) Brunauer, Emmett and Teller, *ibid.*, **60**, 309 (1938).

TABLE IV

THERMODYNAMIC FUNCTIONS AND B. E. T. CONSTANTS

Protein	ΔF_{25}	ΔH	c	a_1	Protein area m.²/mg.	a_2	$(a_s)_{25}$
Nylon, unstretched	− 254	− 510	4.40	1.92	0.068	2.44	12.4
Nylon, stretched	− 244	− 540	5.97	1.76	.062	2.53	8.7
Silk	− 656	−1270	12.78	4.07	.144	8.8	33
Wool	− 975	−1950	11.13	6.58	.233	13.6	41
B-zein	− 605	−1160	13.20	4.10	.145	7.3	29
C-zein	− 604	−1060	12.30	3.78	.138	6.7	29
Salmin	−1450	−1800	64.8	5.28	.187	8.3	..
Elastin	−1000	−2250	11.90	6.22	.220	14.0	47
Collagen	−1620	−3640	17.80	9.52	.337	20.5	62
Gelatin	−1537	−3800	17.40	8.73	.309	19.1	85
Egg albumin, lyophilized	− 892	−1570	11.58	5.65	.200	11.4	47
Egg albumin, unlyophilized	− 956	−1350	11.60	6.15	.218	12.5	51
Egg albumin, heat coagulated	− 814	−1400	13.62	4.97	.176	10.5	36
β-Lactoglobulin, lyophilized	− 917	−1610	9.44	5.93	.210	12.1	53
β-Lactoglobulin, wet crystals	−1020	− 940	8.57	6.67	.236	12.7	78
Serum albumin	−1034	−1450	11.25	6.73	.238	14.6	46
α- and β-pseudoglobulin	−1109	−1830	12.20	7.15	.254	14.6	58
γ-Pseudoglobulin	−1119	−1700	11.83	7.16	.254	14.3	63

relative vapor pressure of about 0.7, at which point there is another up-swing of the ΔH-curves for all the proteins. This increasing slope continues, so far as can be determined, up to saturation. This feature of the ΔH-curves is characteristic and in some cases is very conspicuous.

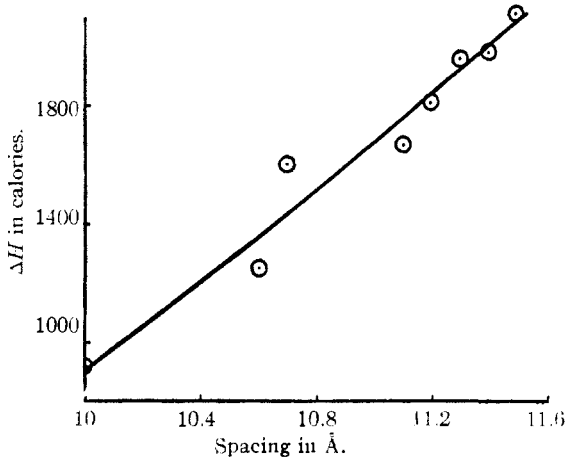

Fig. 3.—Side chain spacings in Å. plotted against the corresponding heats of hydration in gelatin.

The meaning of such changes in the free energy and heat as are described above are obscure. Fortunately, in the case of gelatin, Katz and Derksen[10] have reported a detailed X-ray diffraction study of this protein containing varying amounts of water. The medial spacing of about 10 Å. has been interpreted by various workers as representing the distance between the peptide chains of gelatin in the direction of the side chain residues. As water is added the peptide chains are pushed apart and the X-ray spacing increases. When the spacings in the direction of the side chains are plotted against the corresponding ΔH-values obtained in the present study, the result is a simple linear relation. This indicates that most of the heat up to about 20% water is involved in the combination of the polar side chain residues with water and with the forcing of these residues apart. The plot of ΔH against the corresponding X-ray spacings is shown in Fig. 3.

There is little comment to be made on the total free energy, and heat changes of hydration. In all cases, they are probably a summation of a number of complicated factors.

In an attempt to analyze the water adsorption curves of proteins in somewhat greater detail, the recent theory of Brunauer, Emmett and Teller[9] (abbreviated B. E. T. in what is to follow) has been used. It will be recalled that B. E. T. have extended the Langmuir treatment of monolayer gaseous adsorption to cover the case where more than one layer of molecules are adsorbed on the adsorbent. It has been found that when $P/a(p_0 - p)$ is plotted against x, a straight line is obtained whose intercept on the y-axis is $1/a_1c$ and whose slope is $(c - 1)/a_1c$ where a is the grams of water adsorbed at a water vapor pressure p, and p_0 is the water vapor pressure at saturation. a_1 is the grams of water adsorbed in the first layer on the surface of 100 g. of dry protein while c is related to the heat of adsorption. Such plots have been made for all the proteins studied and the constants a_1 and c evaluated. The values of the constants a_1 and c as given in Table IV are the averages of these constants at 25° and at 40°.

Figure 4 shows the B. E. T. plots for four selected proteins.

In general, the plot of $p/a(p_0 - p)$ against x yielded rather good straight lines between x equal 0.05 and 0.5. Both below and above these relative vapor pressures departure from a linear relation was found; below x equal 0.05 the points fell below the straight line while above x equal

(10) Katz and Derksen, *Rec. trav. chim.*, **51**, 513 (1932).

0.5 they were above the line. This appears to be the usual type of result and is observed for a variety of adsorbates on many different adsorbant surfaces. It can be said that the adsorption of water vapor on proteins obeys the B. E. T. theory quite as well as do non-protein systems reported in the literature. Salmin curiously enough gave a marked break in the line. This break occurred at x equals about 0.25. The two straight lines so obtained can be extrapolated to x equals zero to yield two values for a_1 and two for C. The first value of a_1 is 5.28 while the second is 10.66. The first value of a_1 is thus almost exactly half of the second. The corresponding C constants were 64.8 and 2.53, respectively. For an unknown reason the plot for unstretched nylon is not satisfactory; there is a considerable scattering of points. With these two exceptions the B. E. T. plots were very gratifying.

The question arises as to what interpretation is to be placed on the B. E. T. constants in the specific case of proteins. If we assume[11] that a water molecule adsorbed on the protein surface occupies 10.6 sq. Å., the area of the protein can be calculated from a_1. This has been done for all the proteins and these areas are recorded in Table IV and expressed in square meters per milligram of dry protein.

It is evident that the surface areas calculated for the proteins from a_1 are far too small to correspond to the total surface area of all the protein molecules. It is our belief that a_1 is a measure of the water which is required to complete the first step in the adsorption process. Water which is adsorbed in addition to the amount a_1 is adsorbed with a different average heat of wetting. Presently we shall attempt to attach a more specific meaning to a_1.

The constant C is supposed to be related to the heat of adsorption of the first monolayer of adsorbed molecules. There is, in our case, no clear relation between the heats calculated from C and the heat of adsorption as calculated from thermodynamics. This lack of agreement may be due to the fact that the heat of adsorption of water in excess of a_1 is appreciable and upsets the simple interpretation which B. E. T. place on this constant.

When the free energy involved in the adsorption of the quantity of water a_1 is divided by a_1, the free energy per gram of water adsorbed in the first layer of water is obtained. The affinity for water in this low vapor pressure region is much the same for all the proteins. The average value for the free energy change per gram of water adsorbed at 25° is 50.6 calories and at 40° it is 49.6 calories.

It is our belief that the adsorption of water by the hydrous magnesium aluminum silicate mineral, montmorillonite offers us an analogy for water adsorption by proteins. The water adsorption of montmorillonite has been studied by Hendricks, Nelson and Alexander,[12] who were able to show that this mineral forms a crystalline material one lattice constant of which depends upon the humidity. Adsorbed water penetrates between the molecular layers of the montmorillonite and pushes them apart. Certainly, moist proteins do not have the hardness to support capillaries in the sense that they occur in silica gel. It is only when the moisture content has been reduced to about 6% that the over-all or bulk density of the protein sample, as determined by the displacement of xylene, begins to diminish as the water content is decreased. The work of Neurath and Bull[13] on egg albumin shows this. These considerations mean that the protein molecules cannot occur in a random arrangement; otherwise the free space not occupied by the protein would be very much greater than 6%. This is not to infer that the whole mass of dried protein exist as one crystal but that the solid mass of protein is made up of a great many microscopic or submicroscopic crystals. Significant for this discussion are the X-ray diffraction studies of Boyes-Watson and Perutz[14] on horse methemoglobin crystals. These workers determined the X-ray spacings of crystals of methemoglobin in the dry and the wet condition. They conclude that the methemoglobin molecules are platelets which are about 36 Å. thick, 64 Å. long and 48 Å. wide. In the crystal, the

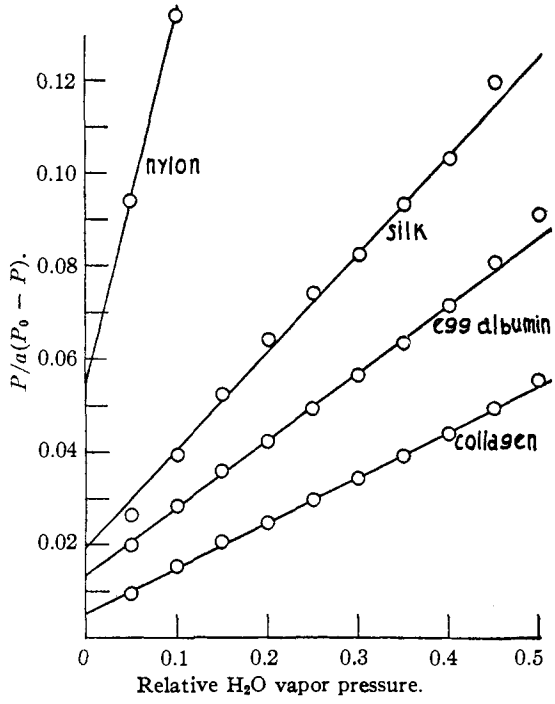

Fig. 4.—The B. E. T. plots for unstretched nylon, for silk (average of wet and dry), for unlyophilized egg albumin and for collagen (average of wet and dry) at 25°.

(11) Livingston, THIS JOURNAL, **66**, 569 (1944).

(12) Hendricks, Nelson and Alexander, *ibid.*, **62**, 1457 (1940).

(13) Neurath and Bull, *J. Biol. Chem.*, **115**, 519 (1936).

(14) Boyes-Watson and Perutz, *Nature*, **151**, 714 (1943).

platelet molecules are linked with their neighbors to form coherent layers which remain parallel at all stages of shrinkage as drying proceeds. The water of crystallization is distributed in sheets between layers of protein molecules. The water of crystallization is thus entirely intermolecular. It is our belief that the results of Boyes-Watson and Perutz give a very strong hint of the mechanism of the hydration of proteins in general.

It will be recalled that globular proteins such as egg albumin, β-lactoglobulin and the serum protein occupy about 0.9 square meter per milligram when spread in a monolayer on a water surface.[15] The surface areas of these proteins in bulk are given in Table IV and as calculated from the B. E. T. constant a_1 is about one fourth of the area when spread in monolayers. The exact meaning of this proportionality is at the present time obscure and it would be premature to attempt an explanation.

The ratios between the bulk and surface film areas for gelatin, collagen and silk appear to be somewhat different from that for β-lactoglobulin, egg albumin and the serum proteins. From a knowledge of the average residue weight of gelatin and from the X-ray diffraction spacings of this protein we can calculate that the spread area of a monolayer of this protein should be about 0.8 square meter per milligram. The ratio of the spread film area to the bulk area is therefore 2.5.

The area of a tightly packed monomolecular layer of silk can likewise be calculated from a knowledge of the average residue weight and from the X-ray diffraction spacings. The area so calculated is 1.15 square meters per milligram of silk which gives a ratio of spread area to bulk area as determined by water adsorption of 7.8.

Water adsorbed in excess of a_1 increases in an approximately linear manner with the water vapor pressure. In the case of gelatin the interpretation of this portion of the adsorption curve is clear and as we have discussed above involves principally the adsorption of water on the polar side chains followed by a separation of side chains from neighboring peptide chains. We suggest that essentailly the same process occurs during the hydration of all the proteins. Nylon is an exception as it contains no side chains of any kind. Perhaps a more general way of expressing this thought is to say that water goes on those groups which are most readily available; in the case of true proteins these are the exposed polar side chains.

The point at which the adsorption curve departs from linearity and swings upward can be estimated fairly closely. In Table IV are shown values of a_2 which is the amount of adsorbed water in grams per 100 g. of dry protein and which represents the completion of the linear portion of the adsorption curve. The values of a_2 were obtained by plotting a/x against x and selecting the point at which a/x reached a minimum. The value of a/x at this point was multiplied by the corresponding value of x to give a_2. It is to be noted from Table IV that a_2 is almost double a_1. In fact, if we exclude the a_2 values for nylon and for salmin, the average ratio between these constants is 2.05.

It seems reasonable to conclude that at point a_2 the exposed polar groups of the protein have become saturated with water and, accordingly, the force of attraction between protein molecules will be greatly decreased. As the water vapor pressure is increased beyond the point corresponding to a_2 the protein tends to go into solution. The upswing of the heat of adsorption which begins at this point can be regarded as a species of heat of solution. In the case of the insoluble proteins such as silk and wool, the heat of solution arises from the freeing of segments of the peptide chain from the restriction in motion which was present in the dry state.

We have noted the ratio of a_2 to a_1 is very nearly 2. We have suggested that a_1 represents the completion of a layer of water molecules between two coherent hydrophilic planes of protein molecules. This layer of water is sheared by neighboring protein planes and is bound rather tightly. We further suggest that a_2 represents the completion of a second layer of water molecules between the protein planes. This provides each side of the plane of protein molecules with a layer of water and means that the still coherent protein molecular planes are held together only through water–water linkages. Upon the addition of more water the protein tends to dissolve in water.

There are a few incidental points to note from Table IV. It is evident that lyophilized egg albumin is not as hydrophilic as is the unlyophilized. This protein apparently cannot be lyophilized without a certain degree of denaturation taking place; there is independent evidence for this.[16] It is also to be noted that heat coagulated egg albumin is not as hydrophilic as is the native egg albumin. The heat coagulation probably involves interaction between the polar groups on neighboring molecules, and, accordingly, there are fewer polar groups available for water binding.

The final question arises as to what if any relation exists between the amount of water held by a solid protein in an atmosphere saturated with water and the amount of water held by a protein when it is dissolved in water. This question is of great importance for the viscosity of protein solutions and for the diffusion of protein molecules. It is our belief that there is no direct relation between these two kinds of bound water. If our ideas, set forth in this paper, are correct, part of the saturation values given in Table IV can be regarded in the nature of water of crystalli-

(15) Gorter, *Ann. Revs. Biochem.*, **10**, 619 (1941); Neurath and Bull, *Chem. Revs.*, **23**, 391 (1938).

(16) Neurath, private communication.

zation of the protein and the amount of water so held will depend upon a number of factors among them being the arrangement of the protein molecules in the undissolved state. The amount of water held by a protein molecule in solution should be principally dependent upon the extent of the total hydrophilic surface exposed to the water. Bull and Cooper[17] have estimated this water held by dissolved proteins from a consideration of viscosity and diffusion and conclude that the volume of water so held is on the average about 28% of the volume of the protein. This would correspond on weight basis to about 21% hydration.

Acknowledgment.—It is a pleasure to acknowledge the financial assistance of the Abbott Research Fund of Northwestern University which permitted this research problem to be completed.

(17) Bull and Cooper, "Surface Chemistry," Am. Assoc. Adv. Sci., Pub. No. 21, page 150 (1943).

Summary

1. The weight of water vapor adsorbed by a series of purified proteins has been studied as a function of the aqueous vapor pressure at 25 and at 40°. These results are reported.

2. The free energy changes and the heat changes of the adsorption of water vapor by proteins have been calculated and reported.

3. It has been found that the theory of multilayer adsorption as proposed by Brunauer, Emmett and Teller is able to describe water adsorption by proteins in a very satisfactory manner. The B. E. T. constants have been evaluated and reported.

4. It is believed that the results obtained are consistent with the view that protein molecules in the solid state are linked together to form coherent planes whose exposed surfaces are hydrophilic. Water is adsorbed between these planes.

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Unsaturated Synthetic Glycerides. VIII. Unsymmetrical Mixed Triglycerides Containing Linoleic Acid[1]

BY B. F. DAUBERT AND A. R. BALDWIN[2]

Many of the earlier contributions on the synthesis of mixed triglycerides containing linoleic acid resulted from the investigations of Grün[3] and Izar.[4] Grün[3] reported the successful preparation of 1-linoleyl-2,3-distearin, m. p. 33.5–34°, and 1-linoleyl-2,3-dipalmitin, m. p. 11.5–13°, from linoleic anhydride and the corresponding 1,2-diglyceride. Because of the uncertainty regarding the structure of the supposed 1,2-diglycerides, which were synthesized by methods conducive to migration, the mixed triglycerides prepared from these intermediates were undoubtedly mixtures of the symmetrical and unsymmetrical isomers. This fact may account for the magnitude of difference in the reported melting points of the homologous triglycerides. The difference is much too large to be attributed to the carbon chain length increase or decrease of the saturated acids in the triglyceride molecule.

The efforts of Grün[3] to obtain the mixed triglycerides mentioned above by heating potassium linoleate and the 1,2-distearyl ester of chlorohydrin were not successful.

Since mixed triglycerides containing linoleic acid are important components of many natural fats and oils, the purpose of the present paper is to report physical and chemical data for a series of 1-linoleyl-2,3-disaturated triglycerides and 1-monosaturated-2,3-dilinoleins.

Experimental

Preparation of Intermediates.—All saturated fatty acids and saturated fatty acid chlorides were prepared by methods described previously.[5]

Linoleyl chloride was prepared from a linoleic acid obtained by debromination of tetrabromostearic acid (m. p. 115°) and oxalyl chloride.[6]

1-Monolinolein was prepared as described in a previous paper of this series[7] and all saturated 1-monoglycerides were synthesized after the method of Malkin and Shurbagy.[8]

Preparation of 1-Stearyl-2,3-dilinolein.—To a solution of 1-monostearin (3 g.) in a mixture of quinoline (5 ml.) and chloroform (30 ml.), linoleyl chloride was added slowly. The mixture, after refluxing on a steam-bath for four hours, was dissolved in petroleum ether (300 ml.) and the solution washed successively with 0.5 *N* sulfuric acid, 5% potassium carbonate solution and distilled water. The solution was then dried over anhydrous sodium sulfate. Repeated fractional crystallizations from petroleum ether and finally 95% alcohol yielded a product melting at 5 to 6°.

Other analytical constants together with the melting points of the saturated analogs obtained by hydrogenation for this compound and for 1-palmityl-2,3-dilinolein, 1-myristyl-2,3-dilinolein and 1-lauryl-2,3-dilinolein are listed in Table I.

Preparation of 1-Linoleyl-2,3-distearin.—1-Monolinolein (4 g.) was dissolved in a mixture of quinoline (5 ml.) and

(1) The authors are indebted to The Buhl Foundation for a grant in support of this investigation.

(2) Nutrition Foundation, Inc., Fellow.

(3) Grün and Schonfeld, *Z. angew. Chem.*, **29**, 48 (1916).

(4) Izar, *Biochem. Z.*, **60**, 320 (1914).

(5) Daubert, Fricke and Longenecker, THIS JOURNAL, **65**, 2142 (1943).

(6) Wood, Jackson, Baldwin and Longenecker, *ibid.*, **66**, 287 (1944).

(7) Daubert and Baldwin, *ibid.*, **66**, 997 (1944).

(8) Malkin and Shurbagy, *J. Chem. Soc.*, 1628 (1936).