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The Adsorption of Water Vapor on Acetyl Derivatives of Collagen and of Silk Fibroin

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The adsorption of water vapor on acetyl derivatives of collagen and silk fibroin has been studied by the isopiestic method and the equation of Brunauer, Emmett and Teller used to analyze the adsorption isotherms. First layer adsorption is appreciably reduced by acetylation, but this is not a specific effect of the amino and hydroxyl groups, which retain their power to coördinate water. It is suggested that a proportion of the first layer water molecules are adsorbed on the polypeptide chain and that it is these molecules which are displaced in the rearrangements accompanying acetylation.

Introduction

In a recent review of the adsorption of water vapor on proteins, McLaren and Rowen[1] have discussed the developments arising from Bull's[2] use of the Brunauer, Emmett and Teller (B.E.T.)[3] equation and from Pauling's[4] suggestion that the amount of first-layer adsorption, V_m, could be interpreted in terms of the number of polar side chains in the protein. Any such connection should be most easily discernible at low relative humidity (a_w) since at higher humidities simple stoichiometric relationships will be obscured by the formation of incomplete multi-layers and the incidence of swelling dependent on the mechanical properties of the substrate. In the region of low a_w it should be profitable to follow the controlled modification of specific protein groups by water vapor adsorption measurements.

Mellon, Korn, Kokes and Hoover[5] have shown that replacement of the polar amino groups of casein by guanidino groups, which are also polar, has no influence on adsorption. On the other hand, Mellon, Korn and Hoover[6] report a reduction in adsorption over the whole range of a_w (0.06–0.93) when the amino groups of casein are benzoylated, a reduction which they ascribe to the blocking effect of the benzoyl radical, so that their results support the idea of localized adsorption on specific sites. However, the direct derivation of V_m from the data of Mellon, *et al.*, is somewhat uncertain because they made only three measurements in the a_w range (0.05–0.40) most favorable for the determination of V_m.

Green[7-9] has shown that, by measuring 8–12 points in this range, fairly precise estimates of V_m can be made. For some proteins, such as silk or casein, even when physical or chemical pretreatment may strongly affect the actual quantity of water adsorbed at a given a_w, the derived value of V_m remains unchanged, the whole result of the modification appearing in the energy term, C. In others, such as collagen, even changes in the method of drying can affect both V_m and C. Any study of the influence of chemical modification on the adsorptive powers of specific protein groups must be made with these conditions in mind.

We describe here the adsorptive properties of a range of derivatives of collagen and silk which have been acetylated at the side-chain amino and hydroxyl groups.[10]

Experimental

Materials.—The collagen used was the standard hide powder of the International Society of Leather Trades' Chemists. Denatured collagen was prepared by immersing standard hide powder in water at 72° for 5 minutes, filtering, washing with cold water, dehydrating with acetone and drying in the air of the laboratory. Pure degummed silk fibroin was obtained from the Nutritional Biochemicals Corporation, Cleveland, Ohio, and was found to be identical as an adsorbent with a sample of silk[9] supplied by the Shirley Institute, Manchester, England.

Acetylation.—The general method of acetylation consisted in treating the oven-dried protein with mixtures of acetic acid and acetic anhydride for periods of from 40 minutes to several days. It has already been reported in the case of collagen[10] that a 5% solution of acetic anhydride in glacial acetic acid effects complete N-acetylation of amino groups in 8 hours without attacking other protein groups. Partially N-acetylated derivatives were made by reducing the reaction time. Higher concentrations of anhydride in the mixture yield derivatives in which all the amino groups and up to 80% of the hydroxyamino acid residues have been acetylated. Similar methods were used with silk fibroin. After acetylation the samples were washed with several changes of acetone and then treated with acetone for two days in a soxhlet apparatus. This method of extraction with warm acetone was shown to give products whose behavior as adsorbents was identical with that of samples washed for long periods in the cold. After extraction the derivatives were spread out in the air of the laboratory until free from excess acetone and then dried to constant weight at 100° *in vacuo* over freshly prepared calcium oxide. They were analyzed for total acetyl by acid hydrolysis, followed by distillation and titration,[10] for free amino nitrogen by the method of Doherty and Ogg[11] and for total nitrogen by the Kjeldahl method. O-Acetyl was taken as the difference between total acetyl and the number of blocked amino groups.

Adsorption Measurements.—Water vapor adsorption isotherms at 25° were measured by the isopiestic method of Robinson and Sinclair[12] in the manner described by Green.[7] In the a_w range 0.05–0.40, duplicate measurements were made for 10–12 points. A few isotherms measured over the whole a_w range proved to be of the familiar sigmoid form apparently common to all proteins and their derivatives.

Results and Discussion

Acetylation.—It has already been shown[10] that the maximum number of N-acetyl groups which can be introduced into collagen is exactly equivalent to the number of lysine and hydroxylysine residues reported by Bowes and Kenten.[13] Similarly about 80% of the total hydroxyamino acid residues can

(1) A. D. McLaren and J. W. Rowen, *J. Polymer Sci.*, **7**, 289 (1951).
(2) H. B. Bull, THIS JOURNAL, **66**, 1499 (1944).
(3) S. Brunauer, P. H. Emmett and E. Teller, *ibid.*, **60**, 309 (1938).
(4) L. Pauling, *ibid.*, **67**, 555 (1945).
(5) E. F. Mellon, A. H. Korn, E. L. Kokes and S. R. Hoover, *ibid.*, **73**, 1870 (1951).
(6) E. F. Mellon, A. H. Korn and S. R. Hoover, *ibid.*, **69**, 827 (1947).
(7) R. W. Green, *Trans. Roy. Soc. N. Z.*, **77**, 24 (1948).
(8) R. W. Green, *ibid.*, **77**, 313 (1949).
(9) R. W. Green, *ibid.*, **79**, 494 (1952).
(10) R. W. Green, K. P. Ang and L. C. Lam, *Biochem. J.*, in press.
(11) D. G. Doherty and C. L. Ogg, *Ind. Eng. Chem., Anal. Ed.*, **15**, 751 (1943).
(12) R. A. Robinson and D. A. Sinclair, THIS JOURNAL, **56**, 1830 (1934).
(13) J. H. Bowes and R. H. Kenten, *Biochem. J.*, **43**, 358 (1948).

be O-acetylated, but the remainder appear to be masked.

The most recent analytical figures for silk fibroin[14] indicate the presence of 0.05 mmole of lysine and 2.39 mmoles of hydroxyamino acids per g. of protein. In this work it was found that fibroin was acetylated much more slowly than collagen, and Table I shows that, when the reaction was complete, although the number of N-acetyl groups introduced was close to the expected value, a high proportion of the hydroxyamino acid residues remained unattacked.

The behavior of fibroin on acetylation can be shown to be consistent with its reaction with anhydrous hydrogen chloride.[15] In that reaction a maximum of 0.79 mmole of HCl is fixed per g. protein and of this quantity 0.13 mmole/g. is accounted for by the basic groups of arginine, histidine and lysine.[14] The remaining 0.66 mmole/g. is combined with serine and threonine residues. Although the protein contains a total of 1.68 mmoles of these two amino acids per g., not more than 0.66 mmole/g. is available for reaction with hydrogen chloride. If we suppose that only these same available residues together with all the tyrosine residues (0.71 mmole/g.) can form O-acetyl derivatives, the total number of O-acetyl groups introduced into silk fibroin should be 1.37 mmoles/g., only slightly in excess of the figure shown in Table I. It seems that about 60% of the serine and threonine residues are masked against both hydrogen chloride and cold acetic anhydride, but that all the tyrosine residues can be made to react with the latter agent.

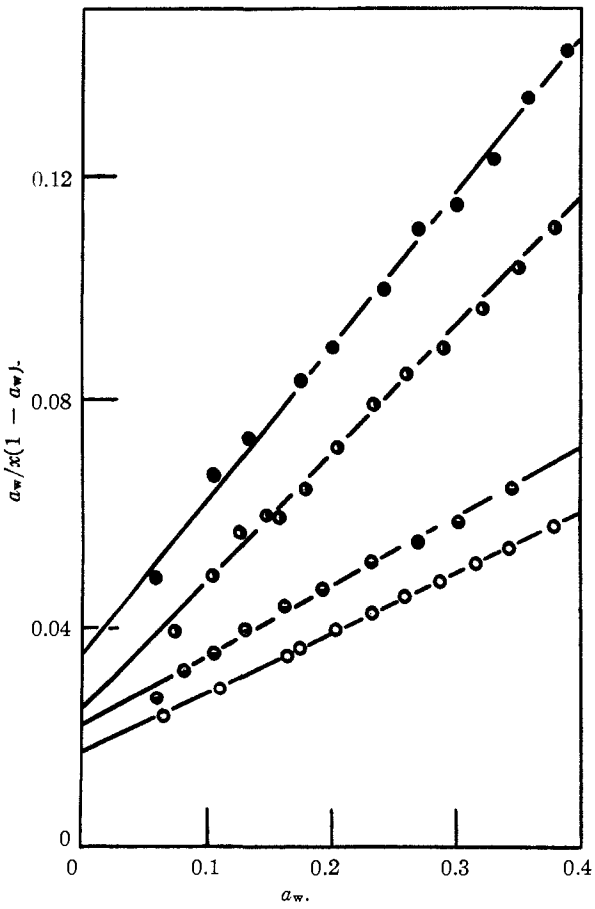

Fig. 1.—B.E.T. functions for protein derivatives plotted against a_w: ●, acetylated fibroin, sample O, Table III; ◑, acetic acid-treated fibroin, L; ⊖, acetylated collagen, H, Table II; ○, acetylated collagen, F.

(14) G. R. Tristram, *Advances in Protein Chemistry*, **5**, 142 (1949).
(15) R. W. Green, *Trans. Roy. Soc. N. Z.*, **79**, 485 (1952).

TABLE I

ACETYLATION OF DRY SILK FIBROIN WITH A MIXTURE OF EQUAL PARTS OF ACETIC ANHYDRIDE AND ACETIC ACID

Time of reaction, days	Mmoles/g. protein N-Acetyl[a]	O-Acetyl[b]
5	0.06	0.25
10	.06	0.66
20	.06	1.30
30	.06	1.30

[a] Lysine content of fibroin: 0.05 mmole/g. [b] Hydroxyamino acid content of fibroin: 2.38 mmoles/g. (Tristram, ref. 14).

Water Vapor Adsorption.—Although the acetylated silk fibroin was indistinguishable in appearance from the native protein, the method of acetylation used here caused collagen to swell and left it in a state closely resembling the heat-denatured material. V_m for collagen is known to be sensitive to changes in the method of preparation,[7] heat denaturation having a particularly marked influence. If, in addition to acetylation, acetic anhydride or acetic acid were to cause some change similar to denaturation, any specific effect at the acetylated groups might well be obscured. Two samples of collagen were therefore treated, one with acetic anhydride and the other with acetic acid for 24 hours, and then washed and extracted with acetone as before. Neither of these reagents alone in the absence of water causes the introduction of acetyl groups into proteins,[10] although the acetic acid resembled the acetylating mixtures in producing an appearance of denaturation. The pure anhydride left the physical condition of the collagen apparently unchanged.

Water vapor adsorption isotherms were measured for both these preparations and also for acetic acid treated fibroin, for a sample of collagen acetylated after heat denaturation, and for a range of acetyl derivatives of both proteins. Examples of the B.E.T. plots are shown in Fig. 1, and the values of the B.E.T. parameters are set out in Tables II and III. For comparison we have included the B.E.T. constants for the two parent proteins[9] and for heat-denatured collagen,[7] all similarly dried. In the preparation of these tables, the values of V_m derived from the B.E.T. plots for acetylated proteins have been corrected by the factor: (total nitrogen content of original protein/total nitrogen content of derivative) in order to allow for the dilution effect of the added acetyl groups. V_m is thus expressed as mmole water per g. original dry protein so that all values are directly comparable. The standard errors of V_m and C were determined by a statistical analysis of four of the B.E.T. regression lines, selected at random, representing a total of forty-five experimental points. The estimates of standard error in Tables II and III are

therefore based on thirty-seven degrees of freedom and can be used with confidence.

TABLE II

B.E.T. CONSTANTS FOR COLLAGEN DERIVATIVES

	Adsorbent	Mmoles/g. protein N-Acetyl	Mmoles/g. protein O-Acetyl	V_m[a]	C[b]
A	Collagen[c]	..	..	5.24	6.2
B	Denatured collagen[d]	..	..	4.56	6.6
C	Acetic acid-treated collagen	..	..	4.58	6.9
D	Acetic anhydride-treated collagen	..	..	4.60	8.8
E	Acetylated collagen	0.24	..	4.51	7.3
F	Acetylated collagen	.39	..	4.54	7.3
G	Acetylated collagen	.40	0.11	4.21	7.6
H	Acetylated collagen	.40	0.64	4.01	6.6
I	Acetylated collagen	.40	1.32	4.10	6.7
J	Acetylated denatured collagen	.40	0.91	4.20	6.4

TABLE III

B.E.T. CONSTANTS FOR FIBROIN DERIVATIVES

	Adsorbent	Mmoles/g. protein N-Acetyl	Mmoles/g. protein O-Acetyl	V_m[a]	C[b]
K	Fibroin[c]	..	..	2.33	7.0
L	Acetic acid-treated fibroin	..	..	2.21	9.9
M	Acetylated fibroin	0.06	0.25	1.98	9.6
N	Acetylated fibroin	.06	0.66	1.96	9.6
O	Acetylated fibroin	.06	1.30	1.93	8.6

[a] Standard error of V_m = 0.06. [b] Standard error of C = 0.34. [c] Reference 9. [d] Reference 7.

Examination of the figures for collagen derivatives confirms the impression of denaturation given by the physical appearance of the adsorbent. Collagen treated with either acetic acid or acetic anhydride alone, or collagen acetylated only at the $-NH_2$ groups shows a value of V_m exactly equal to that for the heat-denatured protein and independent of the number of N-acetyl groups present. It is evident that either of the two components of the acetylating mixture is able to produce the same effect on V_m as does denaturation in water at 72°; and the effect seems to be independent of the duration of the pretreatment, which lasted for 24 hours for samples C and D but for only 40 minutes for the N-acetyl derivative, E. Contrary to the findings of Mellon, *et al.*,[6] in the benzoylation of casein, the acetylation of the ϵ-amino groups of the lysine residues has no specific effect on adsorption of water vapor by collagen. This apparent contradiction may arise from either the different electronic or the different spatial properties of the substituent groups, but in either case it demonstrates that acylation does not necessarily inactivate amino groups as sites for adsorption of water vapor.

When we consider the higher acetyl derivatives of collagen, with substitution in hydroxyl as well as amino groups, another sudden decrease in V_m is observed. Like the change from native to denatured collagen, the change in V_m caused by O-acetylation again appears independent of the number of acetyl groups introduced. The absence of any stoichiometric relationship between V_m and the degree of acetylation indicates that acetylation has no power to block the sites which adsorb water and suggests that this effect, too, is a form of denaturation. This is in qualitative agreement with the behavior of collagen in which the aliphatic hydroxyl groups have been caused to react with anhydrous hydrogen chloride.[9] Furthermore, acetylation of denatured collagen gives a derivative whose B.E.T. constants lie in the same range as those for the derivatives of native collagen.

According to the analytical data of Bowes and Kenten[13] the total number of polar side chains in collagen is equivalent to about 3.87 mmoles/g. If we consider that each of these polar groups contributes one water molecule in the calculation of V_m, we see that the lowest value reported in Table II comes very near this calculated figure of 3.87, while V_m for dried native collagen is as high as 5.24. It already has been postulated[7] that this great discrepancy between the calculated and observed values of V_m for collagen arises from adsorption on the polypeptide chain. This discrepancy would become somewhat greater if the small number of –OH groups (0.40 mmole/g.) already shown[10] to be incapable of acetylation were also incapable of adsorbing water vapor. During denaturation either by heat or in the reactions described here, the refolding and rearrangement of the polypeptide chains must result in a more stable form, in which hydrogen bonds between adjacent peptide groups become more numerous, and the number of points remaining for the coördination of water molecules becomes less. In the extreme case of fully acetylated collagen, hardly any uncompensated peptide groups remain to adsorb water and V_m is reduced to almost the theoretical value. This interpretation is consistent with the view that the acetylated side chains of collagen still retain their power to adsorb water.

The data for silk fibroin pose a slightly different problem. The total number of polar groups according to Tristram[14] is 2.87 mmoles/g., much greater than the observed value, 2.33, of V_m for the dried protein. There would therefore appear, as has been suggested previously,[9] to be no need to postulate adsorption on the polypeptide chain. However, it then becomes difficult to explain the appreciable diminution in V_m accompanying the acetylation of fibroin, since we have already concluded that O- and N-acetyl groups retain their adsorptive power. For fibroin, as for collagen, the fall in V_m is a fairly sudden one and bears no quantitative relationship to the duration of the reaction or to the number of acetyl groups introduced.

The difficulty may be resolved if we take a different view of the composition of V_m for fibroin. It has been shown above that a high proportion of the serine and threonine residues (1.02 mmoles/g.) are not available for reaction with either hydrogen chloride or cold acetic anhydride, and it is likely that these same residues have no power to adsorb water. On this basis, the calculated value of V_m is reduced from 2.87 to 1.85 mmoles/g. and it becomes necessary to admit some adsorption on the polypeptide chain to account for the observed value of 2.33 mmoles/g., *i.e.*, adsorption on the polypeptide chain is responsible for 0.48 mmole/g. If we

suppose that, as for collagen, some kind of rearrangement or redistribution of hydrogen bonds during acetylation reduces peptide adsorption almost to zero, the observed fall in V_m to 1.93 mmoles/g. on acetylation can be made to fit into the general theory. The smaller magnitude of the polypeptide adsorption effect in fibroin appears consistent with the more compact physical structure of this protein.

If our interpretation of the data of Tables II and III is correct, it reopens the whole question of the relationship between V_m and the number of polar groups. Even when the hydrolytic products of the protein contain sufficient amino acids with polar side chains to account fully for V_m, it must still be established that, in the intact protein, these groups are available for ordinary chemical reactions before their number can be used to compute V_m.

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Studies on Coördination Compounds. IV. A Comparison of the Chelating Tendencies of β-Diketones toward Divalent Metals[1]

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The formation constants for the chelate compounds formed by a series of divalent metal ions with a group of β-diketones have been determined and compared. The general order of increasing stability of these chelate compounds using nitrate salts and β-diketones with aromatic ring end-groups in 75 volume per cent. dioxane solution is: Ba, Sr, Ca, Mg, Cd, Mn, Pb, Zn, Co, Ni, Fe, (Cu, Be), Hg (log K_1). The stabilities of the chelate compounds increase with the electronegativities of the metal ions involved and hence are a function of the covalent character of the resulting bonds. The divalent metal ions Fe, Ni, Co, Zn, Mn and Mg form complex salts with acetylacetone (HCh) containing the stoichiometrical ratios of Na^+:M^{2+}:Ch^- = 1:1:3; nickel and zinc with acetylacetone form soluble chelate compounds containing the stoichiometric ratios $NiCh_2$, $ZnCh_2$, NiChCl, ZnChCl and Ni(OH)Ch and the *insoluble* chelate compounds having the stoichiometrical ratios of $NaNiCh_3 \cdot C_4H_8O_2$, $NaZnCh_3 \cdot C_4H_8O_2$, $Zn_2(OH)Ch_3$, and $Zn_5Ch_2(OH)_7Cl$.

Introduction

Information concerning the role of the metal ion in the process of chelation is of primary importance to the understanding of the nature of chemical bonding. In a previous publication[2] the chelating tendencies of β-diketones toward chlorides of a strongly chelating divalent metal ion, copper, one of intermediate behavior, nickel, and one having a relatively low chelating tendency, barium, were compared. Investigations on the formation constants of a series of divalent metal nitrates with a representative group of β-diketones are reported here.

The values of the "measured" chelation constants are dependent upon the salt anions present in solution and the solvent in which the measurements are made. The measurements reported here have been made in an approximately 75 volume per cent. dioxane–25 volume per cent. H_2O solution. Comparisons are based upon the coordinating behavior of the 0.01 M nitrate salt solutions. The equations

$$M(NO_3)_2(H_2O)_x + HCh \rightleftharpoons M(NO_3)Ch(H_2O)_y + HNO_3 + (x - y)H_2O \quad (1)$$

and

$$M(NO_3)Ch(H_2O)_y + HCh \rightleftharpoons MCh_2(H_2O)_z + HNO_3 + (y - z)H_2O \quad (2)$$

represent the equilibria that are involved in determining the first and second formation constants.

(1) A portion of a dissertation presented by L. G. Van Uitert in partial fulfillment of the requirements for the degree of Doctor of Philosophy, 1952.

(2) L. G. Van Uitert, W. C. Fernelius and B. E. Douglas, THIS JOURNAL, **75**, 457 (1953).

Experimental

The experimental procedure and calculations are the same as previously reported.[2] A nitrogen atmosphere was employed to avoid the oxidation of the unstable divalent metal ions. Specific exceptions are noted in the appropriate parts of the discussion. *Anal.* of the precipitate having the stoichiometry Na^+:Zn^{++}:$C_5H_7O_2^-$ = 1:1:3: Calcd. for $NaZn(C_5H_7O_2)_3 \cdot C_4H_8O_2$: C, 47.9; H, 6.15. Found: C, 48.0, 48.2; H, 5.41, 5.68.

Discussion

It has been shown[2] that the plotted points of the logarithms of the first, average or second formation constants (log K_{f_1}, log K_{fav} or log K_{f_2}) of a series of di-ring end-group β-diketones with a given metal salt *vs.* the negative logarithms of the dissociation constants (pK_D values) of the respective β-diketones fall on a common line. Dibenzoylmethane, 2-furoylbenzylmethane and 2-thenoyl-2-furoylmethane are representative of this series. Benzoylacetone and acetylacetone demonstrate the effect of substituting one and two methyl groups in place of aromatic ring end-groups on a β-diketone. The formation constants of these compounds with the nitrate salts of Cu, Be, Ni, Co, Zn, Pb, Mn, Cd, Mg, Ca, Sr and Ba are tabulated (Table I). The values for the perchlorate salts of Fe, Ni and Pb also are listed to allow certain approximations to be made.

The data given in Table I are plotted in Fig. 1. The values for copper that are given are based upon the measured value for $Cu(NO_3)_2$ with acetylacetone and an assumed proportional relationship to the data for $CuCl_2$[2] with the other chelating agents. Comparable values for iron(II), based upon a similar comparison of the chelate