of the components is 13% C_1 (mobility of -0.5×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1}), 12% $C_2(-1.6 \times 10^{-5})$ and 75% $C_3(-3.8 \times 10^{-5})$. The spreading of the peak corresponding to C_3 indicates the presence of additional components. The top fraction analyzed 71% $C_1(-0.68 \times 10^{-5})$, 19% $C_2(-1.92 \times 10^{-5})$ and 10% of a new component $C_4(-2.8 \times 10^{-5})$. The small amount of component C_3 in the top fraction has been included with C_4. Fractionation by electrophoresis-convection resulted in an increase in the relative concentration of C_1 and C_2 by factors of 5.5 and 1.6, respectively. Unpublished results of one of us[10] show that the enzyme has a mobility of about -2×10^{-5} at *p*H 7, which would correspond to component C_2. Although the relative concentration of C_2 was increased by a factor of 1.6 on fractionation, the specific activity of the enzyme in the top fractions was as much as 3 to 5 times that of the crude extract. However, this large enrichment factor was obtained only after transport of 97% of the protein out of the top reservoir, transport of 86–93% of the protein yielding an enrichment factor of only 1.2. Since a large increase in the enrichment factor would not be expected to occur during the transport of the last few per cent. of material out of the top reservoir, it would appear that considerable separation of enzyme from its inhibitor was accomplished during the latter stages of fractionation. Of course, there is no reason to believe that component C_2 is composed entirely of the enzyme, and it is possible that fractionation resulted in an enrichment of C_2 in the enzyme.

The bottom fractions obtained at *p*H 7.0 still possessed considerable activity. With the hope of recovering some of this activity and thereby increasing the over-all yield of purified enzyme, an attempt was made to refractionate at *p*H 7.0 the bottom fraction from run 14. Although 90% of the protein of the starting material was recovered in this experiment, only 5% of the activity was recovered. Similar results were obtained when a composite of bottom fractions was refractionated at *p*H 8.1. As shown in Table II, no serious loss of activity was encountered on fractionation of the crude enzyme extracts. At present no explanation can be given for the loss of activity on refractionation, although large changes in ratio of enzyme to inhibitor in the various fractions might lead to an apparent loss of enzyme.

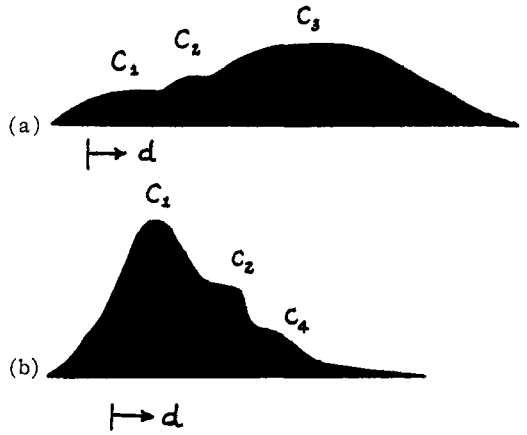

Fig. 1.—Electrophoretic patterns of (a) the crude enzyme extract and (b) a composite of top fractions obtained by fractionation at *p*H 7.0 for 48 hours.

DENVER, COLO.

[CONTRIBUTION FROM THE CHEMISTRY DEPARTMENT, UNIVERSITY OF SOUTHERN CALIFORNIA, LOS ANGELES]

The Surface Areas of Proteins. V. The Mechanism of Water Sorption[1]

BY JERROLD M. SEEHOF, BERTRAM KEILIN AND SIDNEY W. BENSON

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The sorption of water by solid proteins is characterized by the evolution of heat, a peculiar hysteresis, swelling of the solid and an increase in the BET surface area of the solid. Heat evolution is of the order of magnitude of the heat of vaporization of water and in conjunction with the poor thermal conductivity of the protein acts to slow the sorption considerably. The hysteresis is unusual in that it is almost constant over the entire range of partial pressures from about $p/p_0 = 0.05$ to 0.90 and independent of temperature. Although the hysteresis loop is quite reproducible (*i.e.*, in both branches) the sorption–desorption curves do not correspond to states of thermodynamic equilibrium and thermodynamic data calculated from them are apt to be quite anomalous. It is observed that the surface areas of proteins as measured by the BET method show an increase after water sorption which is reproducible and can be interpreted in terms of particle dimensions to give an apparent molar density for water which is anomalously high and indicates rather unusual packing efficiency in the solid. It is proposed that the hysteresis is associated with binding on the free basic groups of the protein. The existing data on sorption do indeed show an excellent correlation between number of free basic groups and the maximum amount of hysteresis and a hypothesis is suggested for such a correlation.

I. **Hysteresis.**—Recent work in these laboratories[2] has shown that whereas dry, solid proteins are capable of adsorbing non-polar gases[3] in a manner characteristic of general physical adsorption processes, polar gases[4,5] fall into a distinctly different category displaying characteristics ranging from chemical reaction to solvation.[5,6] The sorption of water, while similar in many ways to the other polar gases, shows some special features, in particular that of complete reversibility and much

(1) The contents of this paper were presented in part at the Symposium on Colloid Chemistry held by the Division of Colloid Chemistry of the American Chemical Society at Los Angeles, June, 1952.

(2) The authors wish to express their appreciation to the Research Corporation and to the Office of Naval Research for grants which made the present work possible.

(3) S. W. Benson and D. A. Ellis, THIS JOURNAL, **70**, 3563 (1948); **72**, 2095 (1950).

(4) S. W. Benson, D. A. Ellis and R. W. Zwanzig, *ibid.*, **72**, 2102 (1950).

(5) S. W. Benson and J. M. Seehof, *ibid.*, **73**, 5053 (1951).

(6) S. W. Benson and J. M. Seehof, unpublished work, presented in part at National Colloid Symposium, June, 1952.

more rapid equilibrium under comparable conditions,

All of the polar gases show a marked hysteresis in sorption, and there has been considerable study of the hysteresis phenomenon in the case of water.[7,8] In Fig. 1 are presented some data obtained in these laboratories on the water sorption of bovine serum albumin[9] at two different temperatures. The curves are similar to those obtained for other proteins both in these and other laboratories. The most interesting feature is that the amount of hysteresis is almost constant over the entire range of relative pressures and that the hysteresis persists down to extremely low relative pressures. It is also to be noted that the amount of hysteresis is virtually independent of temperature.

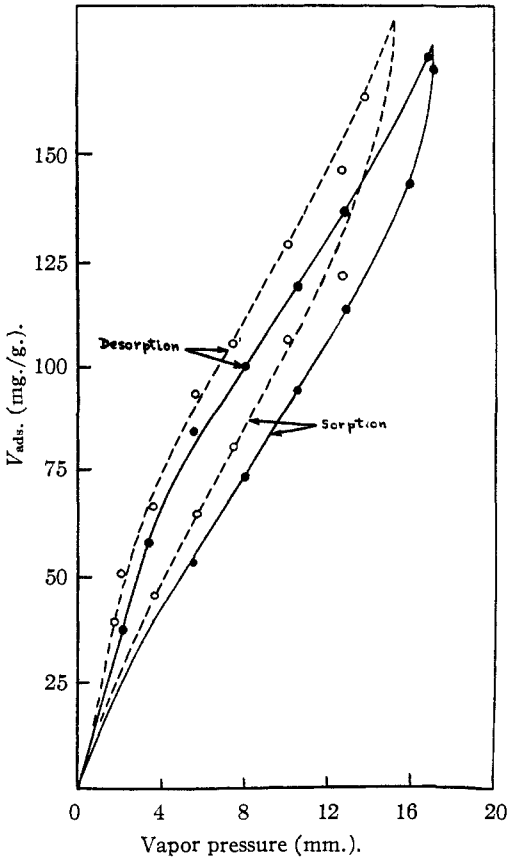

Fig. 1.—Water sorption on bovine serum albumin: – – –, 20°; —, 25°.

Because of this unusual quality of the hysteresis we were inclined to attempt to relate it to the free acid and base groups present in the protein. A similar correlation has been found strikingly successful for the irreversible binding of HCl[5,6] on proteins, which may in many ways be considered comparable to this quasi-constant and persistent hysteresis.

Mellon, Korn and Hoover[7] endeavored to show that hysteresis in casein was independent of the "free" amino content by benzoylating varying percentages of these groups in different casein samples. They found the hysteresis to be virtually constant for all the samples but they did not show that the benzoylated amino group did not partake actively in the hysteresis[10] as well as the "free" amino groups. In an earlier article by the same authors[11] it was shown that the total water sorption at any given pressure depended markedly on the extent of benzoylation (in this way confirming the importance of specific groups in the sorption process). These two findings may be consistent with a polar group interpretation of hysteresis if it is assumed that benzoylation allows bonding of an initial water molecule but hinders multilayer adsorption thereafter.

In Table I is shown a correlation of the maximum amount of hysteresis (last column) in water sorption with the sum of the arginine, histidine, lysine and cystine groupings (next to last column). In the case of tobacco mosaic virus it was necessary to include the 13% of ribonucleic acid bound to the protein portion of the molecule.

TABLE I

CORRELATION BETWEEN MAXIMUM HYSTERESIS AND POLAR GROUPINGS IN PROTEINS

	Mmoles/gram of protein								
	Arg. 1	Hist. 2	Lys. 3	Cys-teine 4		Cys-tine 5	RNA 6	Σ 1–6	Max. hyst.
Casein[a]	0.2	0.2	0.6	...		...	...	1.0	1.1[g]
TMV[b]	.5	...	.1	0.1		...	2.7	3.4	3.5[h]
Insulin[c]	.2	0.3	.2	0.1		0.5	...	1.3	1.2[i]
Collagen[d]	.5	.1	.3	...		...	...	0.9	0.8[j]
BovSAlb[e]	.3	.2	.8	...	0.3[f]	...	...	1.6	1.5[k]
BovPAlb									1.4[l]

[a] Gordon, Semmett, Cable and Morris, THIS JOURNAL, 71, 3293 (1949). [b] C. Knight, *J. Biol. Chem.*, 171, 297 (1947). [c] E. Brand, *Ann. N. Y. Acad. Sci.*, 47, 487 (1946). [d] Chibnall, *J. Intern. Soc. Leather Trades Chem.*, 30, 1 (1946). [e] Lewis, *et al.*, *J. Biol. Chem.*, 186, 23 (1950). [f] W. Stein and S. Moore, *ibid.*, 178, 79 (1949). [g] Ref. 7. [h] B. Katchman and D. MacLaren, THIS JOURNAL, 73, 2124 (1951). [i,l] R. Robinson, *J. Chem. Soc.*, 1803 (1948). [j] R. Green, *Trans. Proc. Roy. Soc. New Zealand*, 77, 24 (1948). [k] Present work.

The arginine, lysine and histidine would be expected to bind water strongly by virtue of their free amino groups. The sulfur linkages of cysteine and cystine are known to be hydrogen bonding and the work of Speakman and Stott[12] on wool indicates that the sulfur linkages are of prime importance in the hysteresis phenomenon. In the case of tobacco mosaic virus, approximately 0.18 mole/g. of ribonucleic acid was present. The ribonucleic acid is composed of *d*-ribose, adenine, guanine, cytosine, phosphoric acid and uracil. These groups contain 15 atoms of possibly reactive nitrogen, 3 hydroxyl groups and 4 carbonyl groups, a

(7) E. F. Mellon, A. H. Korn and S. R. Hoover, THIS JOURNAL, **70**, 1144 (1948).

(8) See A. D. McLaren and J. W. Rowen, *J. Polymer Sci.*, **7**, 289 (1951), for a review of this and other features of water sorption.

(9) The preparation of the samples and the experimental techniques have been described earlier (see ref. 4).

(10) Our experience with Nylon and HCl have shown that the peptide linkage is a quite good base unless hindered by "crystallinity" or prior hydrogen bonding. Thus the apparent lack of basicity of the peptide skeleton is very probably to be attributed to internal hydrogen bonding. The model proposed by Pauling is in this respect quite in accord with the chemical evidence.

(11) E. F. Mellon, A. H. Korn and S. R. Hoover, THIS JOURNAL, **69**, 827 (1947).

(12) J. B. Speakman and C. J. Stott, *J. Text. Inst.*, **27**, T186 (1936).

total of 22 groups.[13] If we had included the latter the value of Σ (Table I) would be 3.96 mmoles/g. instead of 3.4.

While it is still possible that the agreement shown is fortuitous, the total weight of evidence makes this rather unlikely and points strongly to a significant correlation.

II. Protein Swelling and Surface Area.—It was deemed of interest to determine whether the surface area of a protein, as determined by the BET theory with argon as the adsorbate, would vary with the quantity of water sorbed by the protein. Accordingly, the surface area of a sample of spray frozen, lyophilized, dried bovine serum albumin fraction V was determined. A quantity of water vapor (0.31 g./g. protein) was then sorbed on the protein and the surface area was redetermined at liquid nitrogen temperature. The sample was then dried by evacuation and the area determined still again. Assuming an "effective" water density in the sorbed phase and knowing the amount of water adsorbed the volume increase can be calculated. With these data the surface area increase to be expected of spheres or infinite cylinders can be determined and compared with the experimental value. The results are summarized in Table II.

TABLE II

SURFACE AREA—SORBED WATER

Water adsorbed, g./g. protein	Surface area, m.²/g.		
0.00	9.1		
.31	9.8		
.00	9.2		
Increase of surface area, %	7.5		
Protein density (dry)	1.3		
Water density (effective)	1.0	1.3	
Volume increase (calcd.), %	40	31	
Spheres (calcd. area incr.), %	27	21	$A \alpha V^{2/3}$
Cylinders (calcd. area incr.), %	20	15	$A \alpha V^{1/2}$

For the cylinders therefore an effective density of 2.6 would have to be assumed for the water to approach the experimentally observed value of 7.5% area increase while even larger effective densities would have to be assumed for spheres or ellipsoids. Such anomalous densities can be explained either in terms of a partly laminar structure for the solid protein or else a very efficient packing of the water in the protein structure. Work is now under way in these laboratories to extend these measurements to other water contents and to direct observation of the solid protein densities.

III. General Features of the Sorption Process.—In Fig. 2 is presented a graph of the partial molal differential heats of sorption of water calculated from the isotherms by means of the Clausius–Clapeyron equation. It is to be noted that the points for sorption and desorption fall on the same curve, that the heats are less than the heats of condensation of water and finally that the values decrease with increasing sorption. Such anomalies indicate the difficulty of applying thermodynamic arguments to what is essentially a non-equilibrium system. The existence of a persistent hysteresis over the entire range of pressures means literally that the system, though reproducible, is not in true equilibrium. It is further doubtful that any real arguments can be made to show that either branch of the isotherm is of any greater thermodynamic significance and it is our belief that thermodynamic quantities calculated for such systems cannot be simply interpreted.[14]

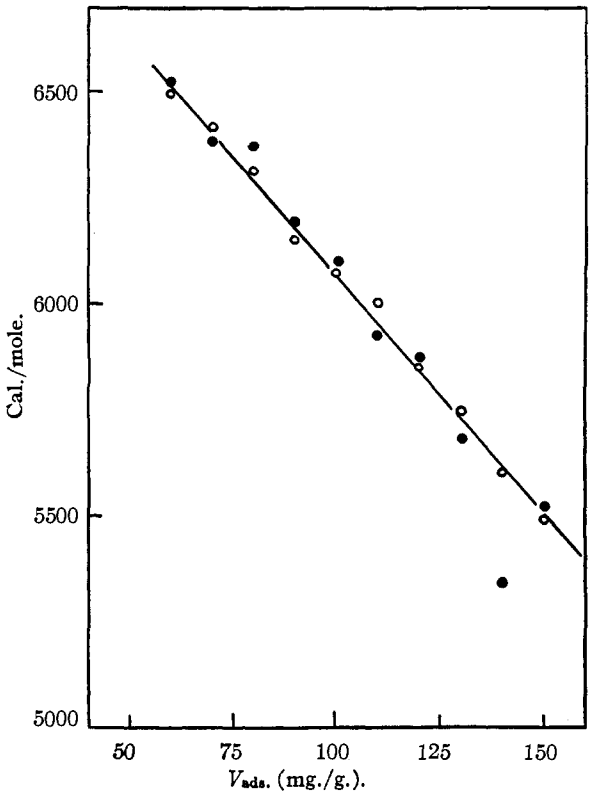

Fig. 2.—Heat of sorption of water vapor bovine serum albumin, 20–25°.

In conclusion we might inquire into the significance of the hysteresis curve and the apparent correlation with free basic groups. We should like to propose that the sorption process is initially a surface adsorption of water, followed (at even low relative pressures) by a rather fast diffusion of water molecules into the interior of the molecule where the strongest bonding sites are the free basic groups[15] in the side chains. This interval diffusion is accompanied by a progressive swelling of the

(13) In Table I we have utilized only the 15 nitrogen groups. It is of some interest to note that the polypeptide, Nylon with a few free amino acid groups shows little or no hysteresis with water, J. B. Speakman and Saville, *J. Text. Inst.*, **37**, P271 (1946); Hutton and Gartside, *ibid.*, **40**, 7170 (1949).

(14) In order to interpret such data a model would have to be constructed for the hysteresis phenomenon. Thus it has been suggested by Mr. Ryden L. Richardson that the low partial molal heats of sorption may be due to the energy absorbed in swelling the protein structure.

(15) It is of course not at all certain that we are dealing with free amino groups rather than with quaternary ammonium ions or a mixture of both; nor is it clear why the free acid groupings do not also contribute to the strong sorption. It may very well be that the acids do contribute but that as Pauling has suggested [L. Pauling, THIS JOURNAL, **67**, 555 (1945)] for the case of salmine, there is coöperative sharing of single water molecules between two close groups. It may also be that such coöperative sorption is made possible by the swelling and subsequent rotation of otherwise non-coöperating groups. This could easily be a mechanism accounting for the specificity of hysteresis and its very existence.

structure, an increase of the binding to these active centers and a concomitant weaker adsorption on to the polypeptide skeleton and finally at higher pressures a multimolecular adsorption equivalent to solvation and eventually leading to solution.

If now the swelling process is not reversible, this would account for the hysteresis and if the co-operative action of the swelling should have affected principally the binding by the free basic groups then the constancy of the hysteresis would also be accounted for. Such a hypothesis appears at present to be quite reasonable and consistent with all of the known facts on the sorption process.

LOS ANGELES, CALIF.

[DEPARTMENT OF CHEMISTRY, FACULTY OF SCIENCE, NAGOYA UNIVERSITY]

On the Heats of Formation and Potential Barriers for the Internal Rotation in Hydrocarbon Molecules

BY KAZUO ITO

RECEIVED MARCH 11, 1952

It has been assumed that intramolecular potential is expressible as the sum of interatomic bonding energies and inter-non-bonding atomic potential energies. On this assumption, it has been shown that the following facts can be explained very satisfactorily: (1) the regularity and irregularity of the heats of formation in paraffins and, in general, those in homologous series; (2) the difference between the heat of formation of isobutane and that of *n*-butane; (3) the heats of formation of cyclohexane series; (4) the heats of formation of cyclopentane series; (5) the relation between the heats of formation of decalin isomers and their molecular structures; (6) energy differences between rotational isomers in paraffin hydrocarbons; (7) the relations between the values of hindering barrier in paraffin series; and (8) the correlations between heats of formation and hindering barriers. From these results the author has concluded that both the origin of the irregularity of heats of formation and the origin of the hindering barriers in hydrocarbons are due mainly to the interactions between non-bonding atoms in a molecule.

The heats of formation of simple organic compounds can be expressed approximately by the sum of interatomic bonding energies. Although this rule is fairly extensive, Rossini and his co-workers[1] have shown that this additivity rule fails for the members below propane in paraffin hydrocarbons and, moreover, the heats of formation of iso-compounds are larger than those of the corresponding normal compounds. Rossini[2] has explained these results with the assumption that the bonding energies of C–C and C–H bonds depend upon the class of carbon atom to which these bonds are attached, but no satisfactory explanation has yet been given for the energy differences between isomers. On the other hand, many investigators have established, mainly on the basis of Raman spectrum and heat capacity measurement, the existence of rotational isomers and have estimated the energy differences and hindering barriers for their transitions. The heat of formation, energy difference between rotational isomers and the hindering barrier of transition between the corresponding isomers are all related to the intramolecular potential and should be explained on common basis although as yet no such extensive treatment has been given.

To explain these experimental results, the following assumptions were made for the intramolecular potential: intramolecular potential can be expressed by the sum of interatomic bonding energies and inter-non-bonding atomic potential energies. The interatomic bonding energy is constant regardless of the class of carbon atom. For example the heat of formation of methane, (C_1), is expressed by the sum of the six H–H interaction terms $6J_{H-H}$ and the four C–H bonding energy terms $4Q_{C-H}$

$$(C_1) = 4Q_{C-H} + 6J_{H-H}$$

In Table I the analogous equations for the heats of formation are given for the members of paraffin series, where each radical in a molecule is postulated to have the staggered configuration. Q is the bonding energy and J is the interaction of the non-bonding atoms attached to the same carbon atom. The interaction between the atoms attached to a given carbon atom and the atom attached to the nth carbon atom from the given carbon atom along the carbon skeleton in *trans* form is designated as ngg, ngg', ntt, ngt and ntg where g, g′ and t specifies the position of an atom relative to the plane of carbon skeleton in *trans* form. g means the position of an atom above this plane, g′ the position below it, and t the position on it. For examples, 3 ggHH means in propane the interaction between the two nearest H's belonging to another methyl radical with each other and 4 ttHH in *n*-butane means (assumed as a straight form) the interaction between the farthest hydrogen atoms attached to another terminal methyl radical with each other (in Table I this term is neglected). The term 2 gg CH_3–CH_3(HH) in 2-methylbutane means the H–H interaction between two methyl groups in 2 gg position (gauche position in ethane) and contains nine terms in all. Members higher than C_4 have more terms than those contained in Table I but all interactions across greater distances are neglected. Since a real molecule has not always the tetrahedral bond angles, the interactions between the same relative positions in ideal molecules used in Table I, bond angles of which are all the tetrahedral angles, are not always the same to one another in real molecules. Hence, in order to make the expression in Table I valid, the molecules should be corrected to the ideal molecules, which are constructed with the tetrahedral bond angles. As for the energy necessary to correct a real molecule to an ideal molecule, it was assumed that it has a proper value for $-CH_3$, $-CH_2$ and $-CH$

(1) F. D. Rossini, *Chem. Revs.*, **27**, 1 (1940).
(2) F. D. Rossini, *J. Research Natl. Bur. Standards*, **13**, 21 (1934).