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The Surface Areas of Proteins. VI. Vapor Phase Titrations with Polar Gases[1]

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The irreversible sorption of HCl, BF_3, CH_3NH_2 and NH_3 on a series of representative proteins has been investigated with the intention of determining the chemical reactivity of the protein as a function of the aforementioned gases. Methods have been developed which distinguish between "free" basic and "free" amino groups and which show promise of being able to elucidate the internal structural features of many proteins and indeed of other large organic molecules. It has been found possible to titrate the free amino groups by two independent methods and the free basic groups by another, the agreement of the free amino groups with chemical analysis being marked. The whole procedure is carried out in a vacuum system and at no time do the proteins come into contact with anything except the reacting gas, water vapor and all solvents being excluded from the process. It is proposed that the irreversible sorption of polar gases is merely the end point of the more general hysteresis phenomenon and that hysteresis itself can be considered as being intimately tied up with the chemical groupings within a protein.[12]

Introduction

As has been discussed in a previous article,[2] the study of the reactions of solid dry proteins with gaseous reagents offers an attractive method of investigation since the results are not complicated by the role played by solvent interactions. Basic work on this subject, excluding water vapor sorption, was undertaken in the thirties[3-9] after which little was completed in the field of gaseous titrations until the last few years.[1,10,11]

The over-all picture of polar gas sorption on proteins as determined by the aforementioned researchers showed the following general characteristics: (1) Large heat evolution, (2) "Flats" on HCl, BF_3 and CH_3NH_2 adsorption curves which indicate compound formation. (3) Relatively large amounts of gas sorbed compared to physical adsorption at similar relative pressures. (4) Independence of amount of sorbed gas on the surface area (as measured by a B.E.T. nitrogen isotherm). (5) Large hysteresis (current hypothesis relates this hysteresis to chemical reactivity of amino groups within the protein[12]). (6) Irreversible sorption of HCl, BF_3 and CH_3NH_2 which indicates strong chemical bonding. Thus it can be seen that sorption of polar gases differs considerably from the typical physical mode of adsorption and in fact exhibits behavior consistent with a chemical method of sorption, or chemisorption. As a result of recent work in which a definite amount of gas (HCl and BF_3) remained "irreversibly" bound (at pressures of 10^{-5} mm. and room temperature) to the protein, it was surmised that an investigation into the amount of various gases sorbed permanently upon a variety of proteins would yield useful information relating to the chemical reactivity and internal structure of the proteins. That this was indeed true will be indicated by the following experimental results.

Experimental Procedure

The proteins utilized were egg albumin (E.A.), bovine serum albumin fraction V (B.S.A.V.), bovine plasma albumin (B.P.A.), edestin (Ed.), casein (Cas.), lactalbumin (Lact.), fibrin (Fib.), silk fibroin (S.F.), and nylon (N) which were obtained from Armour and Co., Delta Chemical Co., and du Pont. Further purification procedures were not employed. The water soluble proteins (E.A., B.S.A.V., B.P.A. and Cas.) were spray frozen[2] into liquid nitrogen to yield a highly porous mass which facilitated the speed of sorption. Ed., Lact. and Fib. were allowed to swell in water for over a day and were then poured into liquid nitrogen and vacuum dried. This procedure increased the porosity appreciably.

The purification procedures for the HCl, BF_3, CH_3NH_2 and NH_3 are discussed in a preceding publication.[2]

The experimental setup is depicted in Fig. 1. As can be seen, runs may be made with six different proteins with the same gas. Each experiment entails determining the weight of vacuum-dried (10^{-5} mm.) protein, allowing the polar gas to come into equilibrium at room temperature with the proteins (48 hr.), and recording the final pressure in the line. The gas is then evacuated at 10^{-6} mm. over a liquid nitrogen trap until the samples come to constant weight (1-2 weeks depending upon the protein). Constant weight is assumed to have been obtained when the rate of loss of gas is less than 0.03 mmole/g./day. The difference between the final weight and the weight of dry protein is the amount of "irreversibly" bound gas.[13]

Discussion of Results

I. Dependence of Irreversible Binding upon the Final Pressure of the Polar Gas.—One of the general characteristics of the sorption of polar gases upon proteins is the phenomenon of hysteresis. A typical curve depicting the sorption of HCl upon E.A. at room temperature (Fig. 2) shows how the final pressure of the adsorption process may affect the value found for irreversible binding of HCl. It has been found that the amount of bound HCl is

(1) Part of the present work was reported on at the 26th Annual Symposium of the Division of Colloid Chemistry (Los Angeles, June, 1950) and the remainder has been reported at the Spring Meeting of the American Chemical Society, Los Angeles, 1952.

(2) S. W. Benson and J. M. Seehof, THIS JOURNAL, **73**, 5053 (1951).

(3) W. D. Bancroft and C. E. Barnett, *J. Phys. Chem.*, **34**, 449, 753, 1217, 1930, 2433 (1920).

(4) W. D. Bancroft and C. E. Barnett, *Proc. Natl. Acad. Sci.*, **16**, 118, 135 (1930).

(5) B. Belden, *J. Phys. Chem.*, **35**, 2164 (1931).

(6) J. Beek, *Bur. Stand. J. Res.*, **8**, 549 (1932).

(7) E. Czarnetzky and C. Schmidt, *J. Biol. Chem.*, **105**, 301 (1934).

(8) L. Tsai and C. Hsiao, *J. Chinese Chem. Soc.*, **2**, 87 (1934).

(9) L. Parks and A. Melaven, *J. Phys. Chem.*, **40**, 471 (1936); **41**, 1101 (1937).

(10) (a) R. W. Green, *Trans. Proc. Roy. Soc. New Zealand*, **78**, 291 (1950); (b) **79**, 494 (1952).

(11) P. A. Marshall and W. J. Moore, THIS JOURNAL, **74**, 4779 (1952).

(12) J. M. Seehof, B. Keilin and S. W. Benson, *ibid.*, **75**, 2427 (1953).

(13) It is clear from this definition that the "irreversibly" bound gas is somewhat arbitrarily defined and it is conceivable that a lower rate of loss of gas or a faster pumping system might lead to different results. It is however the experience of the authors that this would not be the case for most of the albumins (where tests were made), unless the figure of 0.03 mmole/g. protein/day were changed by at at least an order of magnitude. Thus in some of the early work on egg albumin (ref. 2), it was found that, once this figure was reached, no further changes in weight took place over a period of a week. This figure was then arbitrarily decided upon since it is the limit of precision of our weighing techniques.

TABLE I

Data in mmoles gas/g. dry protein; experimental uncertainty ±0.05 mm./g.

		E.A.	E.A. coag.	B.S.A.V.	B.P.A.	Ed.	Cas.	Lact.	Fib.	S.F.	Nylon
1	Arg.	0.34		0.33		0.96	0.24	0.22	0.48	0.04	0.00
2	Hist.	0.16		0.24		0.19	0.20	0.14	0.20	.01	0.00
3	Lys.	0.44		0.79		0.16	0.56	0.69	0.66	.02	0.00
4	1 + 2 + 3	0.94		1.36		1.31	1.00	1.05	1.34	.07	0.00
5	HCl	0.95	1.05	1.35	1.32	1.32		1.10	1.41		
6	BF_3(5)	4.22		4.78			3.77		0.1[a]	.2	5.23
7	$NH_3(BF_3)$	3.31		3.48			2.80		0.2	.2	5.27
8	6–7	0.91		1.30			0.97		−0.1	.0	−0.04
9	BF_3(2)	4.12	3.40	4.21			1.88			.1	2.61
10	$BF_3(NH_3)$	4.97			4.85	2.69	2.56	1.57	1.76		
11	NH_3	0.1			0.2	0.1	0.1	0.2	0.1		
12	CH_3NH_2	0.35		0.40		0.45	0.74	0.74	0.40		

[a] Sample not pre-swollen with water.

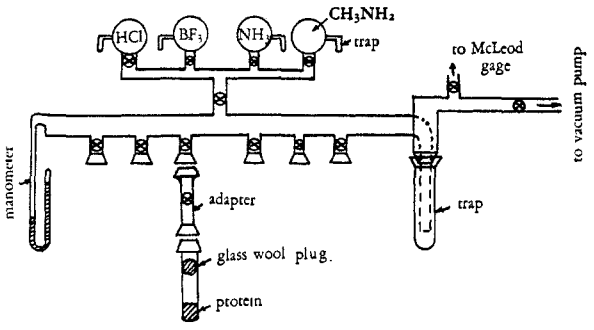

Fig. 1.

independent of pressure over a value of 2 cm. and is equal to 0.95 ± 0.05 mole/g. protein. It is quite obvious that if the adsorption curve is followed only up till a value at point 1 that even a horizontal desorption curve would yield a value lower than the true, pressure independent one. Therefore, the lowest possible pressure which would yield correct results would be at point 2 and even this value would be low since the desorption curves are concave downwards. In order to achieve useful results it is necessary to find experimentally the critical pressure for the desorption process; this being a function of the shapes of both the adsorption and the desorption curves and therefore of both the protein and the gas being utilized. Thus

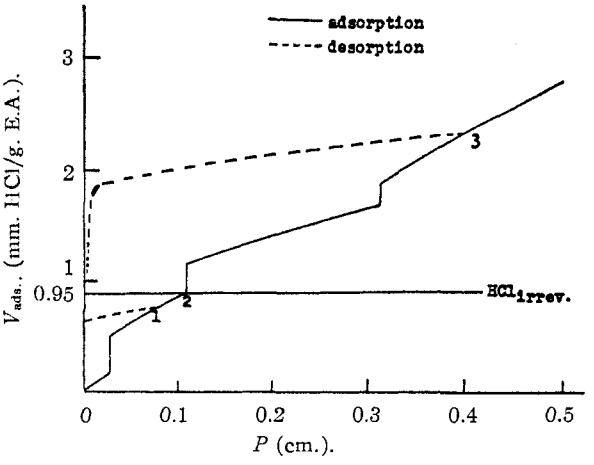

Fig. 2.

for nylon, a typical polypeptide, BF_3 at a final pressure of 2 cm. gave a value for irreversibly bound gas of 2.61 mm./g. and a final pressure of 5 cm. yielded 5.23 mm./g. (see Table I).

II. Irreversible Sorption of HCl: (P_f, 4 cm.).—The weight of HCl irreversibly bound agrees within 10% with the sum of the arginine, histidine and lysine analyses for the proteins except in the case of fibrin which is 15% too low. This may be explained on the basis of the pressure dependence for this protein which has not as yet been tested in this manner. It is also plausible that the fibrin, being a very close-packed structure, does not allow the HCl to penetrate to all of its basic amino groups. Thus, this method allows one to titrate the "free" amino groups of diamino acids arginine, histidine, and lysine and indeed permits a simple method of analysis for these acids in proteins. Good correlations have been obtained for E.A., B.S.A.V., B.P.A., Ed., Lact., Fib. It is of interest to note that the titration of coagulated (boiling one hour in water) egg albumin yields an only slightly higher value for titratable free amino groups, 1.05 ± 0.05 mm./g., thus indicating that such denaturation of egg albumin does not block the reactivity of its free amino groups and does not break many peptide linkages.

III. Irreversible Sorption of BF_3, $NH_3(BF_3)$ and $BF_3(NH_3)$.—The symbol $NH_3(BF_3)$ indicates a NH_3 run made after a complete BF_3 run or the reaction between ammonia and the protein·BF_3 complex. As can be seen from the data (Table I), the pressure dependence for the BF_3 is well demonstrated as in all cases except egg albumin there is a definite increase in the amount of irreversibly bound material, with increase in the total adsorbent pressure (as much as 100%, when the final pressure varies from 2 to 5 cm.).

Since BF_3 is an extremely strong Lewis acid it seems probable that not only could it react with free amino groups but also OH, N, S and peptide bonds; indeed any of the variety of weaker bases in the protein molecule. Because of the large amount of gas bound to nylon (Table I) it seems quite definite that BF_3 is able to titrate peptide bonds since these are the only probable reactive groups in nylon.[14] When an ammonia run was made following the BF_3 run, the amount bound was

(14) The mole ratio of end groups to peptide groups was negligible.

exactly equal to the BF_3 originally tied up indicating that the NH_3 is a stronger base than the peptide group and is able to form a complex with the BF_3 sorbed by the nylon. Thus it appears that the amount of BF_3 in a protein bound to peptide or other basic groups weaker than ammonia should be titratable by subsequent NH_3 addition. Amines being stronger bases than ammonia, it is improbable that these groups would be picked up upon NH_3 addition. In the cases of E.A., B.S.A.V. and Cas. the difference between the BF_3 and the subsequent NH_3 bound was equal to the number of free amino groups in the protein. Thus the ammonia titration following a BF_3 run may be considered to yield the amount of "free" peptide groups plus any other BF_3-base compounds in which the base is weaker than ammonia.[15] The absolute values of the aforementioned quantities have meaning only when the critical pressure for irreversible sorption of BF_3 has been exceeded. Since the difference between the two values is a function of the pressure dependence of the free amino groups and the BF_3, it is apparent that this limit has been passed at 5 cm. and the remaining pressure dependence is in all probability due to peptide and other basic groups. Therefore, another method of titrating free amino groups is available and also, when the critical pressure is exceeded, a method of titration for the sum of the basic groups in the protein. Since some of these groups may be blocked by intramolecular bonding, the exact interpretation of these values is as yet uncertain.

Unlike the HCl titration, the coagulated egg albumin gave a value for BF_3 which was 0.72 mm./g. less than that for the undenatured protein. This indicates that coagulation leads to a removal of electron donor groups and it is likely that in coagulation these basic groups enter into strong intramolecular bonds.

An ammonia titration in all cases left very little irreversibly bound material (approx. 0.1 mm./g.) but a subsequent BF_3 titration yielded values approximately 0.8 mm./g. higher than on the native proteins thus indicating that the NH_3 had broken up to some extent the internal intramolecular bonding of each protein.[16]

IV. Irreversible Sorption of NH_3 and CH_3NH_2. —The values for bound ammonia are uniformly very small and yield no useful information except that there apparently are very few strongly acid groups free in the protein molecule.

Methylamine being a stronger base than ammonia titrates acids in the protein to the extent of 0.3–0.7 mm./g. but as yet no significant correlation between these values and structure has been determined.[17]

Conclusion

While it may be premature to offer a coherent picture of polar gas sorption, the sum total of evidence now available indicates that there are at least three mechanisms operative. The first and perhaps fastest[10] is the interaction of the polar gases with accessible conjugate polar groups, such as may be exposed at the "surface" of the protein molecule. The second process is a slow diffusion of the molecule sorbed at the surface into the protein molecule accompanied by a third process, swelling of the molecule.[10,12] This swelling must involve a breaking or loosening of intramolecular bonds between segments of the molecule, the activation energy for which must be in part provided by the energy of attraction of the diffusing molecule. The extent of hysteresis or of the irreversibility is best explained as being a coöperative process among the adsorbed gas molecules and the swelling of the protein, higher pressures of gas essentially "pushing" already adsorbed molecules into sites within the molecule, from which on the desorption process they appear to be difficult to extract. If the sorbed molecules are sufficiently active (HCl and BF_3) the extraction process cannot be completed even at pressures of 10^{-6} mm. and the amount of gas remaining is termed "irreversibly bound material." With less active sorbates (NH_3 and H_2O) the process of sorption is reversible and a complete hysteresis loop may be traversed, this being true only when the lowest pressure is of the order of 10^{-6} mm.[18]

Thus since the irreversibility is merely the difference between the adsorption and desorption curves at the lowest pressure obtainable (that corresponding to chemical compounds), *i.e.*, the extent of hysteresis, it is evident that hysteresis is at least to some extent a function of the reactivity of groups within the protein molecule.[12]

The swelling itself seems to be in some measure reversible. For water the surface areas before and after sorption are essentially the same[12] while for NH_3 as discussed before, subsequent BF_3 addition indicates a disruption of the internal structure. Water sorption shows the same type of effect, as the process of spray-freezing opens the internal structure to the reactive gases where non-spray-frozen material may react slowly or not at all.

The data obtained with nylons show that the peptide group can act as a strong enough base to bind irreversibly BF_3 and possibly HCl, if not hindered either by steric factors (packing in structure) or more likely H-bonding with adjacent groups.[19] If we can apply these results to the proteins, we would then conclude that the peptide groups in the protein molecule are less available for reaction than those in nylon (H). The data obtained with heat denatured egg albumin and HCl would then indicate that in denaturation not more than 0.1 ± 0.05 mmole of peptide links 1 g. protein are broken since these would be the only source of additional basic groups.[20]

(15) There is no direct evidence as yet of peptide attack in proteins as the sum of the other electron donor groups in all cases exceeds the $NH_3(BF_3)$ titration.

(16) This process may be equivalent to the swelling which is observed upon water sorption (ref. 12).

(17) It should be noted, however, that these values never exceed the total number of radical acid groups in the protein.

(18) If a vacuum system which had a minimum pressure of 0.1 cm. were utilized one could truthfully say that irreversible adsorption was in evidence, the magnitude of which would be the difference between the sorption and desorption curves at the pressure of 0.1 cm.

(19) These two effects would be extremely difficult to distinguish experimentally.

(20) This would imply that the protein peptide bonds are tied up to a greater extent either sterically or through H-bonds. It should soon be possible, through extensions of the present work, to tell precisely what fraction of the peptide bonds in the structure are so bound.

Work is continuing on the investigation of these phenomena in these laboratories, in particular on the swelling process and the role played by protein configuration in determining the sorption behavior. It begins to appear more and more evident that the technique of gas phase interactions may be expected to yield valuable data on the structure and properties of the native protein molecule.

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Some Optical Properties of Molten Silver Bromide–Alkali Bromide Solutions

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The refractive indices of molten binary solutions of silver bromide with lithium bromide and rubidium bromide have been determined as a function of temperature and composition. The molar refractivities of these solutions were found to be an additive function of the mole fraction. The temperature dependence of the absorption edge of pure liquid and solid silver bromide has been determined as well as the temperature dependence of the absorption edge of pure molten rubidium bromide and that of an equal molar mixture of silver bromide and rubidium bromide. The concentration dependence of the absorption edge of molten binary solutions of silver bromide with sodium bromide and rubidium bromide also has been determined. No evidence is found for the existence of "complex ions" in these solutions.

Introduction

In 1932 Hildebrand and Salstrom[2] published a paper which summarized and discussed a previous series of papers on the thermodynamic properties of binary liquid solutions of silver bromide with the bromides of lithium, sodium, potassium and rubidium. The data had been obtained by measuring the e.m.f. of cells which were without transference, consisting of silver and bromine electrodes dipping into fused silver bromide diluted with varying amounts of several alkali bromides. Measurements were made at three temperatures, 500, 550 and 600°, permitting calculation not only of the free energy of dilution but also the enthalpy and entropy, with their dependence upon the mole fraction of alkali bromide. The character of the results is indicated in Fig. 1 where $RT \ln \gamma_1$ is plotted against the square of the mole fraction of alkali bromide, x_2. Here γ_1 is the activity coefficient of silver bromide, *i.e.*, its activity referred to pure liquid, a_1, divided by its mole fraction, x_1.

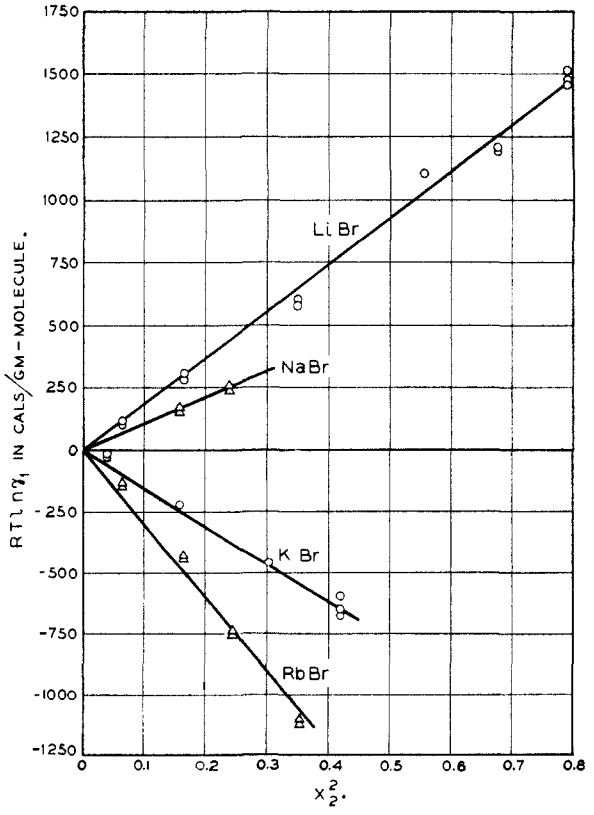

Fig. 1.—The departures from ideality of silver bromide in various molten alkali bromides.

Three highly significant facts are to be noted: first, the lines are all straight, showing that each can be expressed by a simple equation, $\bar{F}_1 - F_1^i = kx_2^2$; second, the points for the widely different temperatures all fall on the same line, whence

$$\left[\frac{\partial(\bar{F}_1 - \bar{F}_1^i)}{\partial T}\right]_x = -(\bar{S}_1 - \bar{S}_1^i) = 0$$

i.e., the entropy is ideal and temperature independent and the heat of dilution is $\bar{H}_1 - \bar{H}_1^i = kx_2^2$; and, third, the deviation from the ideal varies from strongly positive when diluted with LiBr, less positive with NaBr, negative with KBr and strongly negative with RbBr.

Now positive deviations do not surprise us, for that is characteristic of "regular" solutions, where it results from unequal attractive forces, without involving any changes in molecular species. Negative deviations, however, are quite another matter. It requires far greater differences in molar volume than are found in AgBr–RbBr solutions to account for the large deviation in that case by a Flory–Huggins type of entropy. Large negative deviations do accompany the evolution of heat resulting from solvation, but in that case there is ordinarily a diminution in the number of independent molecules, and less than ideal entropy of dilution. Thus,

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(2) J. H. Hildebrand and E. J. Salstrom, THIS JOURNAL, **54**, 4257 (1932).