stant since both have approximately the same distance between the chlorine and nitrogen. The 5-, 6- and 7-chloroquinolines are the strongest bases in the series for in these the chlorine has the least effect upon the electron density at the nitrogen. The 2-chloroquinoline is such a weak base that a constant could not be determined.

Experimental

Absorption Spectra.—The spectra were determined as was reported in the previous paper[3] using a Beckman Model DU quartz spectrophotometer. The concentrations were approximately 0.0002 molar.

Dissociation Constants.—All *p*H measurements were made with a Leeds and Northrup *p*H indicator, Model No. 7664 with glass and saturated calomel electrodes. All measurements were made in a constant temperature room at 25°.

Materials.—The 4-, 6-, 7- and 8-methylquinolines and the 2- and 6-chloroquinolines were Eastman Kodak Co. products and were redistilled before use. The 2- and 3-methylquinolines and 3-, 4-, 5-, 7- and 8-chloroquinolines were prepared in this Laboratory.

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Further Studies of the Interactions of Polar Gases with Solid Proteins and Some Simple Organic Compounds[1-3]

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Studies of the stoichiometric binding of HCl, NH_3, BF_3 and CH_3NH_2 by solid proteins have been extended to a varied series of proteins and concomitantly to some model organic compounds for purposes of comparison. As reported earlier HCl is bound to arginine, histidine and lysine. NH_3 is hardly bound at all while CH_3NH_2 binds less than the expected number of free acid groups. BF_3 can add to even weak Lewis bases and its binding is strongly pressure dependent. In all cases but especially with BF_3, there is a very pronounced effect of particle dispersity indicating diffusion controlled reactions. Strong and weak basic groups can be distinguished in all cases by techniques of back titration. The evidence points to a Zwitterion structure for the residues such that the strong bases are present as $RCOO^{-1}$, the corresponding acids being RNH_3^{+1}. A smaller base strength of the peptide group is found in proteins compared to that in Nylon and is compatible with the α-helix structure for proteins.

Introduction

In earlier studies reported from these laboratories[4-7] it has been shown that the interactions of acidic and basic gases with solid proteins showed a stoichiometric character which could be interpreted in terms of a chemical reaction of these gases with the basic and acidic residue groups in the protein molecule leading to compound formation. Because these reactions are complex and are accompanied by a considerable amount of physical sorption it was decided to extend the work to a large number of proteins of differing structure to gain some information on the important structural features of the interactions. For these purposes the proteins chosen were egg albumin, bovine serum albumin, casein, fibrin, edestin, gliadin, gelatin, lactalbumin, β-lactoglobin, zinc insulin, silk fibroin and pepsin. To aid in the interpretation of the results it was further decided to make a parallel series of studies on some simple organic solids. Glycine, glycyl-glycine, Nylon, histidine, sebacic acid and cetyl alcohol were the model substances chosen for this comparison. The gases used were those already studied in varying detail earlier, HCl, NH_3, CH_3NH_2 and BF_3. Finally because of the related interest in denaturation, heat denatured (coagulated) samples of egg albumin and bovine serum albumin were also included in the studies.

As will be evident from the following discussion, the results are in many cases incomplete and in some cases appear to present conflicting or at least ambiguous features. The results with BF_3 are of a preliminary nature and further work (some of which is now in progress in these laboratories) will be required before a coherent picture of the behavior of this gas with proteins can be obtained. Despite these shortcomings the results are extremely interesting in their implications for the chemical reactivity of the residue groups in solid proteins and indicate a considerably greater coherence of properties than had earlier been supposed.

Experimental Procedure

The details of the preparation of the proteins and gases have been given in earlier papers and will not be repeated here.[8] Similarly the apparatus and techniques of measurement have been described elsewhere.

Except where otherwise noted the temperatures for all the studies were ambient room temperatures (20–26°) it having been shown earlier that the results were not sensitive to temperature changes of this magnitude.

The materials used in the study were either crystalline (cryst.) spray-frozen (SF) or in the case of a number of insoluble substances, water swollen (Sw) and then frozen at Dry Ice or liquid nitrogen temperatures and vacuum dried.

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(3) Some of the material in this paper was presented at the Spring Meeting of the American Chemical Society held in Los Angeles, April 1953.

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(8) The insulin used in the present work was very generously provided by Eli Lilly and Company, E. R. Squibb and Sons and Sharpe and Dohme to all of whom we are much indebted.

TABLE I[a]

		Egg albumin			Bovine serum albumin		
		SF	Cryst.	Coag.	SF	Cryst.	Coag.
(1)	-amino (A + H + L)	0.94	0.94	0.94	1.36	1.36	1.36
(2)	HCl	0.95	0.97	1.05	1.35	..	..
(3)	NH_3	0.1	..	0.2	..	..	..
(4)	NH_3(HCl)	1.21	0.59	0.27	0.56	..	..
(5)	HCl(NH_3)(HCl)	0.99	..	0.43	0.57	..	..
	(4)-(2)	0.26	−0.38	−0.78	−0.79	..	..
	(5)-(4)	−0.22	..	0.16	0.01	..	..
(6)	CH_3NH_2	0.35	..	..	0.40	..	..
(7)	BF_3 (2 cm.)	4.12	..	3.40	4.21	..	..
(8)	BF_3 (3.4 cm.)	..	3.21	..	..	1.44	5.21
(9)	BF_3 (5 cm.)	4.22	..	..	4.78	..	..
(10)	BF_3 (10 cm.)	..	6.28	..	..	3.55	..
(11)	$BF_3(NH_3)$	4.97	..	..	4.87	..	..
(12)	$NH_3(BF_3; 3.4)$	..	2.57	..	..	1.02	..
(13)	$NH_3(BF_3; 5)$	3.31	..	..	3.48	..	..
(14)	$NH_3(BF_3; 10)$	..	5.36	..	..	2.89	..
	(8)-(12)	..	0.64	..	..	0.42	..
	(9)-(13)	0.91	..	..	1.30	..	..
	(10)-(14)	..	0.92	..	..	0.66	..

[a] The data reported in all the tables are in units of millimoles of sorbent per gram of protein or other sorbate.

TABLE II

		Casein		Fibrin				
		SF	Cryst.	SW	Cryst.	Edestin	Gliadin	Gelatin
(1)	-amino (A + H + L)	1.10	1.10	1.34	1.34	1.31	0.32	0.67
(2)	HCl	1.35	1.54	1.34	..	1.32	0.44	0.67
(3)	NH_3	0.1	..	0.1	..	0.1	..	..
(4)	NH_3(HCl)	0.75	1.04	0.75	..	..	0.51	0.60
(5)	HCl(NH_3)(HCl)	0.82	..	0.73	..	..	0.43	0.87
	(4)-(2)	− .60	− .50	− .59	..	..	0.07	−0.07
	(5)-(4)	0.07	..	−0.02	..	..	−0.08	0.27
(6)	CH_3NH_2	0.74	..	0.35	..	0.45	0.34	0.00
(6′)	HCl(CH_3NH_2)	..	..	1.55	..	..	0.73	1.28
	(6′)-(6)	..	..	1.20	..	..	0.39	1.28
(7)	BF_3 (2 cm.)	1.88	..	..	..	..	..	..
(8)	BF_3 (3.4 cm.)	..	0.64	3.14	..	..	4.30	4.21
(9)	BF_3 (5 cm.)	3.77	..	..	0.1	..	..	..
(10)	BF_3 (10 cm.)	..	2.12	..	..	..	..	..
(11)	$BF_3(NH_3)$	2.56	..	1.76	..	2.69	..	..
(12)	$NH_3(BF_3; 3.4)$	..	0.71	2.32	..	..	3.98	3.52
(13)	$NH_3(BF_3; 5)$	2.80	..	..	0.2	..	..	..
(14)	$NH_3(BF_3; 10)$	..	1.79	..	..	..	..	..
BF_3-$NH_3(BF_3)$	(8)-(12)	..	− .07	0.82	..	..	0.32	0.69
	(9)-(13)	0.97	..	..	−0.1	..	..	..
	(10)-(14)	..	0.33	..	..	..	..	..

the electrophoresis results which are described later. We are also grateful for the sample of gelatin they generously provided.[9]

The reproducibility and precision of the HCl results are about ±0.05 mmole HCl/g. protein and that for NH_3 is ±0.1 mmole NH_3/g. protein. The precision of the BF_3 and CH_3NH_2 results are about ±0.05 mmole/g. while the reproducibility has only been established in the case of egg albumin at about the same figure.

Results

The amounts of gas bound by vacuum dried protein solids are tabulated in Tables I, II and III. The first row in each of these columns is the sum from analytical data of the strongly basic groups, arginine, lysine and histidine (A + H + L) which have been isolated in the proteins.[6] The units used are millimoles of groups per gram of dry protein. In subsequent rows are listed the number of millimoles of polar gas irreversibly bound per gram of dry protein. Column I in each of the tables gives the polar gas used and the figures in parentheses refer to the vapor pressure at which the sorption was studied [*e.g.*, BF_3(2 cm.) means BF_3 sorption at a pressure of 2 cm. of Hg].[10]

Table IV is a similar tabulation for the simpler organic molecules. In this case the first row designation, "reactive groups" refers to the number of millimoles/gram compound of α-amino group for

(9) This is a special sample used in the work of the Blood Plasma Substitute Program at the California Institute of Technology and has been carefully characterized and analyzed. Its analysis differs from that of most gelatins (Table II).

(10) The abbreviation NH_3(HCl) represents binding of NH_3 following a run with HCl. HCl(NH_3)(HCl) represents binding of HCl after binding of NH_3 after initial binding of HCl.

TABLE III

		Lactalbumin	β-Lactoglob.	Zn-Insulin	Silk fibroin	Pepsin cryst.
(1)	·amino (A + H + L)	1.05	1.14	1.05	0.07	0.18
(2)	HCl	1.10	1.22	1.35	.40	..
(3)	NH_3	.02	..	..	..	..
(4)	NH_3(HCl)	..	0.64	0.96	0.61	..
(5)	HCl(NH_3)(HCl)	..	0.74	0.87	0.66	..
	(4)–(2)	..	−0.58	− .39	.21	..
	(5)–(4)	..	0.1	− .09	.05	..
(6)	CH_3NH_2	0.74	0.46	0.75	0.20	..
(6′)	HCl(CH_3NH_2)	..	1.70	1.94	1.18	..
	(6′)–(6)	..	1.24	1.19	0.98	..
(7)	BF_3 (2 cm.)	..	..	..	0.1	..
(8)	BF_3 (3.4 cm.)	..	4.12	0.97	0.1	0.37
(9)	BF_3 (5 cm.)	..	..	..	0.2	..
(11)	BF_3(NH_3)	1.57	..	..	0.55[a]	..
(12)	NH_3(BF_3)	..	2.88	0.95	0.2[b]	0.70
	BF_3–NH_3(BF_3)	..	1.24	.02	0	−0.33

[a] This was BF_3 after NH_3(HCl). [b] Same value at 3.4 and 5 cm. of BF_3.

glycine and glycyl-glycine, imidazole for histidine, carboxyl group for sebacic acid, peptide groups for Nylon and OH group for cetyl alcohol.

weight for physical adsorption on highly dispersed proteins. (b) Large amounts of heat evolution estimated to be in the neighborhood of from 6–20 kcal./mole of gas sorbed and resulting in sudden increases in temperature on sorption (as much as 12° for H_2O and 40° for HCl have been observed).[4] (c) Very slow attainment of equilibrium (of the order of hours for H_2O and weeks for HCl). Generally about 80–90% of the gas is sorbed in the first 2 hours, another 5–15% in the next 10 hours and the rest in 1–4 weeks. (d) A considerable hysteresis in the desorption isotherm amounting to some 10–50% of the sorbed material. (e) Swelling of the protein on a macroscopic scale at large amounts of sorption (*i.e.*—in excess of 20% by weight). (f) The formation of protein–gas complexes of reproducible composition with NH_3, HCl and BF_3.[11] No information is available concerning permanently bound water.[12] These complexes are formed very slowly, the rate depending on temperature, partial pressure of sorbed gas and state of dispersion of the protein solid. The reaction thus has all the characteristics of a real chemical process.

TABLE IV

		Glycine cryst.	Glycyl-glycine (SF)	Nylon Fibrous	Nylon Amorphous	Histidine (SF)	Sebacic acid (Sw)	Cetyl alcohol (Sw)
(1)	"Reactive" groups[a]	13.5	7.59	8.85	8.85	6.44	10.00	4.13
(2)	HCl	0.10	4.79	2.73	0.18	5.95	...	..
(4)	NH_3(HCl)	..	6.31	2.76	0.12	4.95	...	..
(5)	HCl(NH_3)(HCl)	..	2.12	..	..	..	...	..
	(4)–(2)	..	1.52	0.03	−0.06	−1.00	...	..
	(5)–(4)	..	−4.19	..	..	..	...	..
(6)	CH_3NH_2	..	0.09	..	..	..	...	..
(6″)	CH_3NH_2(HCl)	..	4.14	..	..	..	...	..
	(6″)–(6)	..	4.05	..	..	..	...	..
(7)	BF_3 (2 cm.)	..	..	2.61	..	..	...	..
(8)	BF_3 (3.4 cm.)	..	0.28	..	..	..	4.75	1.57
(9)	BF_3 (5 cm.)	..	..	5.23	..	..	...	..
(9′)	BF_3 (8.7 cm.)	0.05	..	..	7.74[b]	2.16	5.24	1.26
(10)	BF_3 (10 cm.)	..	..	..	..	4.84	7.35	2.00
(12)	NH_3(BF_3)[c]	..	0.39	5.27(5)	..	2.04(10)	1.07(10)	1.71(10)
	BF_3–NH_3(BF_3)	..	−0.11	−0.04	..	2.80	6.28	0.29

[a] These are explained in text and units are mmoles/gram. [b] This is after NH_3(HCl) which is low [see line (4)]. [c] Values in parentheses refer to BF_3 pressure.

General Discussion

It is well, before launching into a discussion of the results obtained, to outline some of the general features of the sorption process which have become apparent from the work done in this and other laboratories. We distinguish sorption which is of special concern in the present work from physical adsorption. The latter is a property of the exposed surface of a solid, while the former is a bulk property. The polar gases which we have studied have been shown to sorb internally rather than adsorb and so the process is closely akin to solid solution or possibly chemical reaction.

In the sorption of polar gases on proteins the isotherms are characterized by: (a) Rather large amounts of sorption at relatively low partial pressures (*e.g.*, in the neighborhood of 5–50% by weight at partial pressures of from 0.01 to 0.3). This is to be compared with values of 0.01–0.2% by

We see from this description that the sorption process is considerably complicated and we shall have to exercise caution in the interpretation of our results. In particular the irreversible though reproducible nature of polar gas sorption leads us to the view that the process must be looked upon as the fairly strong interaction of a *deformable* solid with a gas reagent, equivalent to solvation for the desorbable gas and chemical reaction for the permanently bound gas.[13]

In interpreting our results we shall have to concern ourselves with a number of effects which tend

(11) The reproducibility depends on the method of measurement as will appear from the later discussion.

(12) Some crude measurements on the exchange of "dry" protein with D_2O done in these laboratories however would indicate that there is less than 0.1% of water in our dry proteins.

(13) The formation of simple hydrates or molecular addition complexes of small molecules is closely analogous to this process. The protein systems' differences from these simpler systems seems to be quantitative rather than qualitative.

to obscure the comparability of the data. These effects can be listed as follows.

(1) **Local Heating.**—Sorption is quite exothermic and the solid–gas systems are not good heat conductors. Local heating may produce structural changes in the protein and possibly chemical reaction. In addition, since the complex formation has a temperature dependence, local heating may alter the rates as well as amounts of gas permanently bound. An independent investigation of these effects is now in process in our laboratories.

(2) **Partial Pressure.**—There seems to be a critical pressure for each temperature and each gas above which the amount of permanently bound gas is independent of pressure. Before stoichiometric significance can be ascribed to protein–gas complexes the pressure dependence of the reaction must be established.

(3) **Diffusion.**—The rate of sorption depends strongly on the state of dispersion of the protein. Anomalously low values of permanent binding may be found if the solid is not sufficiently dispersed or swollen. This is presumably due to the slow rate of diffusion into and especially, out of, the bulk solid.

(4) **Deformation.**—If the protein solid swells on sorption then it may tend to contract on desorption. Where diffusion through the solid is slow it may be anticipated that on desorption the gas will evaporate rapidly from the surface layers. If these contract, subsequent diffusion through the contracted surface layers will be even slower than usual and anomalously high values of protein complex may be found.[7] This effect of course enhances an already slow rate of desorption.[14] The net result as we shall see is to make the rate of desorption strongly dependent on particle size and to lead to very poor results for coarse materials. In general the more strongly bound the gas, the slower the rate of desorption and the more important it is to use well dispersed materials.

These points will be considered in more detail in the following discussion of our results. For convenience these have again been divided into several categories.

I. Polar Gases

A. HCl—The results on twelve of the proteins confirm our earlier[5-8] findings to the effect that the HCl binding can be accounted for in terms of titration of the basic groups in arginine, histidine and lysine. The fact that glycylglycine does bind HCl, if only to the extent of 65% of its total α-amino groups (Table IV) does show that the α-amino group is still strongly basic (or the COO^{-1} ion).

For the proteins tested, the amount of HCl bound was independent of HCl pressure above 2 cm.

Coagulated egg albumin shows an increased binding to the extent of 0.1 mmole/g. This increase may be due to slight hydrolysis occurring during the coagulation procedure of boiling in water for 15 minutes.

For a close packed structure such as nylon fibers (Table IV), an appreciable difference between adsorption and desorption rates might be expected. That this is indeed the case is indicated by the slow desorption of HCl over a period of a few weeks while the sorption process takes place in a period of days. When amorphous material is utilized, the sorption process takes place in a period of days. When amorphous material is utilized, the initial adsorption is large but the amount irreversibly bound reaches zero within two days. This diffusion effect also explains the high values found for silk fibroin and casein or is at least consistent with it.

The low values for glycylglycine and histidine are explained on the basis of incomplete penetration of the structure due to inefficiency of spray freezing.[15] In all probability microcrystals form which are penetrated by the HCl only very slowly.

Kinetic studies at various temperatures might be able to distinguish among basic groupings in the protein. If the overlapping of base strengths is not too great, breaks in the desorption curves corresponding to stoichiometric amounts of each group may occur.[16]

B. $HCl(CH_3NH_2)$.—HCl added after an initial CH_3NH_2 run would be expected to titrate the strong basic groups plus any bound CH_3NH_2 (Tables II and III, lines 6′). This is shown by the fact that $HCl(CH_3NH_2)$–CH_3NH_2 is equal to the sum of the basic groups in most of the proteins tested. Gelatin is an exception having a value double that which is to be expected. This has not been explained.

C. $NH_3(HCl)$.—NH_3 titrations yield very small values showing that in the native proteins the strong acid groups (COOH) are intramolecularly bound[17] or are in zwitterion form. After an initial HCl titration, the NH_3 values are appreciable. Since the ammonia is probably not reacting with the strongly bound HCl, some acid groups must be liberated by the reaction.

That the zwitterion form is present in the protein structure may be inferred by the following possible reaction schemes for the binding of NH_3, HCl and BF_3.

Non-zwitterion formulation

$$R{-}NH_2\ldots HOOC{-}R' \xrightarrow{HCl} RNH_3^+Cl^- + R'COOH \quad (1)$$

$$R'COOH \xrightarrow{NH_3} R'COO^-NH_4^+ \quad (2)$$

$$RNH_2\ldots HOOCR' \xrightarrow{BF_3} RNH_2\cdot BF_3 + R'COOH\cdot BF_3 \quad (3)$$

The HCl reaction is consistent with the known data but it is shown in section I-G that BF_3 bound to COOH is not backtitrated by NH_3. Thus the values of BF_3–$NH_3(BF_3)$ should be equal to the sum of arginine, histidine, lysine *and* liberated COOH group. Since this is not experimentally the case, the scheme does not seem acceptable.[18]

(14) On thermodymanic grounds we may expect the specific rate constants for desorption to be much smaller than those for sorption.

(15) The spray freezing process produces a powder which is extremely polydisperse. No attempt was made in this work to utilize monodisperse systems.

(16) Current results show related effects with BF_3.

(17) From the results of chemical analysis it would appear that most of the excess of COOH groups (excess over basic groups) are probably present as amides which would not be expected to be strong bases.

(18) A further argument against this scheme is that it would predict much higher values of NH_3 binding than are observed.

Zwitterion formulation

$$RNH_3^+ \ldots OOCR' \xrightarrow{HCl} RNH_3Cl + R'COOH \quad (1')$$

$$R'COOH \xrightarrow{NH_3} R'COONH_4 \quad (2')$$

$$RNH_3^+ \ldots OOCR' \xrightarrow{BF_3} RNH_3^+ + R'COO\cdot BF_3 \quad (3')$$

It can be seen that this mechanism is compatible with all of the experimental data assuming that ammonia does not react irreversibly with the RNH_3^+. If the NH_3 did titrate this group then all of the values of $BF_3 - NH_3(BF_3)$ would be smaller than they actually are.

The use of $NH_3(HCl)$ titrations as a structural probe is indicated by the following series from Table I.

	HCl	$NH_3(HCl)$	NH_3
E.A. (S.F.)	0.95	1.21	0.1
E.A. (not S.F.)	0.97	0.59	..
E.A. (coag.)	1.05	0.27	0.2

This would seem to indicate that the binding of NH_3 is strongly sensitive to the dispersity of the sample in contrast to HCl which can penetrate the structure much more easily.

D. $HCl(NH_3)(HCl)$.—All values were approximately equal to $NH_3(HCl)$ showing that HCl was able to back-titrate all of the NH_3 or else react with $RCOO^-NH_4^+$ to give RCOOH + $NH_4^+Cl^-$ (see zwitterion formulation). The NH_3, therefore, was bound to acids weaker than HCl. Since the strongest acid group in the protein is COOH, this is to be expected.

E. NH_3.—The values obtained were all very small (lines 3, Tables I–III). It can be seen from Section C (also lines 4, Tables I–III) above that NH_3 can titrate RCOOH groups. This would seem to imply that there are very few RCOOH groups accessible to NH_3 in the native proteins.[19] It is difficult to believe that this can be simply a matter of physical accessibility in view of the enormous physical adsorption of NH_3 by the proteins at comparable pressures and temperatures.[4] This view is given further support by the fact that CH_3NH_2, a molecule which would be expected to encounter more difficulty than NH_3 in diffusing through the protein can nevertheless be more strongly bound.

F. CH_3NH_2.—Since methylamine is a stronger base than ammonia it can, and does, bind more acid groups than the latter. No significant correlation with internal groupings has been achieved. If the zwitterion structure is accepted we would then have to assume that CH_3NH_2 is reacting with RNH_3^+[1] to liberate the weaker bases RNH_2. The analyses are not extensive enough as yet to attempt an estimate of the stoichiometry of CH_3NH_2 binding.

G. BF_3.—This gas exhibits a marked pressure dependence which has not yet been studied sufficiently to warrant detailed explanation. It may be expected to titrate all basic groups. $NH_3(BF_3)$ will back-titrate only the BF_3 bound to weak basic groups. Therefore the difference between the two values, $BF_3 - NH_3(BF_3)$, should give the strongly basic groups, arginine, histidine, and lysine. This has been proven to be the case in all proteins tested with only two exceptions.

BF_3 titrates available peptides as indicated by values for fibrous and amorphous Nylon. Irreversible binding is far greater than can be accounted for on the basis of oxygen containing groups other than peptides and therefore this reagent gives an indication of the internal bonding of the peptide groups in proteins. Comparative values of the albumins (open internal structures, and high BF_3 values) with silk fibroin (close packed structure and low BF_3 values) bear this out.

Before a quantitative statement can be made relating to the number of "free" peptide groups, the question of pressure dependence mus tbe more nearly completely resolved. However, at any one pressure relative values of this quantity may be determined. And at no time does the value for BF_3 exceed the available reactive groupings in any protein. This gas titrates OH and COOH groups (unstoichiometrically due to presence of microcrystals) as indicated by cetyl alcohol and sebacic acid values.

Back titrations with NH_3 indicate the extent of the weak basic groups. Since both cetyl alcohol and amorphous Nylon are back-titrated to completion by ammonia, both the peptide and alcoholic groups are shown to be part of the weak base of the protein. However only 15% of the BF_3 bound by the sebacic acid was back-titrated by the NH_3. The most probable explanation of this is that the ammonia was not back-titrating the BF_3 at all but was merely reacting with some available COOH groups which the BF_3 did not diffuse to. Thus, it appears that COOH should be classified as a strong base with respect to BF_3. Since the $BF_3 - NH_3(BF_3)$ values indicate the absence of COOH's in the strong basic groups, it appears that the COOH's are strongly tied up in intramolecular linkages. This is consistent with the low values for NH_3 and CH_3NH_2.

At any one pressure, BF_3 values for spray frozen materials are greater than for non-spray frozen proteins. The most striking difference is between normal fibrin (0.1) and swollen fibrin (3.14). Coagulation in the one case tested (E.A.) caused a decrease from 4.12 to 3.40. This may be due to a decrease in the number of available peptides in the structure of the coagulated material. A run made at −85° indicated that very little sorption took place at this temperature. Upon returning to room temperature, an immediate heating took place and sorption was recognizable. Thus it appears that an important process taking place is one of activated diffusion.

H. $BF_3(NH_3)$.—Initial treatment with ammonia served to raise the value of BF_3 by approximately 30% in all cases. The ammonia probably caused the protein to swell and upon desorption left more peptides in a state available for reaction with BF_3.

I. $NH_3(BF_3)$.—NH_3 should be capable of back-titrating all of the BF_3 bound to bases weaker than ammonia. That this is indeed the case is shown by the difference of BF_3 and $NH_3(BF_3)$ being equal to the strong bases of the protein.

II. Low Temperature BF_3 Run

The sorption of BF_3 on a series of proteins at −85° was found to be both slow and slight. Reac-

(19) This is compatible with the zwitterion formulation and the chemical analysis which indicates that the excess acid is tied up as amide.

tion rates were followed with the line manometer. Upon removal of the dewars in which the protein samples were immersed, instantaneous increase in adsorption rate was observed. It appears, therefore, that the diffusion of the BF_3 into the interior of the molecule is a highly activated process which probably involves the spreading apart of peptide chains within the molecule. In line with this work it was found that the HCl was also adsorbed more slowly at lower temperatures with, however, greatly increased values of equilibrium sorption. Since the HCl is a smaller molecule, rate variation is to be expected. The sorption of BF_3 at low temperature was found to be much less than at room temperature and therefore penetration into the molecule must have been severely restricted.

III. Specific Structural Information

A. Albumins (E.A., B.S.A.).—This class of proteins was found to have the most loosely knit structure. The rates of sorption and desorption were greatest of all the proteins and the amount of BF_3 bound had the largest values.

B. Coagulated Albumins (E.A., B.S.A.).—The number of strong basic groups was increased by approximately 10% and was probably caused by slight hydrolysis upon boiling. The BF_3 values were nearly the same for undenatured and coagulated materials. From this and electrophoresis data (Sect. IV) it seems that the strong basic groups do not figure very importantly in the coagulation process. A possible explanation of this phenomenon is given in section I-C and indicates that the intramolecular bonding of the COOH groups is a prime factor.

C. Globulins (Edestin, β-Lactoglobulin, Gelatin).—A more tightly knit structure than the albumins was found to be in evidence.

D. Gliadin, Casein, Lactalbumin.—The same general properties as the globulins were found.

E. Fibrin.—An extremely tightly packed structure which was impervious to BF_3, but not to HCl, exists. Treatment with water to promote swelling of the molecule was found subsequently to promote BF_3 addition.

F. Silk Fibroin.—BF_3 was not able to penetrate the molecule and HCl was only very slowly desorbed (Sect. I-A-4) thus indicating a tightly packed structure.

G. Zinc Insulin.—The values for HCl and $HCl(CH_3NH_2)$–CH_3NH_2 are both appreciably greater than the sum of the arginine, histidine, lysine and N-terminal amino groups reported in the literature. If, however, we assume that the Zn^{+2} ion is tied to two strongly basic ions (*e.g.*, $RCOO^{-1}$) the number of binding bases is raised from 1.05 to 1.22 mmoles/g. which is now consistent with the CH_3NH_2–HCl values and reasonably close to the 1.35 obtained with HCl.

The fact that $BF_3 - NH_3(BF_3)$ equals zero shows that none of the strong basic groups are available for reaction with BF_3. Since HCl is able to titrate these groups and BF_3 is a stronger acid than HCl the effect is a striking example of selective steric hindrance. This indicates that insulin must possess a very rigid structure with spacings of the order of magnitude of the HCl molecule but smaller than the BF_3 molecule. The low value for BF_3 itself indicates the tightness of the structure by the non-availability of free peptide groups.

H. Nylon.—A comparison of sorption values for fibrous and amorphous materials indicates that the fibrous material is much more tightly arranged than the amorphous. Indeed, the number of titratable peptide groups in the amorphous material is 40% greater than in the fibers.

IV. Electrophoresis Results

A. Egg Albumin Hydrochloride.—This material did not dissolve in a veronal buffer at *p*H 8.6 and may have been denatured by the addition of HCl. It must be noted that this material had stood in a screw-capped vial for several months and was therefore subject to the effects of air and water vapor.

B. Egg Albumin·BF_3.—The mobility of this soluble compound was increased from a normal 5.8 to 7.6 with decreasing sharpness of boundaries in the descending limb of the pattern.

C. Spray Frozen Egg Albumin.—Patterns were indistinguishable from lyophilized powder.

D. Bovine Plasma Albumin Hydrochloride.—Very little change from the normal pattern was found.

E. Bovine Plasma Albumin·BF_3.—Boundary spreading and slightly decreased mobility was found.

F. Discussion.—The egg albumin, known to be most sensitive to denaturation, was most greatly affected by the gaseous titrations while the bovine albumin was but slightly altered. Although BF_3 is adsorbed to a greater extent than HCl and on the same groups, the HCl caused a greater effect of denaturation. This can be most readily explained by the fact that the HCl is sorbed more quickly than the BF_3 and therefore causes a more concentrated heat effect. Therefore, the temperature rise with the HCl might be expected to be greater than for BF_3.

Conclusions

The extensive sets of measurements on polar gas sorption described here, point very clearly to the potential importance that this field of investigation may have in probing the structural properties of solid proteins. To the extent that difficulties have presented themselves in the course of the work these same difficulties have been of value in indicating important structural differences between various proteins and in pointing out the direction for future work.

The model of the solid protein which has emerged from this work is that of a deformable lattice containing very strongly basic groups (arginine, lysine and histidine), few free acid groups and very inert peptide groups. It seems at present most plausible to picture the residue base groups to exist in the zwitterion form RNH_2^+ strongly tied to $RCOO^-$ ions of adjacent residues in either the same molecule or neighboring molecules. A weak base such as NH_3 can strongly solvate the structure but not to the point of forming permanent complexes with the protein. In these respects it is very similar to water. Stronger bases such as CH_3NH_2 are apparently capable of permanent binding but the

results do not appear to follow any simple stoichiometry.

An acid such as HCl presents a very simple chemical behavior, forming permanent compounds with the strongly basic residue groups. Where it departs from simple stoichiometry, the results appear to be attributable to slow diffusion in the solid. BF_3 is a sufficiently strong Lewis acid to attack weakly basic groups. Here diffusion has become considerably more important and the results are much less simple to interpret than for HCl. Comparisons with the model substance Nylon are very interesting in showing that the peptide in Nylon is more reactive to BF_3 than the peptide in proteins. This would be expected on the basis of the α-helix model of the proteins proposed by Pauling and Corey.[20]

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A Study of Hysteresis in the Sorption of Polar Gases by Native and Denatured Proteins[1]

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A modified McBain sorption balance has been used to measure the sorption–desorption isotherms of various polar gases, on native and heat denatured proteins at 25 and 40°. The relative sorptive capacities of egg albumin and bovine plasma albumin follow the same trend in binding the following vapors: H_2O > EtOH > Et_2O > EtCl. Apparently the amount of sorption decreases as the adsorbate becomes more restricted in its ability to form hydrogen bonds with the solid protein adsorbent. The same trend was observed with heat denatured egg albumin. The series MeOH, EtOH, and *i*-C_4H_9OH on egg albumin at 25° showed a marked decrease in the amount of sorption with increasing size of the adsorbate molecule. All of the isotherms showed hysteresis loops which could be approximately related to the total amount of sorption and the molar volume of the adsorbate. The hysteresis loops were quite reproducible, even after three successive cycles and sorption–desorption points showed negligible drift in 10–40 hour periods. The dissipation of free energy upon completion of a hysteresis loop was calculated with the aid of the Gibbs–Duhem equation. The values, expressed in kcal./mole protein, for egg albumin at 25° were: H_2O = 66; MeOH = 118; EtOH = 79 and EtCl = 38. Calculation of the heats of sorption and desorption by means of the Clausius–Clapeyron equation for EtOH in egg albumin showed quite normal behavior in contrast to the values reported on earlier for H_2O on bovine plasma albumin.

Introduction

There are marked differences in the interactions of polar and non-polar gases with solid proteins. For example, the adsorption isotherms of non-polar gases[3,4] are reproducible irrespective of path: the adsorption and desorption paths coincide and there is no hysteresis loop. This indicates that proteins have no fine pore structure which could give rise to capillary condensation. Moreover, equilibration rates are relatively rapid and the amount adsorbed can be related to the surface area of the protein on the basis of BET theory.[5] Such evidence emphasizes the fact that the adsorption of non-polar gases by proteins is a surface phenomenon and hence can give only indirect or restricted information concerning the internal structure of protein molecules.

The foregoing limitations do not apply to polar gases. This becomes evident when one considers the following observations: (1) polar gases have BET surface areas that are several orders of magnitude larger than those calculated for non-polar gases, (2) the sorption of polar gases is practically independent of particle size or surface area of the protein adsorbent, (3) the equilibration rates are low, suggestive of a rate-controlling diffusion of the adsorbate through the solid protein, and (4) the sorption isotherms have hysteresis loops. All these features point to interactions within the interior of the protein molecule and not on its surface only.[6-8]

In regard to the hysteresis effect, Seehof[9] has observed that the maximum hysteresis displacement in the water sorption isotherms of several proteins appears to correlate with the arginine, histidine and lysine groups of each protein. For example, the maximum displacement between the sorption and desorption branches of the 25° isotherm of H_2O on bovine serum albumin is 1.5 mmoles/g. This is in close agreement with 1.4 mmoles/g., the sum of the above-mentioned R groups in that particular protein. In view of the capacity for hydrogen bonding of both the adsorbate and the cited polar R troups, it was of interest to examine further the general problem of sorption hysteresis as it relates to a series of absorbates having decreasing capacity for hydrogen bonding (*i.e.*, H_2O, EtOH, Et_2O and EtCl). Another interesting comparison might arise from a series of polar adsorbates of equal capacity for hydrogen bonding but of increasing size (*i.e.*, MeOH, EtOH and *i*-BuOH)). The effects of temperature and denaturation also seemed worthy of study.

Experimental

Materials and Reagents.—The various proteins and polar adsorbates used in this research are briefly described as follows: (1) egg albumin, 2% spray frozen, area 18 m.²/g.; (2) egg albumin, crystalline powdered, Armour and Co., Lot E 90115; (3) heat denatured egg albumin, coagulated

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(2) Part of the material presented in this paper has been included in a dissertation submitted by R. L. Richardson to the Graduate School of the University of Southern California in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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