reproducible, and definitely beyond experimental error. It is, of course, possible that the BET theory provides too simple a picture from which to draw conclusions based on such relatively small differences. In support of this it is certainly probable that the idealization of the solid protein surface by an infinite, homogeneous plane cannot be correct.

A further point is the uncertainty as to the areas to be ascribed to the adsorbed molecules. A change in packing on the surface, as for example from hexagonal to cubic, can be made to account for differences in area (for symmetrical molecules) of 15%. This can be magnified if we envisage an extremely hypothetical case in which a cubic liquid forms a hexagonal close-packed layer or vice-versa. Such extreme cases may provide a factor of 45% in measured surface areas.

If we represent by S the surface area (square meters) covered by a quantity of gas V_m (cc. STP), then S is given by the equation $S = \gamma(M/d)^{2/3}Vm$, in which M = molecular weight, d = density of the liquid, and γ is a packing factor. Table VII shows the values of γ corresponding to different packing in the bulk liquid and on the surface.

TABLE VII

PACKING FACTORS FOR DIFFERENT LIQUID AND SURFACE GEOMETRY

	Liquid structure	
Surface structure	Cubic lattice	Hexagonal close-packed lattice
Square lattice	0.377	0.475
Close-packed lattice	.327	.411

Finally, it is quite possible that the adsorption may be localized in one case and non-localized in another. A change either abrupt or gradual from localized to non-localized adsorption which may be obtained with increased temperature will allow for more efficient packing of adsorbent molecules on the surface at the higher temperature and therefore increased surface area.

While the present results cannot be used to discriminate between the above conditions, they certainly indicate a sufficient lack of sophistication in our understanding of the processes of physical adsorption to merit further study.

Summary

1. Comparative studies have been made of the adsorption isotherms of N_2, O_2, CH_4, A, *n*-butane, SF_6 and neopentane at a series of corresponding temperatures on several samples of dry, lyophilized proteins.

2. Electrophoresis patterns and solubility behavior indicate that the spray-freezing technique does not change the composition of the proteins.

3. The isotherms were treated by the simple BET theory, and resonable and fairly consistent values of the surface areas and net heats of adsorption were obtained. Only SF_6 showed markedly anomalous values of the surface area.

4. The data are such as to indicate that non-polar gases are physically adsorbed by the proteins in a manner which is independent of the specific protein and depends principally on the state of subdivision.

5. The total lack of hysteresis and speed of attainment of equilibrium over a range of molecular symmetries and sizes, together with the small net heats of adsorption past the monolayer region, indicates that the proteins have no fine pore structure.

6. There seem to be definite anomalies in the behavior of the calculated BET surface areas with temperature for certain gases which cannot be understood in terms of the BET model.

LOS ANGELES 7, CAL. RECEIVED AUGUST 3, 1949

[CONTRIBUTION FROM CHEMISTRY DEPARTMENT, UNIVERSITY OF SOUTHERN CALIFORNIA]

Surface Areas of Proteins. III. Adsorption of Water[1]

BY SIDNEY W. BENSON, DAVID A. ELLIS AND ROBERT W. ZWANZIG

The surface areas of a number of dry, lyophilized proteins have been measured by the technique of nitrogen gas adsorption interpreted by the BET theory.[2,3,4,5] The results obtained have been reasonable and self-consistent but are several orders of magnitude smaller than the surface areas obtained from water adsorption isotherms which can also be fitted to the BET theory.[6] Several explanations have been offered for this discrepancy[4,7] but as yet there is a paucity of evidence on which to base conclusions.

It is the purpose of the present paper to describe some recent work on the adsorption of water by proteins and to report some very important differences which have been established between the nature of adsorption processes with respect to such non-polar gases as nitrogen and oxygen on the one hand and polar substances such as water on the other.

The experiments to be reported involve two

(1) This work has been carried out with the aid of two grants, one from the Research Corporation and one from the Office of Naval Research, Contract N6-onr-67900. This paper was presented at the High Polymer Forum of the American Chemical Society during the Fall Meeting at Atlantic City, N. J., September, 1949.

(2) Benson and Ellis, THIS JOURNAL, **70**, 3563 (1948).

(3) Palmer, Shaw and Ballantyne, *J. Polymer Sci.*, **2**, 318 (1947).

(4) Shaw, *J. Chem. Phys.*, **12**, 391 (1944).

(5) Benson and Ellis, article in preparation.

(6) Bull, THIS JOURNAL, **66**, 1499 (1944).

(7) Pauling, *ibid.*, **67**, 555 (1945).

points of interest; one the relation of the equilibrium adsorption of water to particle size, and the other the characteristics of the rate process. For this work the proteins selected were egg albumin and bovine serum fraction V.

Experimental Procedures

1. **Preparation of Proteins.**—Several different samples of egg albumin were prepared from raw eggs by methods described in an earlier paper.[2] Four different samples were used whose surface areas as determined by nitrogen adsorptions ranged from about 6 to 20 square meters per gram.

The bovine serum fraction V was a preparation of the Armour Co. Research Laboratories. Three different samples of this material were used having nitrogen surface areas of about 0.3, 4 and 14 sq. meters/gram.

2. **Measurement of Nitrogen Areas.**—The protein samples were prepared by one of the authors (D. A. E.) who also determined the nitrogen adsorption isotherms and calculated the surface areas from the simple BET equation. The technique of nitrogen adsorption is described in detail in the paper previously referred to.[2]

3. **Apparatus for Measurement of Equilibrium Water Adsorption.**—This consisted of a large Pyrex flask in which several small Pyrex, loosely capped vials containing protein samples could be placed. The large flask could be connected to a high vacuum line by means of a large ground joint and was immersed in a large water thermostat during measurements. This assembly could in turn be connected to a sample of distilled water contained in a small Pyrex flask which was itself immersed in a separate thermostat where its temperature and thus vapor pressure could be separately controlled. The temperature control of both baths was within $\pm 0.02°$.

Measurements were made by first evacuating the proteins to constant weight in a high vacuum apparatus, the samples being kept at room temperature. Within twenty-four hours the residual static pressure of adsorbed gases had been reduced to less than 10^{-4} mm. The samples were then placed in the large flask, brought to constant temperature, exposed to the water vapor for specified periods, air let into the system, the sample weighed and the process repeated.

4. **Apparatus for Rate Measurement.**—The apparatus used in the second set of experiments which were primarily concerned with rates is shown in Fig. 1. The adsorption cell (H, Fig. 1) is first thoroughly cleaned and dried, the stopcock greased with Apiezon L and then loaded with about 0.5 to 1 g. of protein. A 10 mg. plug of Pyrex glass wool is placed above the protein, above a loose fitting glass plug. The ground joint $\overline{\$}$ $^{19}/_{22}$ is then sealed by means of a low-melting water insoluble "Shawinigan Resin." The protein weight is obtained from the difference in weights of the cell full and empty, weighings being made against a tare to the nearest 0.1 mg.

The cell is then attached to the vacuum manifold A (Fig. 1) and immersed in a water-bath whose temperature is controlled to $\pm 0.02°$.

The system is then pumped out by a high vacuum manifold until a static pressure of 10^{-4} mm. is obtained, usually within twenty-four hours. The cell is then closed, the vacuum broken, cell disconnected, cleaned, dried and weighed. In this way the final dry weight of protein can be obtained to ± 0.1 mg. Experiments with blanks show that the measurements are reproducible to within these limits.

The rate studies were made by outgassing water in bulb C (Fig. 1) and distilling it under vacuum into bulb B where it is thermostated independently of the sample. At the beginning of a run, after a static vacuum of 10^{-4} mm. is obtained in the manifold, stopcock E is opened allowing water vapor to fill the system. After a measured time all stopcocks are closed, the vacuum in the manifold broken, the cell disconnected, cleaned, dried, weighed and finally replaced on the line. The cycle is then repeated

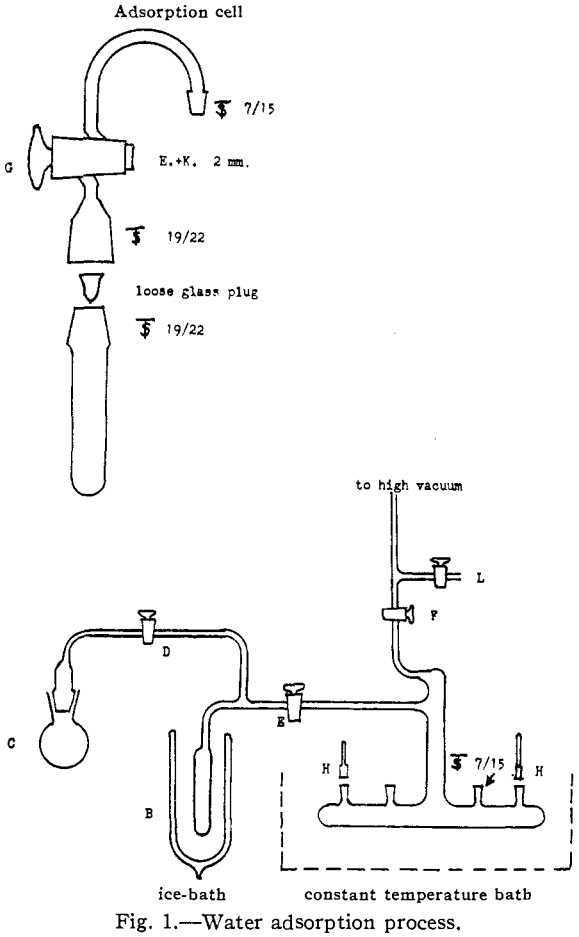

Fig. 1.—Water adsorption process.

until an equilibrium weight is reached. On each cycle the water is outgassed as a precaution against leaks. At no time is the protein cell opened to air once a run has started.

The glass wool was used to prevent the proteins which are in the form of very light, fluffy powders, from being blown into the line. (Blank measurements showed that under the experimental conditions used it adsorbed no water vapor.) Even with the glass plug and the glass wool it was found that extreme care had to be employed in outgassing to prevent mechanical blowover. With very slow initial evacuation this can be avoided. Temperature control of protein sample and distilled water was within $\pm 0.01°$.

Experimental Results

Before describing the results a few qualitative observations are in order. Other workers have observed that a considerable time is needed for equilibrium to be achieved in water adsorptions. For example, Bull[6] found that upwards of six days were needed to reach equilibrium. Frey and Moore[8] working with simple amino acids *in vacuo* found that up to twelve hours was required for equilibrium. Mellon, Korn and Hoover,[9] working under conditions similar to Bull's (samples equilibrated against salt solution in a vacuum desiccator) found they needed from

(8) Frey and Moore, THIS JOURNAL, **70**, 3644 (1948).

(9) Mellon, Korn and Hoover, *ibid.*, **69**, 827 (1947); **70**, 1144 (1948); **70**, 3040 (1948).

TABLE I

RELATION BETWEEN WATER ADSORPTION AND SURFACE AREA

Protein[a]	Temp., °C.	Relative[b] humidity	Equilibrium water adsorption mg. ads./g. Prot.	Equilibrium water adsorption cc. STP/g. P.	N_2 Adsorption[c] at same R. H. cc. STP/g.	N_2 Areas[c] (BET) sq. m./g.
Bovine V, crude	25.0	0.193	82.7	103.1	0.050	0.26
Bovine V, lyo. 2%	25.0	.193	65.8	82.0	1.75	6.88
Bovine V, crude	30.0	.144	48.5	60.4	...	0.26
Bovine V, lyo. 3%	30.0	.144	48.7	60.7	...	3.86
Bovine V, S-F 2%	30.0	.144	50.4	62.5	...	14.1
Egg albumin lyo. 2%	25.0	.193	67.1	83.6	1.31	5.75
Egg albumin S-F 2%	25.0	.193	65.4	81.5	4.65	20.4
Egg albumin lyo. 5.6%	30.0	.144	45.5	56.6	...	6
Egg albumin S-F 2%	30.0	.144	45.5	56.6	...	12

[a] Column I, designations are: crude—lyophilized from concentrated solutions; lyo. 2%; lyo. 5.6%—lyophilized from 2% and 5.6% solutions; S-F 2%—spray frozen at 77.4°K. and evacuated from 2% solutions. [b] Calculated from vapor pressure tables for water at 0, 25 and 30°. [c] Taken from N_2 isotherms at 77.4°K. The ratio of the equivalent BET areas for water to nitrogen are given roughly by the ratio of the cc. STP(H_2O)/g. (column 5) to cc. STP(N_2)/g. (column 6).

six to eight days on adsorption and over eighteen days in desorption.

In contrast to these findings are our own experiences with the lyophilization procedure in which it was found possible to dry as dilute a solution as 2% within twenty-four hours at temperatures of −20 to −50°. Furthermore, proteins which had been allowed to absorb up to 10% moisture by exposure to laboratory humidity over a period of a few weeks could be dried to constant weight in a high vacuum system by pumping at room temperature in never more than ten hours.

In our initial set of experiments on the effect of surface area on moisture content we found that very peculiar rate curves were obtained showing two points of inflection and that it took twenty-four days to reach equilibrium.

In the later sets of experiments it was found that equilibrium could be reached both in adsorption and desorption within from one to three hours.

All of these at first disparate results were finally understood in terms of the inhibition caused by air. A re-examination of the data showed that in all cases in which long equilibrium times were reported, the water adsorption had taken place in a partial pressure of air. This was confirmed as a cause for slow diffusion in a series of unplanned experiments in which it was found that even small partial pressures of air could inhibit the rates by several orders of magnitude. This is an experience which has been already observed in connection with the rates of adsorption of other gases on catalysts.[10]

In Table I is shown a summary of the data found for the values of the equilibrium adsorption of water on different proteins. The first four samples were run in the equilibrium apparatus which had long equilibrium times (twenty-four days) while the last two samples represent data taken in the rate apparatus (Fig. 1) with equilibrium times of one to two hours.

(10) Brunauer, "Physical Adsorption," Princeton University Press, Princeton, N. J., 1943, Chap. XIII.

For purposes of comparison we have included in Table II the values obtained by interpolating Bull's data for the adsorption of water at comparable temperatures and partial pressures for egg albumin.

TABLE II

COMPARISON OF WATER ADSORPTIONS OF DIFFERENT PROTEIN SAMPLES

Protein	Temp., °C.	Relative humidity	Water adsorption (Bull's data) mg. H_2O/g. prot.	Water adsorption (Present work) mg. H_2O/g. prot.	Surface area[a] BET (Bull's data), sq. m./g.
Egg albumin (unlyo.)	25.0	0.194	57.4	..	218
Egg albumin (lyo.)	25.0	.194	51.6	66.0	200
	30	.144	42.8	45.5	...

[a] Calculated by Bull from H_2O adsorption isotherm.

The precision of the water adsorption data is about ±1%. Maximum observed deviations from the mean have never exceeded 3% and have usually been considerably less. For the results at 30.0° shown in Table I, each point represents the mean of at least three different observations.

Discussion

The most striking feature of the water adsorption data (Table I) is the virtual independence of the equilibrium moisture content and the nitrogen surface areas. For the last two samples of egg albumin shown, where the surface areas differ by a factor of 2 there is no measurable difference in water uptake. For the first two samples of egg albumin, having areas in the ratio of 3.5 to 1 the difference is 2.5% which was within experimental error in this particular case. For the extreme case of Bovine fraction V having nitrogen areas in the ratio of 26 to 1 the difference in moisture content is only 26%. It should be noted that in both of these preceding cases the differences are in the opposite direction to that expected, namely, the sample absorbing the larger amount of water is the one absorbing the smaller amount of nitrogen.

The most striking evidence is provided by the very careful experiments done under conditions of excellent reproducibility on the three Bovine V samples at 30.0. The crude and lyo. 5.6% samples whose areas are in the ratio of 15 to 1 agree within experimental error whereas the spray-frozen sample of highest area absorbs only 4% more water than the crude sample whose area is in the ratio of 1 to 56.

It thus seems clear that the water and nitrogen adsorptions are fundamentally different phenomena. Within quite narrow limits the conclusions may be drawn that whereas nitrogen adsorption probably represents a bulk property of the material typical of its state of subdivision[2,5] quite the contrary is true of water adsorption. The latter appears to be almost independent of the state of subdivision of the protein and is probably a specific molecular property of the protein itself. Similar conclusions have been expressed by other workers based on different types of evidence.[7,9]

Rate data were obtained on both egg albumin samples and bovine albumin samples using samples of each of different surface areas. Attempts were made to analyze the data according to a first order law but semi-log plots showed pronounced curvature throughout, many times greater than any conceivable experimental error. A test of the bulk diffusion equation for constant external concentration and zero initial internal concentration[11,12] gave calculated diffusion constants which varied markedly and had values of 10 Å./day which is an unreasonable value. Film diffusion and the Langmuir kinetic theory yield first order kinetics and so too must be eliminated from consideration.

After considerable trial it was found that the data could be fitted rather well to a second order equation of the form

$$dx/dt = k(X_{eq} - X)^2$$

in which X_{eq} = amount adsorbed at equilibrium. However, it was found that k the specific rate constant was higher for lower surface area materials. This observation together with the lack of any reasonable theoretical justification for the form of the empirical rate equation seemed to make the rate data extremely suspect until finally a plausible explanation was found in a series of related papers by Cassie and King[13,14,15] on the behavior of textiles in water adsorption.

Cassie and King, measuring the rates of water adsorption in textiles, found that there was considerable heat evolution in the process and that this latter was the rate determining step in the reaction. They were able to observe temperature rises of 45° on exposing carefully dried wools to water vapor.[16] On repeating our own measurements with a thermocouple placed in the protein samples it was found that the temperature rose about 8–10° and in fact the temperature–time resembled the rate curves rather well. As a final check the rate measurements were repeated in an all-aluminum cell with pieces of aluminum wire placed through the sample to increase heat conductivity. Under these conditions equilibrium was achieved in about one-fourth the time, namely, from forty to seventy minutes. Our rate findings on these proteins may thus be taken as confirming the observations of Cassie and King on wool fibers, namely, that the rates of adsorption of water are probably immeasurably fast and that the observed rates are the rates of thermal conductivity of the material. The observed temperature increase also puts a rather crudely calculated lower limit of about 6 kcal./mole water for the heat of adsorption of water. The correct value is almost certainly quite a bit higher.

These results indicate the inherent difficulty and objections to making rate measurements in heterogeneous systems where reactions are accompanied by even small heat changes. In particular rate measurements involving organic solvents and polymers (*e. g.*, rates of swelling, etc.) are almost certain to involve similar heat changes and thus give spurious results.

Summary

1. Measurements have been made of the effect of surface area of two different dry, lyophilized proteins on their absorption of water. It has been shown that within quite narrow limits, the water absorption is independent of surface area in the range below one-fourth saturation pressure. It is inferred from this that water absorption is qualitatively different from nitrogen adsorption and probably takes place at specific sites on the protein molecule.

2. In the presence of even small amounts of air the rate of water adsorption by proteins is enormously inhibited.

3. Detailed studies of the rates of water adsorption have confirmed the findings of Cassie and King in indicating that in the absence of air the rates of adsorption are immeasurably fast, the observed rates merely being the rate of heat loss to the bath.

4. The findings suggest that all kinetic processes in heterogeneous systems which involve even small heat changes are inherently limited by the thermal transport properties of the media and that such data be scrutinized very carefully.

LOS ANGELES 7, CALIF. RECEIVED AUGUST 3, 1949

(11) Boyd, Adamson and Meyers, THIS JOURNAL, **69**, 2836 (1947).

(12) Barrier, "Diffusion in and through Solids," The Macmillan Company, Cambridge, England, 1941.

(13) Cassie and King, *Trans. Faraday Soc.*, **36**, 445 (1940).

(14) Cassie, *ibid.*, **36**, 453 (1940).

(15) Cassie and Baxter, *ibid.*, **36**, 458 (1940).

(16) A practical joke which seems to be well known among textile chemists and is related to the large heat effects accompanying water absorption is to take a piece of wool from a desiccator containing phosphorus pentoxide and hand it to some unsuspecting party. The wool becomes so warm on a day of normal humidity that the victim has to drop it.