Ferry, J. D., and Eldridge, J. E. (1949), *J. Phys. & Colloid Chem.* (now *J. Phys. Chem.*) *53*, 184.
Flory, P. J. (1960), *Brookhaven Symp. Biol. 13* (*BNL 608* (*C 22*)), 230.
Flory, P. J., and Garrett, R. R. (1958), *J. Am. Chem. Soc. 80*, 4836.
Flory, P. J., and Weaver, E. S. (1960), *J. Am. Chem. Soc. 82*, 4518.
Grassmann, W., Hannig, K., and Schleyer, M. (1960), *Z. Physiol. Chem. 322* 71.
Harrington, W. F., and Von Hippel, P. H. (1961a), *Arch. Biochem. Biophys. 92*, 100.
Harrington, W. F., and Von Hippel, P. H. (1961b), *Advan. Protein Chem. 16*, 1.
Katchalski, E., Berger, A., and Kurtz, J. (1963), *in* International Symposium on Protein Structure, Ramachandran, G. N., ed., New York, Academic, p. 205.
Katz, J. R., Derksen, J. C., and Bon, W. F. (1931), *Rec. Trav. Chim. 50*, 725.
Klotz, I. M. (1960), *Brookhaven Symp. Biol. 13* (*BNL 608* (*C 22*)), 25.
Klotz, I. M., and Frank, B. H. (1962), *Science 138*, 830.
Klotz, I. M., and Franzen, J. S. (1960), *J. Am. Chem. Soc. 82*, 5241.
Linderstrøm-Lang, K. (1958), *Symp. Protein Struct. Paris, 1957*, 23.
Mikkelsen, K., and Nielsen, S. O. (1960), *J. Phys. Chem. 64*, 632.
Nielsen, S. O. (1960), *Biochim. Biophys. Acta 37*, 146.
Piez, K. A., and Carrillo, A. L. (1964), *Biochemistry 3*, 908.
Piez, K. A., Eigner, E. A., and Lewis, M. S. (1963), *Biochemistry 2*, 58.
Piez, K. A., and Gross, J. (1960), *J. Biol. Chem. 235*, 995.
Ramachandran, G. N., Sasisekharan, V., and Thatkachori, Y. F. (1961), *in* Central Leather Research Institute Symposium on Collagen, Ramanathan, N., ed., New York, Interscience, p. 102.
Rice, R. V. (1960), *Proc. Natl. Acad. Sci. U. S. 46*, 1187.
Rich, A., and Crick, F. H. C. (1958), *Recent Advan. Gelatin Glue Res., Proc. Conf. Univ. Cambridge, 1957*, 20.
Rich, A., and Crick, F. H. C. (1961), *J. Mol. Biol. 3*, 483.
Schellman, J. A. (1955), *Compt. Rend. Trav. Lab. Carlsberg* (*Ser. Chim.*) *29*, 223.
Schmitt, F. O., Gross, J., and Highberger, J. H. (1955), *Symp. Soc. Exptl. Biol. 9*, 148.
Smith, C. R. (1919), *J. Am. Chem. Soc. 41*, 135.
Steinberg, I. A., Harrington, W. F., Berger, A., Sela, M., and Katchalski, E. (1960), *J. Am. Chem. Soc. 82*, 5263.
Thompson, H. W. (1963), *Pure Appl. Chem. 7*, 13.
Veis, A., Anesey, J., and Cohen, J. (1962), *Arch. Biochem. Biophys. 98*, 104.
Veis, A., and Cohen, J. (1960), *Nature 186*, 720.
Veis, A., and Drake, M. P. (1963), *J. Biol. Chem. 238*, 2003.
Von Hippel, P. H., and Harrington, W. F. (1959), *Biochim. Biophys. Acta 36*, 427.
Von Hippel, P. H., and Harrington, W. F. (1960), *Brookhaven Symp. Biol.* (*BNL 608* (*C 22*)), 213.
Von Hippel, P. H., and Wong, K. (1963), *Biochemistry 2*, 1387, 1399.
Yaron, A., and Berger, A. (1961), *Bull. Res. Council Israel, Sect. A: 10*, 46.

Hydrophobic Interactions in Proteins: Conformation Changes in Bovine Serum Albumin below *p*H 5*

ARNOLD WISHNIA AND THOMAS PINDER

From the Department of Biochemistry, Dartmouth Medical School, Hanover, N. H.

Received April 13, 1964

The effect of *p*H, temperature, and partial pressure on the solubility of butane and pentane in solutions of bovine serum albumin has been studied. The binding of alkanes to bovine serum albumin is very sensitive to the conformation of the protein. The so-called *F* form, produced, in 0.15 M NaCl, in a single step around *p*H 4.1 marked by sharp but small changes in $[\alpha]$ and $[\eta]$, binds only one-fourth as much butane and one-fifth as much pentane as native bovine serum albumin. In perchlorate solutions, the solubility reduction, like the changes in $[\alpha]$, occurs in two stages. The temperature dependence of the solubility indicates that butane is bound directly to some of the hydrophobic regions of bovine serum albumin, and the pressure dependence indicates that these regions are large. A variety of considerations leads to the conclusion that the binding sites are inside the bovine serum albumin molecule (i.e., most, if not all, the apolar side chains of these regions are not in contact with solvent). These data support Foster's model of bovine serum albumin: these regions, formed by the interaction between the hydrophobic surfaces of several substructures, would be disrupted when these substructures, without much internal rearrangement, separate to make the *F* form.

The role of hydrophobic interactions in proteins has come under increasing scrutiny in recent years, from a variety of theoretical and experimental points of view. In this laboratory the emphasis has been on studies of the solubility of the shorter (C_2 to C_5) *n*-alkanes in protein solutions as perhaps the most direct way of investigating these interactions. We found that the binding of ethane, propane, and butane to BSA[1] and human hemoglobin was quite strong (Wishnia, 1962); observed enthalpies, and, for dodecylsulfate micelles (Wishnia, 1963a), entropies also, agreed with theoretical predictions (Kauzmann, 1959; Némethy and Scheraga, 1962). Moreover, the early studies of BSA, hemoglobin, and lysozyme (Wishnia, 1962) suggested that the specific behavior of these proteins was a reflection of their characteristic structural features, so that alkane-binding provided a new means to investigate such features. Subsequent work on hemoglobin and myoglobin, on ribonuclease (A. Wishnia and T. W. Pinder,

* Supported in part by funds from National Science Foundation grants (G-13973 and GB-1446) and a United States Public Health Service grant (RC-8121). Some of this work was reported at the 7th annual meeting of the Biophysical Society, New York, February, 1963.

[1] Abbreviations used in this work: BSA, bovine serum albumin; SDS, sodium dodecylsulfate.

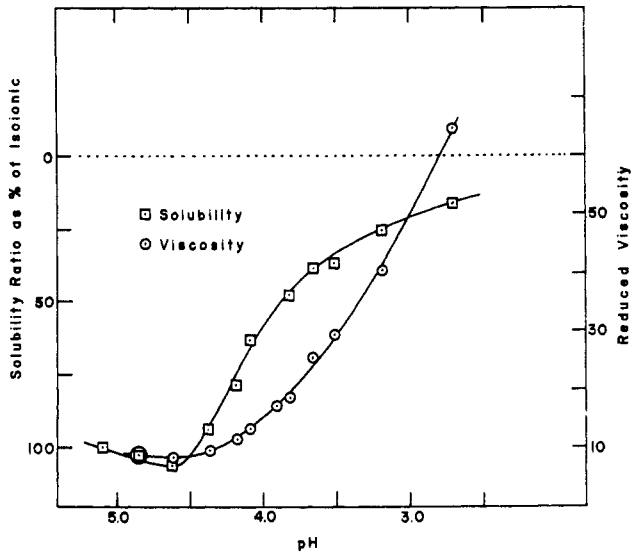

FIG. 1.—The effect of *p*H on the binding of butane to BSA at 25°, in the absence of added salt. ⊡, 100 × $(S_p/S_w)_{pH}/(S_p/S_w)_{pH5}$. ⊙, reduced viscosity.

unpublished observations), and on β-lactoglobulin (Wishnia, 1964), has strengthened this notion.

In particular, we showed that the binding of butane to BSA decreased drastically (Wishnia, 1963b) as the protein underwent its well-known acid expansion. Since then, Wetlaufer and Lovrien (1964) have reported that a transition in BSA near neutral *p*H most readily observed through changes in optical rotation (Leonard *et al.*, 1963) is accompanied by a distinct increase in the binding of butane, and that the expansion in alkali is accompanied by a decrease in butane binding.

The purpose of this paper is to examine in some detail the interaction between butane, pentane, and BSA in the region of the acid transition, to attack jointly two problems: What is the nature of the structures within a given molecule that give rise to a particular kind of alkane-binding behavior? What are the structural changes that occur in the different stages of the acid modification of BSA?

We would like to review some of the behavior of BSA between *p*H 5 and 2. The acid transitions are obviously driven by electrostatic forces, and are strongly dependent on both ionic strength and specific counterions (Foster, 1960). Near *p*H 4 a new species appears, characterized by a greater electrophoretic mobility (the *F* form; Aoki and Foster, 1956), and viscosity (the "expandable" form; Tanford *et al.*, 1955a), and by different relations between charged groups (Tanford *et al.*, 1955b; Foster and Clark, 1962). At still lower *p*H, depending on ionic conditions, large-scale expansion of the molecule begins (Tanford *et al.*, 1955a; Yang and Foster, 1954). However, even below *p*H 3 the viscosity is considerably less than for a random coil (Tanford *et al.*, 1955a), and the hydrogen-ion equilibria may be accounted for by an expanded shell penetrated by solvent surrounding a still substantial compact nonpolar core (Tanford *et al.*, 1955b). In 0.15 M NaCl the sharp transition to the *F* form near *p*H 4 is marked by readily observed but rather small changes in $[\alpha]$ (Leonard and Foster, 1961) and $[\eta]$ (Tanford *et al.*, 1955a) paralleled by changes in the ultraviolet spectrum equivalent to exposure to solvent of 20% of the 70% of "buried" tyrosine residues (Herskovits and Laskowski, 1962) and by striking changes in the nature of the binding of dodecylsulfate to BSA (see Foster, 1960, to which we will refer later). At lower ionic strength in

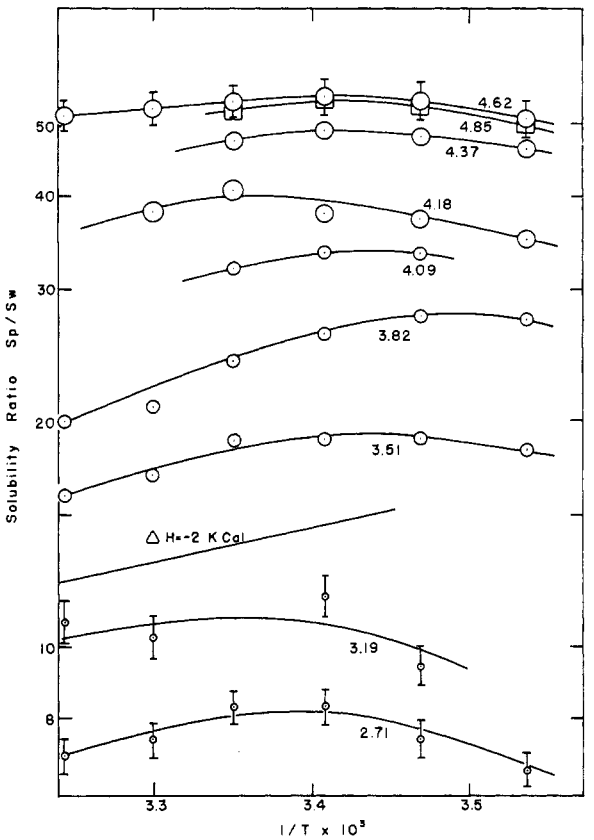

FIG. 2.—The effect of temperature on the binding of butane to BSA in the absence of added salt. Ordinate, (S_p/S_w) on a logarithmic scale. Abscissa, inverse absolute temperature. *p*H as shown. The line $\Delta H = -2$ kcal is included for reference.

thiocyanate or perchlorate the change in $[\alpha]$ occurs in two discrete steps: the *F* form appears at the higher *p*H, and the exposure of aromatic residues occurs at the lower (Leonard and Foster, 1961).

Foster (1960) has proposed a subunit model of BSA, resembling a club sandwich, with several slices of bread held together by butter; he suggests that the first few acid modification reactions correspond to the disruption of one or more of these hydrophobic interfaces without any serious unraveling of the slices themselves. We intend to show that the alkane-binding data, measured as functions of pressure of *p*H, support such a model, and as a corollary, that for BSA, but by no means for proteins generally, butane and pentane are bound in large hydrophobic regions inside the protein molecule.

EXPERIMENTAL

Bovine Serum Albumin.—Crystalline BSA (Pentex lot BX 3, lot 4, lot 8) was treated as follows: A 6–7% solution was brought to *p*H 2.5–3.0 for 30 minutes at room temperature, filtered through a Millipore HA filter, then deionized, first batchwise and then on a column, with mixed Dowex-1-X8-OH^- and Dowex-50W-X8-H^+ resins. Several stock solutions were deionized without prior acidification. No systematic differences in behavior between these and the usual preparations were observed. The protein solutions contained no fatty acids, as determined by the method of Dole (1956), although added sodium laurate (2 moles/mole protein) could be recovered quantitatively. Ultracentrifugal analysis of one such solution (Spinco Model E, 22°, 50,740 rpm, 0.10 M NaCl, *p*H 5.5, diluted to 1%) showed a major component with an $s_{20,w}$ of 4.3

S, and 9% of minor component, $s_{20,w}$ = 6 S (Bro *et al.*, 1955). The reduced viscosity of this preparation (0.04 M $KClO_4$, *p*H 5, 25.0°, 2.1%) was 0.042 dl/g. We used an absorptivity of 6.67 (1%, 279 mμ) (Leonard and Foster, 1961) in determining BSA concentrations.

Butane and Pentane.—Butane-1,2-^{3}H and pentane-1,2-^{3}H (New England Nuclear Corp.) were diluted with cold butane (Phillips Research Grade) or pentane (Matheson, Coleman, and Bell Chromatoquality) to specific activities of 5 mc/ml, and treated as described.

The validity of the solubility measurements rests on the radiochemical purity of the hydrocarbon. It was therefore a matter of some concern (Wishnia, 1963a) that several samples appeared to contain a highly water-soluble contaminant of moderate vapor pressure. The alkanes are routinely transferred in a vacuum system with the receiver in liquid nitrogen and the source in melting ice. Typically, the impurity (^{3}HOH ?) was transferred to the first reservoir from the original bulb along with the alkane but remained behind in the aqueous phase on subsequent transfers. In the most recent instance this residue constituted about 0.5% of the total radioactivity; more than 99% of this residue remained in the aqueous layer following partition against isooctane. Our current procedure calls for scrubbing the hydrocarbon sample with water, then with alkaline permanganate, then twice with water, monitoring the partition behavior of residual radioactivity at each step; finally, a test solubility run is made, the distribution of radioactivity in the gas phase is determined by gas chromatography, and the partition behavior of the radioactivity in the aqueous phase is examined.

Although the problem was recognized early, it is possible that the various remedies tried during the course of these investigations were not always successful, and that some of the variability between runs is due to residual contaminants. We also tried to test the hydrocarbon for purity within the framework of the actual solubility experiment by using dodecylsulfate solutions as well as water as reference solutions, but dodecylsulfate solutions proved not to be ideal standards.

Measurement of Gas Solubility.—The manometric technique has been described before (Wishnia, 1962). The radioactive tracer method (Wishnia, 1963a) was modified slightly. The solubility cell consists of eight 95 × 14-mm tubes, selected to fit the stoppers of Becton-Dickinson 3204 Vacutainers snugly, joined by a manifold. [^{3}H]Butane or pentane from a reservoir was allowed to expand into the evacuated cell at a temperature appropriate to the desired final pressure, 4 ml of solution or solvent was injected into each tube of the sealed cell through the stoppers (in an alternating or solvent–solution 1–solution 2–solvent pattern), and the cell was rocked in a thermostat for 1–2 hours. Aliquots (0.2 ml) were withdrawn (through the stoppers) into weighed syringes, and the weighed contents were delivered, with rinsing, into 20 ml of Bray's scintillation fluid (Bray, 1960) containing blank protein solution or solvent as indicated. (Except for the heme proteins, however, no effect of protein on counting rate was observed.)[2] The vials were counted in a Nuclear-Chicago Model 703 or a Packard Instruments Model 3214 liquid scintillation counter for at least 4 × 10^4 counts.

For pressure runs the partial pressure of alkane was reduced by stepwise replacement with air, until the counting rates were 5–10% of their initial values. The effect of *p*H on solubility was determined at low partial pressure of alkane (0.1 atm butane, variable pentane); the pressure for a given series was relatively constant because the volume of the gas phase, which contains most of the alkane, was kept relatively constant. For the *p*H runs two stock solutions (low *p*H, isoionic *p*H) of the same ionic strength and protein concentration were prepared; after the solubility samples had been withdrawn the *p*H in each leg was determined (Radiometer PHM 4, Beckman 14400 external probe assembly electrodes) on 2-ml aliquots temporarily removed for this purpose; in order to vary the *p*H in each leg successive increments of the opposite stock solution were added when these samples were returned to the cell. Aliquots were also removed at the beginning, middle, and end of the experiment for determinations of protein concentration (the BSA concentration (4–5%) does not usually change during the course of an experiment).

Treatment of the Data.—All eight samples of a given set are presumed to have reached equilibrium at the same temperature with the same gas phase. We define the following explicit functions of T and p: $c_w = c_w(T,p)$ counts/g solvent/second; $c_s = c_s(T,p)$ counts/g solution/second; $R(T,p) = c_s/c_w$; $S_{soln}(T,p)$, $S_w(T,p)$, and $S_p(T,p)$ = solubility increment, mmoles alkane/10^3 g solution, solvent, or protein. Let f_w and f_p be weight-fractions of solvent and protein, respectively; then, since S_p is defined by $S_{soln} = f_w S_w + f_p S_p$, we may calculate the solubility ratio $S_p/S_w = (R - f_w)/f_p$. To calculate S_p and S_w explicitly it is necessary to determine the specific activity of the gas in terms of the experiment. For each butane sample duplicate experiments on pure water were performed, in which the gas pressures as well as the counting rates were measured. (The efficiencies of the counter and the particular batch of scintillation fluid were taken into account in these and all other experiments by assaying standard vials made up for each run with stock [^{3}H]phenylalanine solution.) The solubility of butane in water at 25° and 1 atm is 1.25 mM (Wishnia, 1963a); the concentration of free butane in solution at any temperature and pressure is then given by $S_w = 1.25 \times c_w(T,p)/c_w$ (25°, 1 atm) mM. The extent of binding, r, in moles alkane/mole protein, may be computed from $r = (mw)\,(S_p/S_w)(S_w)$, taking the molecular weight of BSA as 65,000 (Tanford *et al.*, 1955a).

The natural mode of expressing the tracer data, is as S_p/S_w, since this quantity is computed directly from the counting ratios, and since part of the convenience of the method lies in not being required to control the precise composition of the gas phase. If the solubility obeys Henry's law, S_p/S_w is, of course, independent of the partial pressure of alkane; in any case S_p/S_w is relatively independent of pressure at low pressures, when either saturation phenomena, or effects of alkane-binding on the protein if they appear at higher pressure, will be minimal. When the point at issue is, for example, the unperturbed *p*H profile of a conformation change, or an association, low pressures of alkane ought to be used. Of course, if what is being studied is the nature of the binding groups of the different isomers or the effects of the alkane itself on the course of the transition, determination of the actual binding, S_p (and hence of c_w^0 [1 atm, 25°] and S_w), is necessary.

Finding deviations from Henry's law is the point of the pressure runs, and plotting S_p/S_w, rather than S_p, against S_w, magnifies such deviations (this plot is about as sensitive as a Scatchard plot of $1/[S_p/S_w]$ against S_w for this purpose; an Eadie-type plot, S_p against S_p/S_w, would be more sensitive). It will be seen that the BSA

[2] We have been removing precipitated heme proteins by centrifuging the vials, but are now studying the use of a toluene-based scintillation liquid. At the moment some fundamentally trivial but perhaps excessively inconvenient difficulties have been encountered.

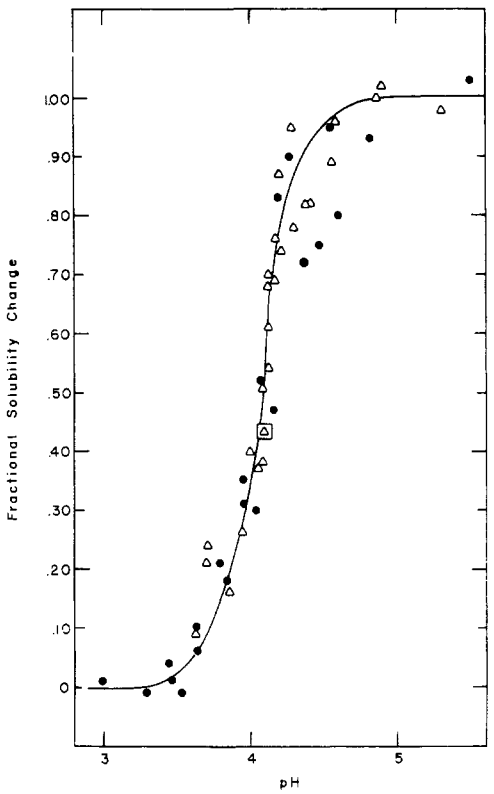

FIG. 3.—Butane binding in the *N-F* transition. 25°, 0.15 M NaCl. Ordinate, fractional solubility change for butane: $[(S_p/S_w)_{pH} - (S_p/S_w)_{pH3}]/[(S_p/S_w)_{pH\,5.5} - (S_p/S_w)_{pH3}]$. Abscissa, *p*H. ● and △ represent runs, about eight months apart, using different samples of protein and gas. The □ indicates the pressure point (Fig. 5) that preceded the second run. The data were normalized to minimize apparent systematic differences between the two runs: the values of (S_p/S_w) at *p*H 5.5 and 3.0 are, respectively, 50 and 13, and 61 and 16 for the two sets.

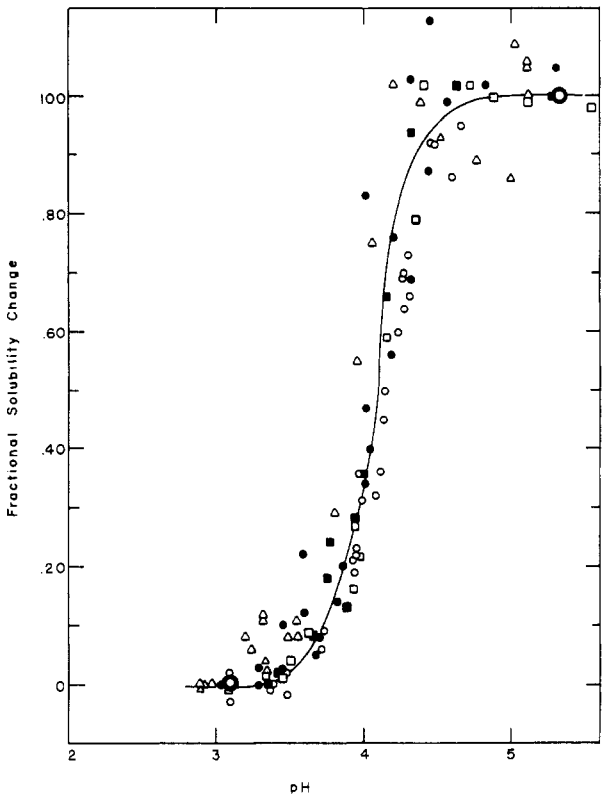

FIG. 4.—Pentane binding in the *N-F* transition. 25°, 0.15 M NaCl. Ordinate, fractional solubility change for pentane (see Fig. 3). Abscissa, *p*H. The line through the data is taken from Fig. 3, to show the correspondence with changes in butane binding. The various points represent separate runs on different samples of alkane and protein. Here (S_p/S_w) took on values of 140 ± 10 at *p*H 5.5 and 28 ± 3 at *p*H 3.0 for the several series.

data show at most small, and perhaps insignificant, deviations from Henry's Law.

RESULTS

The early manometric data (Wishnia, 1963b) showing the effect of *p*H on the solubility of butane in BSA solutions are given in Figures 1 and 2. Under these conditions (absence of added salt, hence low ionic strength increasing toward lower *p*H) the *N-F* transition and the expansion of BSA are not well separated (Tanford *et al.*, 1955a; Leonard and Foster, 1961); the solubility decrease coincides with the viscosity increase (in fact, $-\log S_p/S_w$ varies linearly with $\log \eta_{red}$). The point which we would like to emphasize is the very slight temperature dependence of S_p/S_w at all *p*H. For the native structures, for the structures that remain, and hence for the structures that disappeared during the course of the transition, the enthalpy of butane binding is small (near zero at 25°); this behavior strongly resembles that of dodecylsulfate micelles (Wishnia, 1963a). Binding of butane to BSA is an entropy-driven process.

In 0.15 M NaCl the *N-F* transition is quite sharp, and is essentially over before any major expansion has begun (Tanford *et al.*, 1955a; Leonard and Foster, 1961). If we examine the data of Figures 3 and 4 two facts become clear. First, the butane and pentane data coincide: both sets of experiments show the same transition. Second, the sharp decrease in butane and pentane binding occurs in the *N-F* transition, not at the lower *p*H of the large-scale expansion. Furthermore, the *F* form, while still compact, is able to bind only one-fourth or one-fifth as much butane or pentane as isoionic BSA: this is perhaps the most dramatic change in the properties of the new species. The solubility transition in perchlorate occurs at lower *p*H, as expected (Leonard and Foster, 1961) (Fig. 5); the sharpest change occurs at the upper step, along with the appearance of the *F* form. Note, however, that the decrease is only about half as great as in chloride. In these experiments the second step is not yet complete by *p*H 2, presumably because the higher ionic strength (0.10 μ) required to bring 5% BSA to this *p*H, and the additional anion-binding that this entails, decrease the electrostatic repulsion that drives the transition. These results suggest, in addition, that changes in alkane binding may be used to investigate relatively subtle alterations in the nonpolar regions of proteins.

In Figures 6 and 7 the pressure dependence of solubility of alkane is shown (as the dependence of the solubility ratio S_p/S_w on the concentration of free alkane, S_w). The usual first step in the analysis of such data is a graph of one of the linearizations of $K = (n - r)c_{solute}/r$, the expression for the dissociation constant of n independent, equivalent sites on a macromolecule. Such a procedure is meaningful for β-lactoglobulin (Wishnia, 1964), which has the capacity to bind only 2 or 3 moles of butane per 18,000 g monomer (depending on *p*H and temperature); K is close to 1×10^{-3} M between 0 and 25°, increasing slightly with increasing temperature in this range. Now, the binding of butane to BSA and β-lactoglobulin, at 25° and 1 atm, is roughly the same: if native BSA had as few as seven or eight sites, as required for $K = 10^{-3}$ M, then saturation be-

havior between 0.1 and 1.0 atm would be as obvious as it is for β-lactoglobulin. In fact, the contrast could not be more striking: native BSA shows no significant dependence of S_p/S_w on S_w. If we define a saturation parameter a ($0 \leq a \leq 1$) for a given alkane pressure by $a = (S_p/S_w)/(\lim_{p \to 0}(S_p/S_w))$, it is easy to show that $n = S_p/(1 - a)$. The lowest admissible value of a (1 atm, 25°) is 0.9, and 1.0 is equally likely; n is therefore not less than forty sites per molecule of BSA.

We also looked for an effect of butane on the *N-F* equilibrium (favoring *N* if the effect is like dodecylsulfate [Foster, 1960]; favoring *F* if the effect is like heptane [Alfsen, 1963]),[3] which would appear as an increase (*N*) or decrease (*F*) in S_p/S_w. At the most likely place, *p*H 4.1 (the steepest part of the curve), there is no evidence for such a shift at butane pressures up to 1 atm. In the absence of saturation behavior at higher *p*H there is no reason to suspect that an increase in solubility arising from a shift to the *N* form is exactly balanced by a decrease arising from saturation of sites; neither is a shift to the *F* form taking place. By *p*H 3.1, where expansion is considerable, there is perhaps a suggestion of some refolding.

There are two conclusions which we would like to draw from these data. First, that there is no change in the *character* of alkane binding during the *N-F* transition. Second, that with $n > 40$ (perhaps $>> 40$), the notion of fixed sites either covered or available is unrealistic, and it is much more likely that the binding regions are large, not restricted by the binding of a molecule of alkane, and perhaps expandable.

In earlier work the extent of binding of butane to BSA at 1 atm and 25° (S_p[1, 25°]) was given as 64 mmoles/1000 g BSA (Wishnia, 1962). The value in Figure 2 is 66. These numbers are derived from measurements of the excess butane disappearing from the gas phase over BSA solutions. The tracer experiments (Figs. 3, 5), which measure the excess butane *contained* in BSA solutions, give 63 and 76 mmoles/1000 g BSA for two series. Using mw = 65,000, we may convert these numbers to moles of butane bound per mole of BSA; these numbers become 4.2, 4.4, 4.1, and 4.9. We can offer no explanation for the discrepancy between these data and the values, 2.0 and 2.7 moles/mole, based on the excess butane *extracted* from BSA solutions, recently reported (Wetlaufer and Lovrien, 1964).

Discussion

Any proposed structure or mechanism for binding butane and pentane to BSA must have the following features: (1) a high capacity (from the observed pressure dependence), (2) low heat of binding (temperature dependence), and (3) disappearance in the *F* form of the structures responsible for binding, in a way consistent with what is known about the *N* and *F* forms (*p*H dependence). The mechanism may be common, but must not be general, since the behavior of proteins is not. Finally, it should predict an increase in binding by factors of 2–3 for each additional methylene group in the series ethane-propane-butane-pentane (Wishnia, 1962; 1963a). We propose that large hydrophobic clusters in the interior of BSA are responsible for the observed behavior, in accordance with Foster's model. We will proceed by dichotomy.

The most likely sites of alkane binding in proteins are structures involving the apolar amino acid side chains directly; we cannot take electrostatic or polar binding

[3] If the parenthetical remark of Strauss and Strauss (1958) is correct, either 1–2 molecules of heptane have a profound effect on BSA, or Alfsen's data results from surface denaturation.

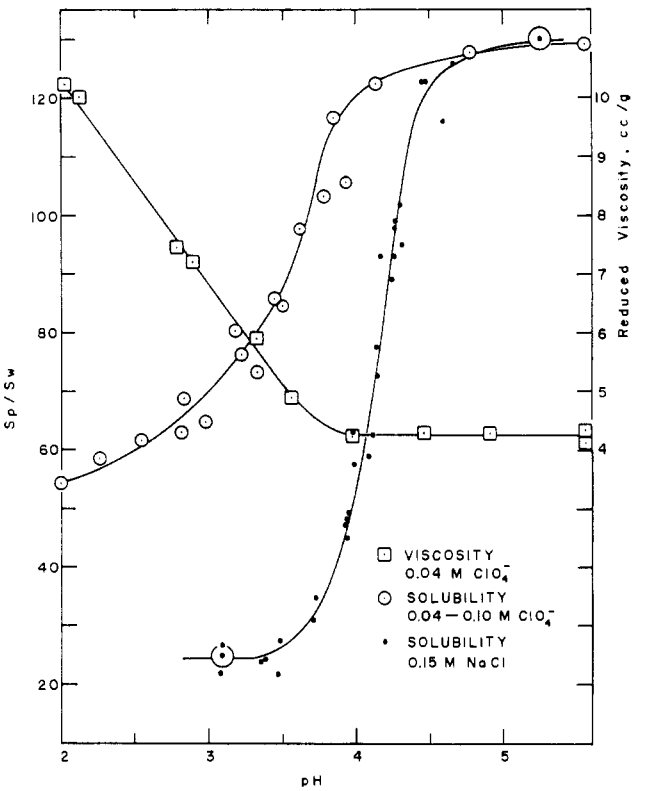

FIG. 5.—The effect of perchlorate on pentane binding. 25°. ⊙, (S_p/S_w) in perchlorate. ⊡, reduced viscosity. . . . ., the most recent run in 0.15 M NaCl. The ionic strength in the solubility run increases more or less uniformly from 0.04 M ($KClO_4$) at *p*H 5 to 0.10 M (protein$^+$, H^+, ClO_4^-) at *p*H 2.

mechanisms very seriously. Wetlaufer and Lovrien have proposed alternatives, which they concede to be unlikely, in which the binding of alkanes, by lowering the local dielectric constant, strengthens the interaction between ionic or dipolar groups. The fact remains that the transfer of charges, dipoles, or ion pairs from a medium of higher to one of lower dielectric constant *always* has a positive ΔF, and such a situation cannot facilitate binding. The question is rather whether electrostatic interactions will seriously interfere with the normal mechanisms of binding apolar solutes. Tanford (1957b) has shown that the energy of interaction of charged groups in proteins is almost independent of the volume of the low-dielectric-constant sphere in which they are embedded. If the alkane penetrates to an interior site without changing the effective depth of the charges or the distance between them, ΔF_{el} will be zero; if the distance between charges changes slightly, there will be a quite small change in the electrostatic free energy of the system. It may be that this argument can be extended qualitatively to surface binding, which can be considered to change the local radius of curvature of the protein. If we use Lovrien's model (1963) correctly,[4] we can calculate that binding a

[4] Lovrien's energy estimates are too high by a factor of 4π. (In transforming Stratton's equations expressed in m.k.s. units and using inductive capacities ϵ, in farads/meter, into forms containing electrostatic units and dimensionless dielectric constants, an extra 4π appears to have been retained. The proper estimate of the work of introducing a sphere of radius r, dielectric constant D_2, into a medium of dielectric constant D_1, at a point of distance b from two like charges, using his model, is $W = (D_1 - D_2)/[D_1(D_2 + 2D_1)]$ $(q^2/2\pi\epsilon_0)(r^3/b^4)$ joules/molecule or $W = 3.86\ R^3/B^4$ kcal/

(Continued on following page)

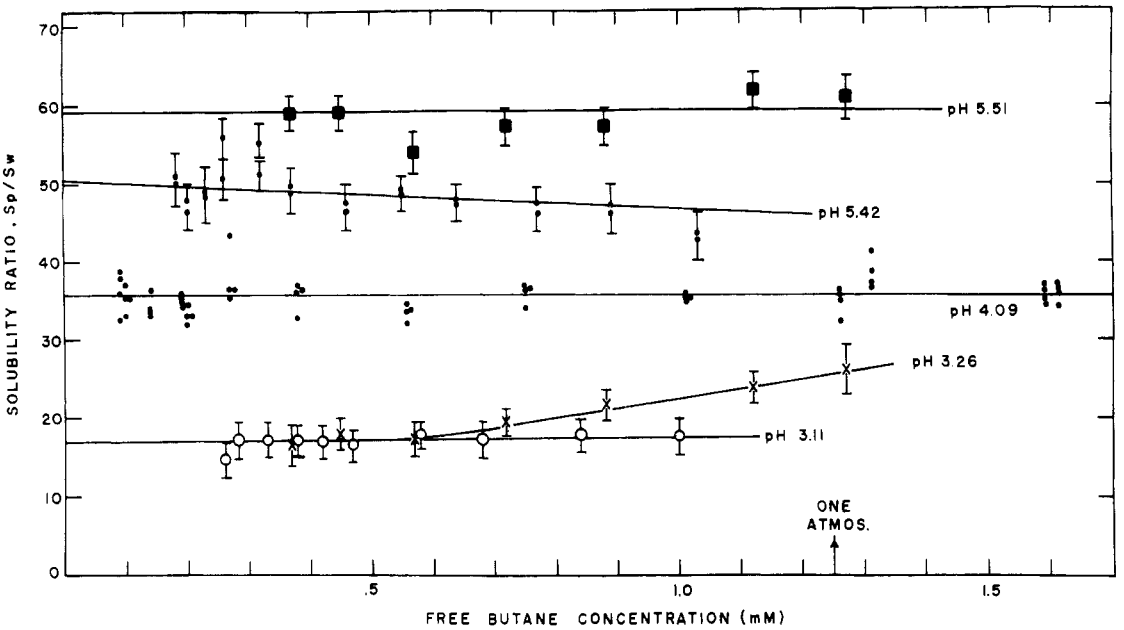

FIG. 6.—Butane binding to BSA as a function of butane concentration. 25°, 0.15 M NaCl. *p*H as indicated. Ordinate, (S_p/S_w). Abscissa, calculated free butane concentration, mM. The divergence between the *p*H 5.51 and 5.42 data comes from systematic differences between samples of gas and protein (see text and Fig. 3).

molecule the size of butane to the surface between two like charges would involve electrostatic energies less than 0.4 kcal when their separation exceeds 6 A. If increased solubility arose from the interaction of highly polarizable solutes with the fields of ionic groups on proteins, one would expect such "binding" to be quite general (the forces involved would be of much shorter range than coulombic, so that the interaction would be essentially between individual charged groups and the polarizable solute). In fact, neither ribonuclease nor lysozyme binds butane to a measurable extent, and the behavior of β lactoglobulin is very different from that of BSA; we should also expect the solubility of alkanes in water to increase with salt concentration, contrary to fact (Morrison and Billett, 1952). There is no evidence that BSA has any, much less many, regions of unusually high charge density (see Tanford, 1957a).

The mechanisms of binding involving apolar residues are of two general kinds. The first requires direct contact between the alkane and some apolar region of the protein, accompanied by a net decrease in water-hydrocarbon contact; in the second, a layer of water molecules intervenes, and the interaction is between the alkane and partial clathrate cages surrounding the apolar residues on the protein surface. (Kauzmann, 1959, would approach the problem from the first point of view; Klotz, 1958, 1960, from the second.) Models for these two cases are, respectively, transfer of alkane from water to detergent micelles (Wishnia, 1963a), and transfer to the crystalline gas hydrates (see Van der Waals and Platteeuw, 1959). These models are, of course, extreme; we would not expect a molecule bound to protein to be as unrestricted as one contained in a micelle, nor would we expect the precise geometry of the gas hydrates. Rather, we can construct a series: penetration of alkane into the protein interior, penetration into surface clusters, binding in a surface crevice, binding on the protein surface with formation of a more favorable ice cage, and binding with completion of a partially formed clathrate cage. Nevertheless the actual binding will resemble, more or less, one of the extremes; we must determine what kinds of behavior are characteristic of each.

The negative free energy of transfer of alkanes from water to dodecylsulfate arises almost entirely from the entropy term (Wishnia, 1963a), as one might have expected (Kauzmann, 1959; Némethy and Scheraga, 1962). For butane at 25°, $\Delta H_{trans} = 0$, $\Delta S = +17$ eu. The transfer of butane from water to BSA (the quantity S_p/S_w) has the same temperature dependence, even to the slight curvature of the van't Hoff plots that indicates a small negative ΔH above 25° and a small positive ΔH below 25°. The gas hydrates, on the other hand, are characterized by large negative heats of formation: methane, −14.5 kcal/mole solute (26.0 atm, 0°); ethane, −16.3 (5.2 atm); propane, −32 (1.74 atm) (Van der Waals and Platteeuw, 1959); and, since ΔF is zero, by correspondingly large negative entropies. Of course, at these pressures, $-\Delta H$ exceeds $-T\,\Delta S$ below 0°, while the reverse is true above 0°. If we accept the calculation of Platteeuw and Van der Waals (1958), the heat content of the water lattices is the same as ordinary ice; the greatest part of ΔH comes from the heat of fusion of ice. The reaction ethane (5.2 atm) + (approx) 7 $H_2O_{(l)}$ → ethane·7$H_2O_{(s)}$ may be separated into several processes:

(1) $n\ H_2O_{(l)} \rightarrow n\ H_2O_{(s)}$, $\Delta H_1 = -n(\Delta H_f) = 10$ kcal
(2) ethane$_{(g)}$ → ethane$_{(apolar\ solvent)}$, $\Delta H_2 = -2$ kcal
(3) ethane$_{(apolar\ solvent)}$ → ethane$_{(aq\ soln)}$, $\Delta H_3 = -2$ kcal
(4) ethane$_{(aq\ soln)}$ + $n\ H_2O_{(s)}$ → ethane·$n\ H_2O_{(s)}$, $\Delta H_4 = -2$ kcal

Propane does not fit the small cavities of the type I lattice; for the type II lattice n and $-\Delta H_1$ are much larger. Hydrates of butane and pentane have not been reported. We have specifically included step 3 for comparison, because the reverse of step 3, with small

mole where q is the electronic charge in coulombs, ϵ_0 is the permittivity of free space in farads/meter, and r and b are in meters; R and B are in angstroms. If $R = 3$ and $B = 4$ A, $W = 0.4$ kcal; the distance between the charges would be twice $[4^2 - 3^3]^{1/2}$, or 5.3 A, if the charges are at the surface of a plane, and less if the charges are below the surface. As Lovrien says, the simplifying assumptions break down at short distances, so that these numbers are approximate. The average distance between charges of any sign on BSA is 8 A (Tanford, 1957a): for that value, $W \leq 0.17$ kcal.

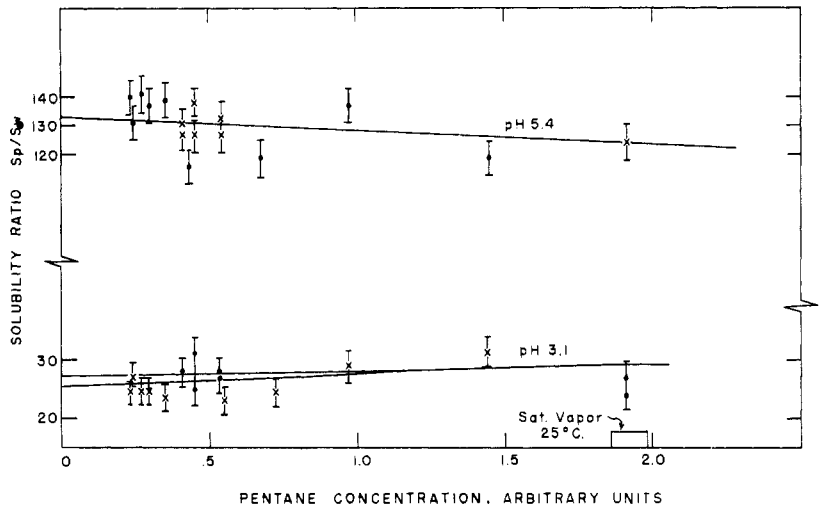

FIG. 7.—Pentane binding to BSA as a function of pentane concentration. 25°, 0.15 M NaCl. Ordinate, (S_p/S_p). Abscissa, free pentane concentration in arbitrary units (approximate concentration in equilibrium with saturated vapor is shown).

positive enthalpy and large positive entropy, corresponds to the first binding mechanism. The combination of steps 1 and 4 is the analog of the second type of binding. Now, there are not more than forty preformed cages (unstable with respect to ice at 0°, certainly with respect to water at higher temperatures) on BSA, yielding only the equivalent of ΔH_4 when one is occupied by butane. If butane is bound by the clathrate mechanism it can be only by completion of partial cages. The point of this analysis is that the formation of such structures necessarily involves a net decrease in the heat content of the water forming the cage with respect to liquid water, over and above the decrease brought about by introducing the alkane in step 3, so that, even if ΔH_4 is small, the binding of butane by this mechanism would be marked by a substantial negative ΔH, contrary to what is observed.

All the direct-contact mechanisms should exhibit the observed temperature dependence, provided, on the one hand, that surface binding did not increase cage formation (which would have the consequences discussed), and on the other hand, that the conformation changes induced by penetration were limited to the apolar region and did not require rearrangements of the protein framework (for which, near the isoionic *p*H, there is no evidence [Wetlaufer and Lovrien, 1964]).

The van der Waals interactions between alkyl residues are not strongly directional, in contrast to hydrogen bonding; given the constraint imposed by attachment to the peptide chain, the configuration inside a cluster is adjusted primarily to minimize free volume and contact with water. The ability to refold to include a molecule of alkane would, in general, but not in every instance, increase with the size of the cluster. If the internal relationships of other parts of the protein were not disturbed (for example, because the peptide chains were folded into the rigid slabs of Foster's model), the energy requirements for such refolding would be quite small. Binding of alkanes inside BSA is not unreasonable, but we have not yet shown that it occurs.

It is necessary to consider, first, whether exposure of apolar residues to the solvent contributes to the thermodynamic stability of proteins, and second, the first considerations notwithstanding, whether the surface of BSA contains patches of apolar residues of sufficient extent to account for the observed pressure dependence of butane and pentane binding.

The transfer of an alkyl residue on a random coil, fully exposed to the solvent, to the hydrophobic interior of a compact protein is analogous to the transfer of an alkane from water to the interior of a micelle; the relative restrictions on the translational motion of the residue probably come close to cancelling. If the vibrational and internal rotational motions of the residue are not greatly restricted inside the protein, the transfer will contribute 15–20 eu toward stabilizing the compact configuration. Leaving the residue on the surface of the protein, still partly in contact with solvent, would contribute some fraction of the total entropy gain. Neither of these transfers has any really stringent steric requirements. To estimate the thermodynamic quantities for the transfer of an isolated alkyl residue exposed to solvent to a set of cooperating solvent cages, we return to the gas hydrates. At equilibrium at 0° the overall ΔF is zero; if ΔF_1 is also zero, as Platteeuw and Van der Waals (1958) maintain, and $\Delta F_{2,3} = +4.3$ kcal for ethane (5.2 atm) → ethane (aq), then $\Delta F_4 = -4.3$ kcal for ethane (aq) → ethane (clathrate), suggesting that the analogous transfer of an isolated residue would be favored. Note that the favorable ΔF for (1) and (4) comes from (4), and is mostly entropic, like the comparable transfer of ethane from water to hydrocarbons. However, once again, because of the contribution of the heat of fusion to ΔH, the temperature dependence of this transfer would be strong, and we would expect striking melting phenomena at temperatures somewhere between 0° and, say, 50°. However, there is a further point. The equilibrium pressure of the three-phase system (gas, water, hydrate) increases so steeply with temperature that most hydrates have a maximum temperature (5.7° for propane, 14.5° for ethane, 29.5° for H_2S). This temperature also corresponds to the intersection with the gas-liquid-water phase diagram; at higher temperatures, in part because of a new positive ΔF_1, transfer of solute and water from the hydrate to separate liquid phases is favored. To estimate the relative contribution of these phenomena to the stability of clathrates in proteins we must leave the safe shore of phase equilibria for the swamp of pseudophases in proteins. Our guess is that cage structures may be important in special cases where the geometry is good and several "Hilfsgase" analogs like –SH, –OH, and $-CH_3$ are present (as Klotz, 1960, implied), but not in general. What can be said, quite strongly, is that the *N-F* transition, which is almost independent of temperature,

cannot be accompanied by any increase or decrease in such structures, if they exist.

Therefore the enormous solubility decrease on going over to the *F* form requires either the disappearance of hydrophobic surfaces, if these are the site of binding, or the disruption of hydrophobic volumes, if the alkanes are bound therein. The disappearance of hydrophobic surfaces cannot arise through cohesion of several of such regions, since the resulting apolar volumes of demonstrated flexibility would necessarily bind alkane quite well. The process of cohesion would have a negative ΔF derived from a positive ΔS, requiring the *N* form to be unstable with respect to the *F* form even without electrostatic repulsions; however, since the tendencies in BSA are clearly in close balance, we do not wish to rely heavily on this consideration. An effective decrease in binding to surfaces could be brought about by separating the individual apolar residues and exposing them to solvent. It is difficult to see how such changes would not require unfolding that part of the peptide backbone, resulting in much greater changes in $[\alpha]$ and $[\eta]$ than observed. It should also be recalled that studies of solvent-perturbation difference spectra (Herskovits and Laskowski, 1962) indicate that the "equivalent exposure" to solvent of aromatic residues increases from 30% in the *N* form to 50% in the *F* form, leaving the equivalent of 50% still buried.

What remains is a discussion of the dodecylsulfate binding data (see Foster, 1960). At low concentrations SDS appears to be bound to native BSA at ten strong sites. Higher concentrations of SDS induced marked structural changes in BSA; the binding curve has been interpreted as showing the appearance of 100 weaker sites. The model for the *F* form contains only the 100 weak sites. Now, the notion of 100 independent sites for binding $C_{12}H_{23}OSO_3^-$ on a molecule the size of BSA is hardly realistic (100 molecules of dodecylsulfate would form a complete shell around a BSA sphere). It is much more likely that hydrophobic surfaces have been exposed in the *N-F* transition, and that the conformation change in native BSA at high levels of SDS also involves disruption of hydrophobic regions; under the conditions (e.g., 2 mg/ml BSA, upward of 0.1 mg/ml SDS), the critical micelle concentration for the system SDS-everted protein would have been exceeded, because the exposed hydrocarbon residues can nucleate micelle formation and decrease the critical micelle concentration in the way that many other solutes (e.g., lauryl alcohol) would. The disruption of hydrophobic clusters would have the opposite effect on alkane binding: one would not expect cooperative behavior akin to phase separation from butane and pentane at these pressures and temperatures, while the original binding sites have disappeared.

To summarize, we can best explain the interaction of butane and pentane with native BSA in terms of binding within relatively large (or with less likelihood, relatively many) hydrophobic regions. The integrity of these regions is disrupted in the transition to the *F* form. Although several proteins fit such a model, this mechanism is not universal. The tryptophanyl residues of lysozyme (Laskowski, *et al.*, 1962) are fully exposed to solvent (although nothing is known about the aliphatic residues); lysozyme does not bind butane. The apolar groups of myoglobin are truly hydrophobic (few appear on the surface, and large interior clusters are present [Kendrew, 1963]). Myoglobin, in its several states, binds alkane as well as hemoglobin. (However, the pressure dependence is not yet known.) Ribonuclease appears to have apolar regions in its interior (for example, Anfinsen, 1962), but does not bind butane; perhaps these structures are too constrained to enfold another molecule. The behavior of β-lactoglobulin is strongly indicative of constrained sites; binding in crevices or in small surface clusters is very likely (Wishnia, 1964).

References

Alfsen, A. (1963), *Compt. Rend. Trav. Lab. Carlsberg 33*, 415.

Anfinsen, C. B. (1962), *Brookhaven Symp. Biol. 15*, 184.

Aoki, K., and Foster, J. F. (1956), *J. Am. Chem. Soc. 78*, 3538.

Bray, G. A. (1960), *Anal. Biochem. 1*, 279.

Bro, P., Singer, S. J., and Sturtevant, J. M. (1955), *J. Am. Chem. Soc. 77*, 4924.

Dole, V. P. (1956), *J. Clin. Invest. 35*, 150.

Foster, J. F. (1960), *Plasma Proteins 1*, 179.

Foster, J. F., and Clark, P. (1962), *J. Biol. Chem. 237*, 3163.

Herskovits, T. T., and Laskowski, M., Jr. (1962), *J. Biol. Chem. 237*, 2481.

Kauzmann, W. (1959), *Advan. Protein Chem. 14*, 1.

Kendrew, J. C. (1963), *Science 139*, 1259.

Klotz, I. M. (1958), *Science 128*, 815.

Klotz, I. M. (1960), *Brookhaven Symp. Biol. 13*, 25.

Laskowski, M., Jr., Herskovits, T. T., and Williams, E. J. (1962), Abstracts 142nd meeting, American Chemical Society, September, p. 55c.

Leonard, W. J., and Foster, J. F. (1961), *J. Biol. Chem. 236*, 2662.

Leonard, W. J., Vijai, K. K., and Foster, J. F. (1963), *J. Biol. Chem. 238*, 1984.

Lovrien, R. (1963), *J. Am. Chem. Soc. 85*, 3677.

Morrison, T. J., and Billett, F. (1952), *J. Chem. Soc.*, 3819.

Némethy, G., and Scheraga, H. A. (1962), *J. Chem. Phys. 36*, 3401.

Platteeuw, J. C., and van der Waals, J. H. (1958), *Mol. Phys. 1*, 91.

Strauss, G., and Strauss, U. P. (1958), *J. Phys. Chem. 62*, 1321.

Tanford, C. (1957a), *J. Am. Chem. Soc. 79*, 5340.

Tanford, C. (1957b), *J. Am. Chem. Soc. 79*, 5348.

Tanford, C., Buzzell, J. G., Rands, D. G., and Swanson, S. A. (1955a), *J. Am. Chem. Soc. 77*, 6421.

Tanford, C., Swanson, S. A., and Shore, W. S. (1955b), *J. Am. Chem. Soc. 77*, 6414.

Van der Waals, J. H., and Platteeuw, J. C. (1959), *Advan. Chem. Phys. 2*, 1.

Wetlaufer, D. B., and Lovrien, R. (1964), *J. Biol. Chem. 239*, 596.

Wishnia, A. (1962), *Proc. Natl. Acad. Sci. U. S. 48*, 2200.

Wishnia, A. (1963a), *J. Phys. Chem. 67*, 2079.

Wishnia, A. (1963b), Abstracts, 7th annual meeting, The Biophysical Society, February, p. MA11.

Wishnia, A. (1964), *Federation Proc. 23*, 160 (abstr. 357).

Yang, J. T., and Foster, J. F. (1954), *J. Am. Chem. Soc. 76*, 1588.