Protein Topography by Calorimetry

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Usage of the calorimeter to characterize spatial arrangement of proteins has been limited, in part, by the unavailability of the micro-level ($\lesssim 10^{-6}$° temperature change) instrumentation required by the scarcity of working material. In greater part, difficulty in the development of suitable theoretical models has been at issue. Instrumentation of appropriate sensitivity and suited to protein characterization now exists. Both adiabatic and isothermal devices have been designed (though for some heat capacity measurements greater sensitivity in consonance with high accuracy is needed and apparently beyond the ability of present technology). Topographic studies of proteins have been based either upon the discrimination of alternative theoretical models or upon the analysis of forces which stabilize the native protein structure. The literature includes studies of lysozyme, chymotrypsin, hemoglobin, and, less extensively, many other proteins and polypeptides. Analogous studies of nucleic acids, carbohydrates, and other biological molecules have served to provide a technical basis and a theory appropriate to the study of protein topography *per se*.

Calorimetry, employing no less formidable a tool than the Bunsen ice calorimeter, provided experimental support for the thesis that quantitative methods of physics and chemistry could be applied to biology. The fact that the mouse liberated heat proportionate to the caloric intake of food served in an important way to disprove the then dominant vitalistic view of biology. Thermodynamics, direct as well as indirect, has been applied to most problem areas of biochemistry; hence, to suggest here that thermal measurements can provide evidence for topography of a protein hardly represents a radical idea. Though the literature is relatively limited, much work can be shown to bear upon the problem; for the most part this work consists of indirect measurements and thermodynamic calculations, but there is a firm beginning of direct calorimetry.

To avoid the variety of complications which reside with the terminology of protein spatial relationships, it would seem useful to use the term denaturation, as Kauzmann (1959) does, as "a process in which the spatial arrangement of the polypeptide chains within the molecule is changed from that of a typical protein to a more disordered arrangement." Since small changes in spatial arrangement would hardly be recognized by conventional tests of denaturation, it is useful perhaps to refer to all such changes (configuration, conformation, state of folding, and subunit aggregations) as topographic.

The sparsity of direct measurements which could be applied to the logical choice of alternative models for protein topography or in other ways could be used to serve in establishing topography by calorimetry has been, in a great part, the result of the difficulty of developing suitable theoretical models. It may be illustrative of the difficulty to allude to the conjectural points which have plagued the attempt to know, in physiologically useful terms, the hydrolysis heats of phosphate esters. This relatively simple problem has proved itself fraught with hazard when the attempt has not been to make a physical chemical measurement within well-defined conditions, but rather to allude to a physiologically plausible situation. It is commendable, however, that despite difficulty, thermodynamicists have built models of proteins which can be tested by calorimetric measurement.

Topographic studies of proteins have been based largely upon the discrimination of alternative theoretical models for the purpose of describing protein denaturation or upon a rational analysis of the forces impingent upon protein stabilization. The potential of the tool with respect to proteins can be partially defined also by reference to nonprotein model systems which seem to offer appropriate illustrations of the technology. Nucleic acids and carbohydrates have been studied by direct calorimetry and the problems encountered and the data obtained may be analogous to aspects of protein-topographic calorimetry.

Application of calorimetric measurements to characterization of proteins of biological interest has traditionally been limited also by the unavailability of micro-level ($\lesssim 10^{-6}$° temperature change) instrumentation required by the scarcity of working material. Instrumentation of appropriate sensitivity and suitable to many aspects of the characterization of proteins now exists (though for some heat capacity measurements—to be made with extremely dilute solutions—greater sensitivity in consonance with high accuracy is needed and apparently often beyond the ability of present designs). Both adiabatic and isothermal devices of good sensitivity and useful handling characteristics have been designed and applied to the characterization of biologically significant molecules.

TYPES OF MEASUREMENTS

General. Evaluation of spatial relationships in proteins using thermal measurements may be based upon choice of alternative models of known character or it may involve the inductive analysis of forces which maintain the form of the

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native protein. The latter approach is probably the more aesthetically satisfying, but there are, of course, many different kinds of functional groups present in proteins; presumably in the aqueous environment of the cell, resolution in terms of the nature of the force (or forces) represented is difficult. Several different types of internal bonds are involved in the stabilization of the protein molecule, and the relative importance of these types may be expected to differ in different proteins.

In general, the assumption has been made that if there are two functional groups which adhere to one another in an aqueous environment, then these same two groups on a protein should tend to be in association at equilibrium. Hence, using the equilibrium constant of the reaction, the classic relationship, $\Delta G = -RT \ln K$, provides a quantitative measure of the strength of the bond. All of the reasoning which can be extrapolated from this relationship has been invoked in various studies. (One must make the rather broad assumption that there are no interfering phenomena which occur.) Where protein stabilizing forces are to be evaluated, thermal change, change of pH, or other physical parameters can be used, as appropriate, to involve the bonds responsible for maintaining the spatial arrangement of the protein. The forces include hydrogen bonds, hydrophobic bonds, electrostatic forces, primarily ionic bonds, cross linkages (usually disulfide bridges), stabilization by electron delocalization, dispersion (London) forces, and possibly others.

The former of the two approaches mentioned above is based upon situations in which a configurational change is known (*e.g.*, from optical rotatory dispersion or other instrumental studies) and thermal data is then related to the known configurational state. This category also extends to experiments in which distinct change of state is brought about thermally (melting) or chemically (*e.g.*, urea denaturation).

Establishing a spatial arrangement change as a preliminary to interpreting thermodynamic properties is an approach which offers a very great variation in the experimental mode. This variety of experimental attack is best illustrated by description of data, though some generalization here may be useful. The greatest effort has involved heat capacity calorimetry wherein a temperature profile over a scanning range was recorded. There is an association of increase in heat capacity with unfolding of the protein molecule, presumably as a result of exposure of nonpolar residues to the aqueous medium. Figure 1 is a diagram illustrating a heat capacity change at the temperature range in which helix→coil transformation occurs.

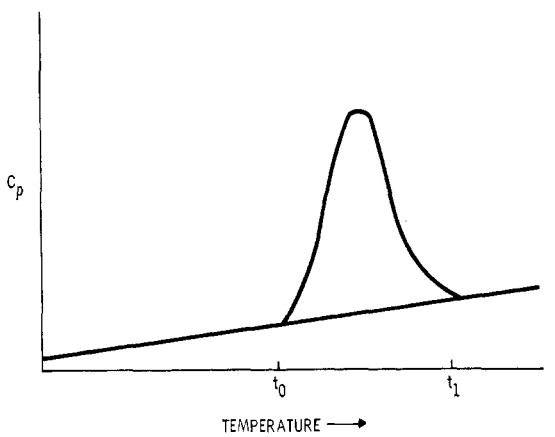

Figure 1. Temperature dependence of heat capacity is evident in the temperature range ($t_0 \rightarrow t_1$) of the transition

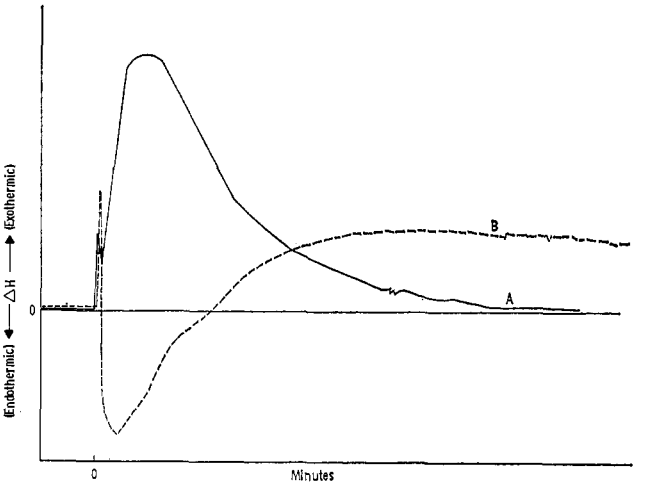

Figure 2. (A) Calorimetric tracing of exotherm produced when asparagine was hydrolyzed *via* asparaginase catalysis; (B) In a similar experiment the protein was restricted to a polymeric matrix [see text] from Stasiw and Brown (1970)

Variations on these two main themes, choice from alternative models or analysis of stabilizing forces, have been described. One such useful experiment involves enzymatic reactions which proceed with retention of configuration of the substrate. Such studies allude to protein topography, though elucidation of the enzyme mechanism was the stated goal of investigators. Inhibitor binding has been studied in an extension of this same logic (Canady and Laidler, 1958; Bjurulf *et al.*, 1969, 1970). These workers altered the structure of inhibitors which were bound to the enzyme active centers and correlations were made between the heat effects and conceptions of the mode of binding. Since it is probable that configurational changes of the protein accompany bindings to substrates or competitive inhibitors of the type used, it is also probable that heat capacity measurements for specific couplings will provide information concerning configurational changes as a result of the bindings.

A more or less unique approach in our laboratory, still qualitative, lends some promise. Catalytic proteins can be restricted by forming derivatives with polymeric matrices. Depending upon the number of bonds between the enzyme molecule and the matrix, the protein is restricted and its ability to change its spatial interrelations is lessened. Figure 2(A) is a calorimetric tracing (Stasiw and Brown, 1970) of the exotherm produced when asparagine was hydrolyzed by asparaginase. In a similar experiment, [Figure 2(B)] the protein molecule was restricted to a support matrix (Hasselberger *et al.*, 1970). The tracing may be described as a brief exotherm—the reaction catalyzed by asparaginase solution in the reaction vessel, followed by an endothermic binding heat of substrate to the polymer, and finally, the exotherm resulting from asparagine deamidation catalyzed by the restricted enzyme. No doubt what is primarily involved in this result is a consequence of the restriction of the approach of the substrate to the protein and hence a lowering of the affinity of the substrate, which converts a relatively rapid reaction to a slower one. Because protein configurational change can be restricted while allowing the molecule to remain catalytically active, the technique may be appropriate to this question.

Stabilizing Forces. HYDROGEN BONDS. X-Ray crystallography and ir spectroscopy have provided direct evidence for the existence of interpeptide hydrogen bonds as significant intramolecular forces in the solid state. Where water provides competing groups, the stabilizing effect of such bonds is

uncertain. The intrinsic stability of bonds between the oxygen atoms of the carbonyl groups and the hydrogen atoms of the amide groups of peptide linkages in water actually has not been accurately measured. Schellman (1955a) calculated the free energy of formation using aqueous urea solutions as a model. Klotz and Franzen (1960) compared adsorption of model amides in various inorganic solvents *vs.* water systems, and concluded that aggregations to hydrogen bonding are essentially nonexistent as long as enough water molecules are available to occupy the N—H and C=O groups. This is not an entirely satisfying conclusion in terms of attempts to build models involving hydrogen bonds as stabilizing groups. It is, however, in essential agreement with the conclusion of Schellman (1955b) that "hydrogen bonds, taken by themselves, give a marginal stability to ordered structures, which may be enhanced or disrupted by interactions of side chains." Other more recent papers have provided enthalpy values for the adducts of phenol derivatives or aliphatic alcohol with carbonyl molecules and ethers which were determined by combinations of calorimetric measurements and spectroscopically determined equilibrium constants. Duer and Bertrand (1970) have compared methods (Arnett *et al.*, 1967; Drago and Epley, 1969; Lamberts, 1965) of determining hydrogen-bonded heats of formation using systems in which inert solvents are essential. While these papers provide a basis for the construction of testable models in calorimetric measurements based upon the assumption that hydrogen bonding is a force in the stabilization of protein interspatial relationships, the development of such models must preclude a truly aqueous environment for the protein. Although many of our suppositions about intracellular relationships justify this, we should certainly be subject to charges of academic apartness were such models to be employed without a suitable juxtaposition to proteins in physiological-like situations. If proteins in the biological cell are stabilized by interpeptide hydrogen bonds, then the present evidence would indicate they must largely be protected from water. This restriction to the design of calorimetric experiments may not be insurmountable, but it is certainly significant.

Hydrogen bonds other than those between peptide links have been considered by Laskowski and Scheraga (1954) and Nemethy *et al.* (1963). They have discussed the effect of side-chain hydrogen bonding upon the reactivity of polar groups in proteins. In their theoretical models the enthalpy of formation of hydrogen bonds has been related to competitive hydrogen bonding with water, except for groups surrounded by nonpolar groups. In aqueous environments the polar groups form more than one hydrogen bond with water. Various other effects of the presence of nonpolar groups and changes in the water structure were factors considered. Generally, it was concluded that hydrophobic interactions enhance the strength of hydrogen bonds.

This sort of theoretical evaluation can certainly be of use in the construction of models for calorimetric testing involving hydrogen bonds between reactive elements of the side-chains. Free energy of bond formation involving both hydrophobic interaction and hydrogen bond formation may range, according to the calculations of Nemethy *et al.* (1963), from about -0.5 to -2.4 kcal/mol. It may reach much more highly-negative values for large structures. The corresponding ranges of the net enthalpy and entropy formation were calculated to be -1.5 to 0.5 kcal/mol and -3 to $+7$ eu, respectively.

Hydrophobic Bonds. Hydrophobic bonding is the adherence to one another of nonpolar groups of proteins in aqueous environments. Since amino acids with nonpolar

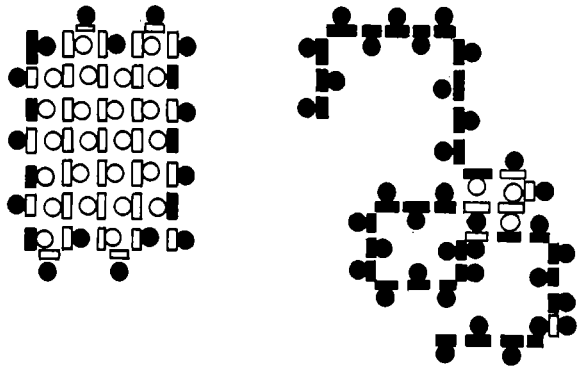

Figure 3. Schematic diagram of native and unfolded states of a protein molecule. The change in the relationship of the internal groups to solvent is illustrated (modified from Tanford, 1964). Reprinted with permission of: *J. Amer. Chem. Soc.* 86, 2050 (1964)

side-chains (valine, leucine, isoleucine, phenylalanine; perhaps proline, alanine, tryptophan) represent roughly 40% of most proteins, this tendency to form intramolecular aggregates is by no means inconsequential, and it is probable that the hydrophobic bond stabilizes the folded configuration in many proteins. Thermodynamic changes which occur in the transfer of hydrocarbons from a nonpolar solvent to water are exothermic (*e.g.*, $\Delta H = 2500$ cal/mol CH_4 transferred from CCl_4 to H_2O; Frank and Evans, 1945) for the aliphatic compounds and have $\Delta H = 0$ for aromatic ones, invariably accompanied by a very large decrease in entropy. Hence, unlike the situation which would arise if hydrogen bonds were broken by the introduction of the nonpolar molecule into water, the low affinity of nonpolar portions of proteins for water is not caused by an unfavorable energetic situation but rather is associated with a large entropy change. It has been estimated (Kauzmann, 1959) that for each nonpolar aliphatic side-chain transferred from an aqueous environment to a nonpolar region of the protein, a gain of 20 eu occurs; the phenomenon is endothermic (1–2 kcal/mol group) and the free energy is exergonic (3–5 kcal/mol group). The characteristics of the dissociation of subunits of proteins, discussed at length by Klotz *et al.* (1970), in some instances appear to represent a similar phenomenon. Figure 3 is a schematic representation of a native and unfolded protein molecule. The diagram puts forward the great change in hydrophobic interaction which can result from the change in relationship of internal groups to the solvent.

Electrostatic Forces. Like hydrophobic bonds, salt linkages and other electrostatic interactions are stabilized by entropy rather than energetic effects. However, fortunately for the calorimetrist, the experimental design can be constructed to descriminate electrostatic interactions from hydrophobic interactions. This expectation is based upon the fact that these forces should respond in opposite directions to the addition both of electrolytes and nonpolar substances to the medium. Salt linkages should be strengthened by lowering the dielectric constant of the medium, while hydrophobic bonds are weakened by adding a nonpolar substance to the aqueous medium. Because electrolytes decrease the solubility in water of nonpolar portions of proteins, they should serve to strengthen hydrophobic bonds but weaken salt linkages. Although the importance of electrostatic forces, *e.g.*, between positively charged amino and guanidino groups and negatively charged carboxyl groups, has been debated (Jacobsen and Linderstrøm-Lang, 1949), the relative ease with which we should be able to identify their contribution calorimetrically may make the discussion academic to the experimentalist.

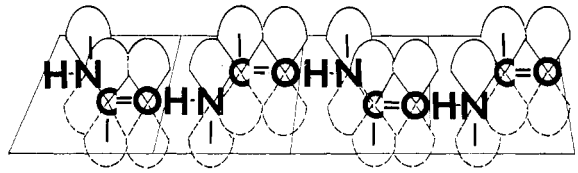

Figure 4. π Orbitals with axes perpendicular to peptide groups can be assumed to be available on N, C, and O atoms of each peptide bond. Molecular orbitals constructed from them serve to present an illustration of electron delocalization [drawn from the treatment of Evans and Gergley (1949) as synthesized by Kauzmann (1959)]

CROSS LINKAGES. Cross linkages reduce the total number of configurations available to a protein chain so that a smaller entropy gain is made in going from a folded configuration to a random coil form. Hence, if a configuration is possible when cross linkages are introduced, then that configuration is stabilized by the cross linkages (Schellman, 1955b).

ELECTRON DELOCALIZATION AND DISPERSION FORCES. Evans and Gergely (1949) have made an estimate of the effects of delocalization based upon a molecular orbital approach (Figure 4). These authors concluded that stabilization by electron delocalization contributed significantly to the energy of the chain configuration of proteins. The magnitude of this contribution depends upon the number of peptides that are hydrogen bonded to one another. This contribution could conceivably be as high as several hundred calories per peptide residue. The studies of Scatchard *et al.* (1939a,b, 1940) indicate that proteins having aromatic rings (phenylalanine, tryptophan) and aliphatic side-chains (leucine, valine, alanine) will tend to have clustering of these groups as a function of so-called London (dispersion) forces (Waugh, 1954). Hence, chain configurations which allow segregation of aliphatic groups from aromatic groups should, based upon this reasoning and experimental evidence, be more stable than comparable configurations which do not allow such segregations.

INSTRUMENTATION WHICH CAN BE BROUGHT TO BEAR UPON ELUCIDATION OF PROTEIN TOPOGRAPHY

Reaction Calorimeters. INSTRUMENTS CAN BE CONSIDERED UNDER THEIR GENERIC TYPES: ADIABATIC. There is no true adiabatic instrument commercially available which appears suited to work at the micro-level. Good working designs have been published by Sturtevant (1940), Buzzell and Sturtevant (1948, 1951), Privalov (1969), and Ackermann (1969a,b). The instrument of Izatt and Christensen, which is now available commercially from Tronac, Inc., though "macro" in proportions, may be modifiable to utility for fairly low heat-level work. Like other precision adiabatic microcalorimeters, this instrument requires elaborate circuitry for the provision of compensatory heat for that lost from the reaction cell. Although the intricacies of effective true adiabatic reaction microcalorimeter design have proven sufficiently expensive to retard commercial interest, pseudo-adiabatic instruments are relatively inexpensive and may, for many applications, represent an appropriate choice. The instrument described by Berger *et al.* (1968) and Davids and Berger (1969) is offered by Science Products. In this design (essentially a downscaling of the familiar Lange-type macrocalorimeter) the departure from genuine adiabatic behavior has been analyzed by a computer program, and a program has been developed to correct the data (Berger and Davids, 1965).

ISOTHERMAL. True Isothermal. The true isothermal instrument is not being actively used by most microcalorimetrists, though good armchair designs are easily made, and

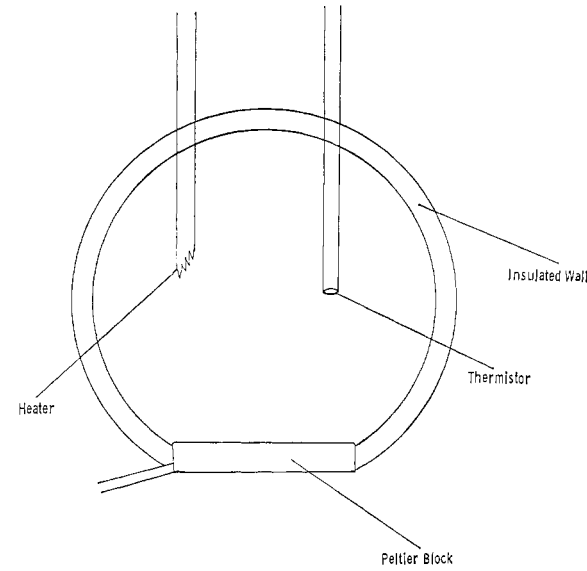

Figure 5. Elements of an isothermal design using the continuous heat pumping action of a Peltier unit. The diagram is a schematized version of the design discussed by Izatt and Christensen (1969)

the intrinsic merit of the isothermal condition for many types of studies leads us to hope that the renaissance is not far away. An interesting new unit (Izatt and Christensen, 1969) which may point the way is based upon the heat pump action of a commercial Peltier cooling block (Figure 5). In operation the container is in contact with the cold junctions of the Peltier unit so that heat is pumped out at a constant rate. The rate always exceeds that of heat production in the reaction; thus, measurement of the heat produced in the reaction is the difference between the heat added to the cell (by a low inertia heater) in the absence of a reaction and that added during the reaction. The potentiality of the design for the construction of a simple micro-isothermal calorimeter exists, though difficulties in the use of a point source detector and its consequent reliance on the stirrer must be overcome. This cell, which presently requires 10 ml of solution, may soon be available from the Utah group.

Pseudo-Isothermal. An instrument which might be classified with the pseudo-isothermal instrument is the crystal thermometer offered by Hewlett-Packard. This has been applied in microcalorimetry configurations. Although the appropriate accessories for its use as a calorimeter are not stock items, presumably they can be supplied by the manufacturer. This design makes use of the temperature dependence of a crystal in a conventional radio frequency oscillator circuit. The change in frequency is read following frequency to voltage conversion by digital voltmeter directly on a temperature scale.

The British Microscal instrument is used as a pseudo-isothermal device. It is a Wheatstone bridge in which the thermistor is the sensing element. Use of the thermistor in a very small cell configuration has obvious limitations, and no doubt the instrument departs widely from isothermicity. It is nonetheless a highly sensitive device and the restrictions imposed by this physical arrangement are not insurmountable. In its present configuration one of the reactants must be solid and the other a liquid; however, the manufacturer plans a liquid–liquid flow system and the present instrument would appear to be modifiable to this use.

Conduction-Type (Pseudo-Isothermal) Instruments. The Benzinger (Beckman Instrument Co.) heat-leak calorimeter, the Wadso (LKB) microcalorimeter, and a version of the

original Calvet instrument (Imass, Inc.) are available commercially, and the prototype of a fourth has been assembled by Aminco; all are of the conduction-type. Used conventionally, it is heat flow across the thermopiles that is measured in these instruments. Thus, by definition, they are not truly isothermal. There is a heat pumping effect of a large inert heat sink at the cold junction of the thermopile. Heat produced by the reaction rapidly leaks from inside the container to the heat sink. Since the sink is (ideally) infinitely large by comparison with the vessel, the temperature changes only to a negligible extent. Conduction-type calorimetry data is generally treated as if the system were truly isothermal. Departure from the ideal, for a properly designed instrument and experiment, is negligible. The principal design features of an instrument of the conduction type are indicated in Figure 6.

Heat-Capacity Calorimeters. The title "Heat-Capacity Calorimeters" is, of course, a statement of method of use rather than instrument design. The term usually implies that a temperature scan is made and that the change in heat capacity at any given instant is indicated by the quantity of heat retained by the cell containing experimental material.

It is apparent that ΔH values which accompany changes in spatial relationships of proteins in solution can be calculated from measurements of the heat capacity of the sample as a function of temperature in the transition region. The adiabatic calorimeter has been used almost exclusively for measurements of this kind. Both single- and twin-vessel instruments have been used. With the single instrument (single-vessel calorimeter) it is necessary first to determine the total heat capacity of the calorimeter vessel and its contents by measurement of the rise in temperature which results from the addition to the system of a known quantity of electrical energy. Recording instruments are used, and hence a direct determination of heat capacity may by obtained by continuous heating of the calorimeter; that is, energy, at constant rate, is placed into the system and measurement is made both of the power and the rate of heating. The heat capacities of the vessel and specimen are ultimately compared to those obtained in a reference blank experiment under identical conditions. As in all single beam instrumentation, the greatest stress is upon the precision of elements of the instrumentation. Hence, for the most part, comparison calorimetry—or more exactly stated, twin-vessel adiabatic calorimetry—has become the more usual practice.

In the twin-vessel calorimeter, the energy applied to one of the vessels is adjusted to equalize the heating rate. In present usage, the recording adiabatic twin calorimeter—suited to heat capacity measurements of protein and other biomolecules—is invariably constructed with multiple adiabatic shields, and either an active detector (thermopile) or passive device such as a thermistor (the quartz crystal element has also been used with success) provides the input to a microvolt electronic amplifier. High precision microcalorimeters properly placed in this category have been described by Ackermann (1969b), Privalov and Monaselidze (1964), Buzzell and Sturtevant (1948, 1951), and Clem *et al.* (1969).

SPATIAL RELATIONSHIPS OF PROTEINS STUDIED CALORIMETRICALLY

Lysozyme. Since Fleming's (1922) description of hen egg white lysozyme, the enzyme, because of its relatively easy isolation and ubiquitous distribution, has been the object of intensive study. It is now probably as well characterized as any biopolymer of its size. It is a basic protein containing 129 amino acid residues with a molecular weight of approximately 14,400. Its catalytic activity is the hydrolysis of

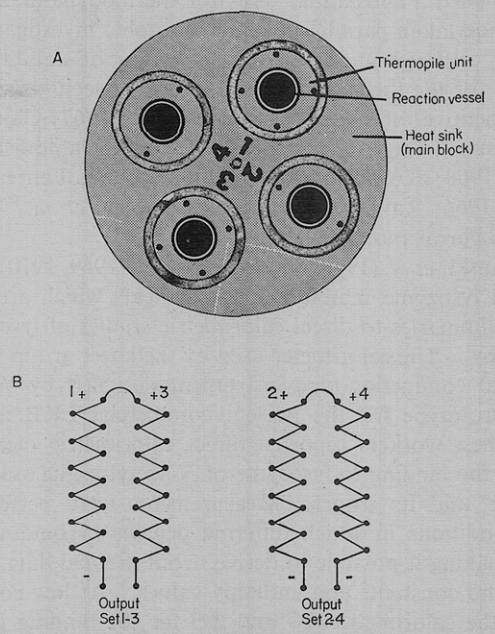

Figure 6. Conduction-type calorimeter using four elements as a double zero blank (double-twin) unit (Brown, 1969). Reprinted with permission of: "Biochemical Microcalorimetry," Academic Press, New York, N. Y., 1969

glycosidic bonds in complex polysaccharides. The amino acid sequence is known; secondary and crystal structure have been well established by the work of many investigators. The present state of knowledge of this protein has recently been reviewed by Raftery and Dahlquist (1970). The power of contemporary instrumentation has been brought to bear directly upon questions of the spatial relationships of lysozyme [Blow and Steitz, 1970 (X-ray crystallography); Glazer and Simmons, 1966 (circular dichroism); Dahlquist and Raftery, 1969; Meadows *et al.*, 1967; McDonald and Phillips, 1967 (proton magnetic resonance)].

Such is the state of the art that lysozyme, probably as well as any natural protein, is appropriate to the development of calorimetric methods for protein spatial relationship studies. The tools at hand appear limitless, for the effects of many agents upon the stability of lysozyme have been described by data obtained with a variety of physical instrumentation. Examples of this are the descriptions (Hamaguchi and Imahori, 1964) of the effects of formamide, acetamide, dimethyl urea, tetramethyl urea, and dimethyl acetamide on the optical rotatory characteristics of the molecular difference spectra, optical rotatory dispersion, and circular dichroism applied to urea denaturation (Yutani *et al.*, 1968). Denaturation was completely reversible ("lysozyme reoxidized by the renaturation treatment . . . described . . . could not be distinguished from the native lysozyme by measurements of the difference spectrum and enzymatic activity").

Tanford and associates (Tanford *et al.*, 1960; Tanford and De, 1961; Tanford, 1962; Weber and Tanford, 1959) have discussed the forces involved in lysozyme stabilization, principally in terms of hydrophobic interactions, though the results of work cited above would indicate that the internal fold of cross-β conformation probably results from the combined contribution of hydrophobic and hydrogen bonding.

Rupley *et al.* (1967) have considered the energetics of spatial relationship alteration of lysozyme and have emphasized that evaluation of the denaturation-type experiments in solutions containing urea, detergents, and alcohols must be made with regard to crystallographic information. This extensive

work provided a substantial basis for thermodynamic study which, undertaken parallel to crystallographic investigations, has been centered upon the determination of equilibrium constants for the formation of complexes between lysozymes and competitive inhibitors (Chipman *et al.*, 1967; Chipman and Schimmel, 1968; Dahlquist *et al.*, 1966; Dahlquist and Raftery, 1968; Kowalski and Schimmel, 1969; Lehrer and Fasman, 1966; Raftery *et al.*, 1969; Rupley *et al.*, 1967; Sykes and Parravano, 1969).

Vichutinskij *et al.* (1969) and Bjurulf *et al.* (1969, 1970) have published lysozyme–inhibitor coupling heats which are welcome preliminaries to direct calorimetric studies of lysozyme topography. The calorimeter used by the latter group was a differential conduction-type instrument designed by Wadsö (1968) (prototype for the present commercial LKB instrument). These workers reported direct calorimetric measurements of the binding to lysozyme of *N*-acetyl-D-glucosamine, its dimer, and its trimer. Measurements were performed under conditions in which differing degrees of binding occurred, making it possible to derive, from thermal data, both equilibrium constants and enthalpy values. At low concentrations the calorimetric ΔG and ΔH for the binding of the acetylated glucosamines to lysozyme are in agreement with those of equilibrium measurements. For the monomeric acetyl glucosamine, the results indicate a stepwise addition of several inhibitor molecules to the enzyme. However, caution should be used because complexing of the enzyme with substituted acetamides resulted in endothermic effects which could not be interpreted in terms of a simple 1:1 stoichiometric complex formation.

As the trimer concentration was increased, an apparent equivalence point was indicated by a sharp bend between two arms of their data plot (heat *vs.* concentration). Hence, the formation of a comparatively strong 1:1 complex of lysozyme to the 3-residue oligomer is indicated. Wadsö and associates are presently attempting to relate structures known from X-ray crystallography and their thermodynamic quantities by the development of model experiments involving the transfer of characteristic groups between different media. It is interesting to note that experiments of Vichutinskij *et al.* (1969), made under different conditions of temperature and pH, present results which increase the alternatives available in explaining binding data. This fortunate complementation of two independent studies makes obvious the necessity for more extensive investigation of the factors impingent upon interpretation of thermodynamic data.

Ogasahara and Hamaguchi (1967) and Delben and Crescenzi (1969) [using a commercial differential scanning calorimeter (Perkin-Elmer)] studied the heat effects accompanying thermal denaturation of lysozyme. The choice of the environments for denaturation was based upon an attempt to identify the forces contributory to the spatial relationship change. Studies were made of the protein in urea, guanidine, HCl, and hexamethylene tetramine solutions. Hexamethylene tetramine, which is thought to influence phenomena driven by hydrophobic forces, had no effect measurable with this instrumentation. It would appear that under the conditions of the denaturation the contribution of hydrophobic bonds was either nonexistent or at least considerably less significant than should have been predicted.

Chymotrypsin–Chymotrypsinogen. Chymotrypsin and chymotrypsinogen have been among the most studied of all proteins. The activities were first described in the mid-1800's by Kuhne (1867) and Heidenhain (1874), and the proteins were extensively characterized by Kunitz and Northrop (1935). The amino acid sequence was described by Hartley (1964), and contemporary knowledge of the structure of α-chymotrypsin worked out primarily by X-ray analysis has been excellently presented in the paper of Birktoft *et al.* (1970). These authors have described in detail the structure in relation to the probable existence of stabilizing hydrogen bond with regard to the conformation of the side chains. Chymotrypsin, like the other well-characterized proteins which have been studied, lends itself to the development of calorimetric approaches to protein topography because other chemical and instrumental methods have established with reasonable certainty the nature of the molecule under a variety of conditions. Calorimetric studies of chymotrypsin conformation have usually been predicated upon the thesis of two-state transition (Lumry *et al.*, 1966) as an attempt to establish sets of thermodynamic data which may ultimately be interpreted in terms of the forces involved in stabilization of protein spatial relationships. The two-state transition, as an all or none concept (Johnson *et al.*, 1954), is extremely convenient for the development of experimental models. It describes a discontinuity in the thermodynamic states so that one state is stable below and another stable above a transition temperature. Hence, α-chymotrypsin has been evaluated by a number of workers with respect to the thermodynamic changes of folding from the fully random coil polypeptide. Heat capacity changes in the unfolding of chymotrypsin are highly temperature-dependent and the useful theoretical interpretations of Jackson and Brandts (1970) and Biltonen (1969) [see also Lumry *et al.* (1966) and the earlier observation of Forrest and Sturtevant (1960)] indicate that the temperature-dependent part of the heat capacity change is assignable to the transfer of nonpolar chains of the polypeptide from an interior position into bulk water (Brandts, 1964, 1969). Hence, the hydrophobic bond contribution has been isolated by Brandts and associates by measurement of the temperature dependence of the heat capacity change in the transition and by suitably using the effect of pH and ionic strength of the medium to allow an evaluation of the lesser contributions of hydrogen bonds, ionic linkages, electron delocalization–dispersion forces, and so forth. They studied properties of chymotrypsinogen in dilute solution in the pH 2–3 range. Heat capacity measurements permitted estimates of $\Delta H°$, and ΔC_p for the unfolding process. Calorimetric value of $\Delta H°$ varied from 100 kcal/mol at 42° C (pH 2) to 140 kcal/mol at 54° C (pH 3). Comparison of these values with estimates based on a two-state analysis showed no discrepancies, and hence the authors concluded that the chymotrypsinogen unfolding could be described by a two-state approximation. In their discussion, Jackson and Brandts (1970) mention that Biltonen obtained similar data by using pH to induce denaturation. A heat equal to that for thermal denaturation was calculated by subtracting values associated with the titration of carboxyl groups.

A set of succinct rules for the test of validity of the two-state approximation has been given by Biltonen (1969): optical properties must show coincidental changes in terms of standard free energy as a function of temperature; the apparent standard enthalpy change from the van't Hoff plot must be a monotonic function of temperature; and the van't Hoff heat and the calorimetric heat must be identical (Figure 7). This represents such a comparison.

Skerjanc and Lapanje (1969) have measured enthalpies of denaturation of chymotrypsin in concentrated urea. This experiment is, in essence, insistence by the investigators upon a two-state phenomenon since they transferred the protein from 2 *M* urea, in which all molecules can be assumed to be in the native state, to 8 *M* urea, where they are all fully denatured.

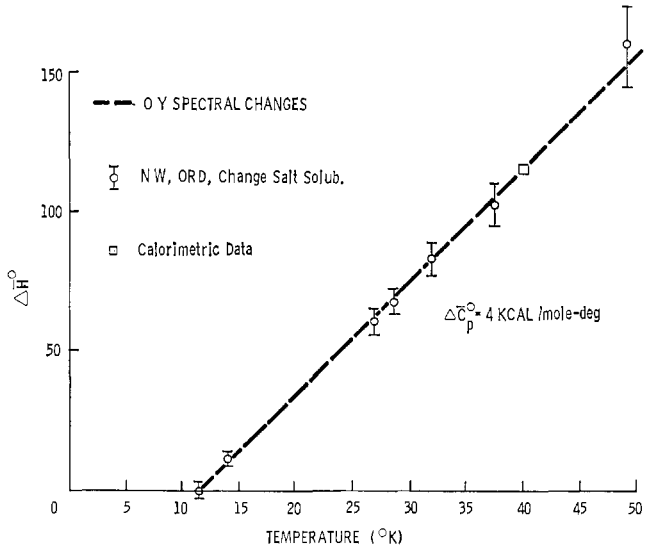

Figure 7. Temperature dependence of $\Delta\bar{H}^\circ$ for the thermal unfolding of α-chymotrypsin (modified after Biltonen, 1969)

This transition is rapid for chymotrypsinogen with a ΔH of -131 kcal/mol. The value is higher than anticipated and these authors interpret this to indicate a great contribution of hydrogen bonding to the stabilization. It is worthwhile at this point to refer to the study of ζ-lactoglobulin by these authors which, comparably, had a $\Delta H = -118$ kcal/mol. However, unlike the chymotrypsinogen, the van't Hoff's enthalpy for the transition under identical conditions is quite different from the measured value. The discrepancy $\Delta H = -20$ kcal/mol *vs.* the measured $\Delta H = -118$ kcal/mol indicates the possibility that this is a multi-state rather than a two-state transition.

Hutchens *et al.* (1969) considered the absolute entropies of chymotrypsinogen calculated from measured heat capacities. This and other derived thermodynamic functions were obtained for the anhydrated forms of the proteins as a function of temperature. The relationship of the protein to water was considered, and the fact that heat capacities for hydrated samples were not elevated in the 250–275° K range was taken as an indication that there was no fusion of ice. The average value for the entropy of formation of a peptide bond in chymotrypsinogen-A was 9 cal/deg^{-1} bond. These authors also measured the same phenomena using as models the individual amino acids of the chymotrypsinogen molecule. The paper of Canady and Laidler (1958), though not directly concerned with conformation, provides useful background to future work with elucidation of spatial relationships as the goal. Their measurements of heats of substrate–inhibitor binding to the protein were rationalized in terms of the physical forces involved. These authors used a Tian-Calvet microcalorimeter to measure the heat of binding of hydrocinnamic acid to α-chymotrypsin over pH range 5.1–7.8. The heat evolved varied from 5 to 28 kcal/mol. Entropies of binding were calculated. Results were explained by reference to the thesis that the active center of the enzyme contained, in addition to acidic and basic sites, a negatively charged group that interacts electrostatically with the anion of hydrocinnamic acid. Similar binding studies have been actively carried forward by other authors using different model proteins (*e.g.*, Bjurulf *et al.*, 1969, 1970; Lovrien, 1970; Sturtevant and Lovrien, 1971), and the collective data provide additional structure upon which an approach to conformation can be built. It seems probable that the analysis of conformational change, especially where it is to be based upon evaluation of multiple forces involved, will of necessity be delimited by just such ancillary measurements as binding heats which serve to allow a discrimination of the forces that play a part in the stabilization of the protein molecule.

Calorimetric Studies of Other Proteins. Tsong *et al.* (1970) have studied ribonuclease denaturation and outlined two mathematical systems by which the calorimetric measurements may be analyzed; one presents good agreement with van't Hoff calculations while another, with a deviation from the theoretical baseline at low temperatures heavily weighted, presents somewhat higher values. The mechanism of the ribonuclease transition has been studied by Beck *et al.* (1965). These authors have stressed the value of direct measurements, drawing attention to the fact that the usual calculations based, for example, on equilibrium constant require that a reaction mechanism be postulated in order to allow the calculations to be made, whereas direct calorimetric determination of the heat of transition provides the necessary information to differentiate between, at least, simpler possible reaction mechanisms. A heat of transition of 70 kcal/mol was recorded in repeat measurements of the same solution, thereby confirming the reversibility of the transition. The heat capacity change upon transition was found to be 0.66 kcal/mol-deg. The measured values differed significantly from those obtained by van't Hoff-type computations. The calorimetric data confirms other indirect studies (Scott and Scheraga, 1963) which indicated that a single step phenomenon is not involved here. Hence, the ribonuclease transition is not a simple helix→coil-type transition nor it is a transition which consists of a single step or several independent steps with significantly different transition temperatures. Data of Beck *et al.* (1965) support a conclusion that the transition consists of more than one closely interdependent step. Kresheck and Scheraga (1966), in an extended series of studies, have used a Benjamin adiabatic calorimeter in an experimental design intended to allow the identification of the various forces involved in the complex ribonuclease denaturation. It is apparent from their studies that a number of quite distinct events may occur which can contribute significantly to both measured heats and calculated thermodynamic parameters. Among other interesting products of their analysis, they conclude that irreversibly denatured material has some residual structure which can be further unfolded with a small heat. Oxidized ribonuclease must, they believe, possess some noncovalent structure and undergo a conformational change upon protonation. Perhaps, too, some noncovalent structure may be present in the unprotonated form of reduced ribonuclease and may be responsible for the correct disulfide pairing which occurs during oxidation. A noncovalent interaction involving tyrosyl residue 115 has been so implicated.

Buzzell and Sturtevant (1952), studying the heat of denaturation of pepsin, related aspects of the protein's behavior to its loss of enzymatic activity and showed that the enthalpy change accompanying alkali denaturation is strongly pH dependent. Helix→coil transitions of proteins were studied by Aldoshin *et al.* (1962) with hemoglobin, serum albumin, ovalbumin, and α-globulin. The authors used adiabatic calorimetry and correlated the degree of helicity obtained by optical measurements with the calorimetric values for the enthalpy of melting. The changes measured in their experimentation correlated with the melting of hydrogen bonds in the helical parts of the protein molecule. Their calculations led them to attribute the major heat contribution to hydrogen bond melting, yielding a value of 1400 cal/mol/hydrogen bond.

Largely for the practical reason of availability in the

amounts needed by macrocalorimeters and at high levels of purity, hemoglobin has been the basis for a substantial body of descriptive work. Forrest and Sturtevant (1960) measured the reversible acid denaturation of ferrihemoglobin. The denaturation was strongly exothermic at 15° and weakly endothermic at 25°. Their corrected heat, subtracting buffer protonation, is −88.5 kcal/mol at 15° and +10 kcal/mol at 25°. The denaturation process at 20° yields a $\Delta C_p = +9850$ cal/mol deg^{-1}. A value close to this was described in the apparent heat capacity of bovine serum albumin in solution when the pH was dropped from 5 to 3. This increase in heat capacity as a function of denaturation has been discussed above, but it is of interest that Forrest and Sturtevant (1960) had drawn attention to it and deduced that the process is accompanied by molecular expansion.

As might be predicted, the polypeptide has proven to be an attractive model to investigators, particularly in making measurements involving transition variations in heat capacity. Writings which give useful examples of this literature include those of Karasz *et al.* (1964), Ackermann and Rüterjans (1964a,b), Ackermann and Neumann (1967), Karasz and O'Reilly (1966), and Ackermann (1969c). The subject area has been reviewed by Ackermann (1969a). Polybenzyl glutamate has been studied by Doty and Yang (1956), Calvin *et al.* (1959), Zimm *et al.* (1959), Karasz *et al.* (1964), and Karasz and O'Reilly (1966). Theoretically, of course, this transition, like those involving larger molecules, has been treated as a cooperative first-order change. The fact that the transition is spread over a number of degrees indicating a helix→coil equilibrium in the vicinity of the transition temperature is contrary to theoretical prediction. Karasz and O'Reilly (1966) approached questions of solvent interaction using deuterated entities for a study of the thermodynamics of the transition. Their measured results were evaluated in terms of the Zimm-Bragg (Zimm and Bragg, 1959) cooperative parameter. The results indicate that the formation of an interruption in the helical sequence, even though the total number of hydrogen bonds is constant, involves changes in the energy of the system as well as a large decrease in the configurational entropy arising from immobilization of additional residues. The possibility that the enthalpic change arises from modifications of dipole interaction at or near newly-formed interfaces was suggested. The alternative possibility that the helix→coil transition involves changes in the protonation of the polypeptide amide group other than changes in hydrogen bonding was also recognized. Dependence of these systems upon solvent concentrations has been pointed out by Ackermann and Neumann (1967). Though correspondence of the measured values with predictions of the Zimm-Bragg theory is good, a number of minor peculiarities appear, particularly when the statistical mechanical predictions and calorimetric measurements are compared with optical data (Ackermann and Neumann, 1967).

OTHER BIOMOLECULES STUDIED CALORIMETRICALLY

Most of the primary assumptions made about protein molecules as preliminary to their calorimetric study have also been made for the nucleic acid molecules. Since a chain of atoms such as DNA should exist as a random coil, it must be assumed that a large amount of stabilizing energy, in the form of intra- and intermolecular interactions, must be available to constrain the polymer to its native conformation. Hence, the nucleic acid polymers, like proteins, derive their native conformation from the interplay of thermodynamic influences. Since very great effort has been put forward in purifying and characterizing the nucleic acids, they present themselves as a rather exceptional model system for defining the measurable thermodynamic parameters which influence the spatial interrelationships of biopolymers.

From the dimensions of the cells in which the nucleic acids are isolated, it is apparent that their *in vivo* helical structure must be extensively coiled and folded. Hence, though we shall discuss studies which significantly relate to the conformational states of purified nucleic acids in solution, very little can be said with surety about the meaning of this data in terms of specific tertiary conformation *in vivo*. Perhaps the classic study of thermodynamic forces stabilizing the DNA molecules is that of Sturtevant *et al.* (1958). They used calorimetric and ancillary data to examine DNA stability in terms of response to changes to pH, ionic strength, temperature, and various chemical agents. Their model of acid denaturation was based upon the opening of many gaps rather than the picturesque but aesthetically unsatisfying visualization of the entire molecule unzipping. Primarily, the Sturtevant study illustrated that hydrogen bonds were not the sole source of stabilization for the molecule. Rather, the double helical structures are stabilized by a number of interacting forces. It is therefore pertinent to determine the energy changes accompanying the formation of these structures. Hence, a number of workers have measured the heats of formation of the polynucleotide complexes.

Working with the model structure proposed by Rich and Davies (1956) of a doubly-stranded helix stabilized by two adenine–uracil hydrogen bonds between the C_6-amino group of adenine and the C_6-carbonyl group of uracil and the other between the N_1 ring of adenine and the N_1 ring of uracil, Kitzinger *et al.* (1962) measured the interaction of synthetic polynucleotides. Pairing of poly-A with poly-U liberated 5300 cal/mol of mononucleotide in 0.5 *M* KCl at pH 6.5. It is, of course, important to recognize that these early calorimetric studies of Kitzinger *et al.* (1962) and Rawitscher *et al.* (1963) contributed further to the conclusion of Sturtevant *et al.* (1958) that the Watson-Crick supposition of a double helical DNA stabilized essentially by hydrogen bonding was an incorrect simplification. It is now clear with considerable distance and a large literature that factors other than hydrogen bonding between interstrand base pairs are quantitatively considerably more important in maintaining the DNA native form. These do not differ in kind from the forces stabilizing proteins (discussed above) but the weight of the relationships, of course, is necessarily somewhat different (see the review of Josse and Eigner, 1966). Rawitscher *et al.* (1963) extrapolated from their data the melting temperature of poly-A + poly-U for the heat of interaction of random coil poly-A with random coil poly-U to form a 1:1 helical complex at pH 7 with a value of approximately −8700 cal/mol base pairs^{-1}. Later workers (*e.g.*, Neumann and Ackermann, 1967) continued the study of transition enthalpies in the polynucleotide systems, particularly poly-A + poly-U, in the effort to define the forces involved in DNA stabilization. These experiments were illustrative of the energetic interactions (so-called stacking energy) in the ordered nucleotide structures. The base stacking forces probably consist of summative values of dipole–dipole and induced-dipole electrostatic energies, as well as dispersion energies (London forces) which result from interactions of the DNA bases as they stack in a helical array.

Krakauer and Sturtevant (1968) have considered the helix→coil transitions of poly-A–poly-U complexes in terms of the effects upon the system of metal ions. They assume that the poly-A–poly-U system, like many others which have been elaborately studied, has an equilibrium between ordered (helix) and disordered (loops) regions of a long chain. Their

experimental data indicate a dependence of measured heats on concentrations of sodium and potassium to be interpretable by a concept of binding of counterions to polyelectrolytes. Privalov *et al.* (1965) have studied calorimetric denaturation of DNA, with emphasis upon the thermodynamics of the separation of the two strands and they have established that the change in enthalpy on thermal denaturation is a linear function of the heat stability of the macromolecules. The value calculated from their measurements gives a ΔH for the enthalpy of transition of a pair of residues from the ordered to the disordered state of about 8300 cal/mol and ΔS (entropy of the transition of a pair of residues) of the order of 23 cal/deg mol.

One would like to be able to say that smaller molecules, particularly carbohydrates, which lend themselves to the modeling of the conformation (Reeves, 1951, 1954; Reeves and Blouin, 1957) have been extensively described calorimetrically. This has not proved to be true, though the existing literature is fascinating, and is an adequate demonstration of opportunity lingering expectantly. Much of the thought in the field can be traced to the assumption, hopefully correct, that the pyranose ring, like the cyclohexane ring (Margrave *et al.*, 1963) is more stable in the chair than in the boat form.

Heats of mutarotation of sugars were calculated as early as 1904 by Hudson (1904). Sturtevant (1937, 1941) made direct measurements of the heat of mutarotation of D-glucose. Kabayama *et al.* (1958) and Kabayama and Patterson (1958) measured the heats of mutarotation of D-xylose, cellobiose, D-glucose, lactose, and maltose in an extension of the line of research initiated by Sturtevant with particular emphasis on sugars undergoing an opposing first-order mutarotation. These authors found a surprisingly large range of values for ΔH and ΔS since the sugars chosen, excepting D-xylose, were identical in the character of the anomeric carbon. D-Fructose was studied by Anderson and Grønlund (1965). Recently Takahashi and Ono (1966) and Takasaki (1967) extended this area of study to other sugars and to other conditions. The discipline has been reviewed by Ono and Takahashi (1969).

The paper of Kabayama and Patterson (1958) is a most interesting exposition of the theoretical considerations which can be brought to bear upon a calorimetric spatial relationship study for a biomolecule. Their arguments were based upon evidence for hydrogen bonding between the sugar hydroxyl groups and adjacent water molecules. Drawing upon the relatively limited number of possible conformations (from Reeves' studies), they reached conclusions about the packing of the water molecules around the sugar molecule to allow prediction that an equatorial hydroxyl group will be more strongly hydrated than an axial group. Their studies using the several sugars indicate that the reducing hydroxyl of the sugar is bonded to an adjacent water molecule in the same way with only slight variations in bond length. They attribute the large value of ΔS for mutarotation to changes in the frequency of torsional oscillation of the reducing hydroxyl group about the $C_{(1)}$–0 axis. A simple model of hydrogen bonding between functional group and water was used to estimate the order of magnitude of the torsional oscillation frequency, and it proved to be low enough to give a large contribution to the entropy. With the same model, the order of magnitude of the variation of ΔS and ΔH was calculated and the slope of ΔH *vs.* ΔS estimated theoretically. Estimation of ΔG for the mutarotation was made by Kabayama and Patterson using the thermodynamic study of Angyal and McHugh (1956) on cyclitol. (Because the pyranose ring contains an oxygen atom, while the cyclitol does not, a significant difference in interactions exists and this extrapolation is tentative.)

While a very important body of knowledge about water has been developed since these limited calorimetric studies of mutarotation were completed, they nonetheless remain as models for approach against which we may now proceed armed both with a greater theoretical insight and additional instrumentation.

CONCLUSION

Protein chemists have been wont to decry the lack of an instrument for ΔC_p measurements of sufficient sensitivity to deal, accurately and precisely, with small quantities of protein in solution in concentration below, wishfully far below, 1%. Whether such instrumentation can be said to exist depends upon the nature of the experiment and the heat level which can be predicted for the particular situation. Hence, we are on the fringe of our ability to deal with this one approach to protein topography. This hardly constitutes an overwhelming deterrent, for very good micro-level instrumentation is available and, if used with appropriate design of the experimentation, it will allow us to elucidate protein topography in terms of interactions either of the protein with itself, the protein with its solvent, or the protein with a third component.

Protein conformational characterization by calorimetric measurements has been accomplished using those entities which were best known from studies based upon chemical methods or upon other instrumentation, and those which were available in high purity. Hence, lysozyme, chymotrypsin, hemoglobin, and the somewhat easier targets, polypeptides and nonprotein models (nucleic acids, carbohydrates) have received first attention. It is probable that microcalorimetrists will continue to develop the discipline with the best available characterized proteins. However, the need of the working protein chemist to describe topographic characteristics of his own protein (even those derived from economic plants and animals) may provide useful stimulus to attack the problem in imaginative ways. The study, particularly of those topographic characters that relate to the protein's potential for interaction, may lead us to find that well characterized systems are not an absolute requirement and that ΔC_p must not invariably be known. Certainly experimental design must take cognizance of the interferences that can be expected in lesser characterized and impure systems, but the availability of calorimetric devices, convenient and rapid in use, helps to make the necessity for experimentation less burdensome and the goals appear—for many interesting proteins—achievable.

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