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Thermodynamic and thermal analysis of a synthetic protein analog.

Roy Pickard McKnight
University of Massachusetts Amherst

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THERMODYNAMIC AND THERMAL ANALYSIS
OF A
SYNTHETIC PROTEIN ANALOG

A Dissertation Presented
By
ROY PICKARD MCKNIGHT

Submitted to the Graduate School of the University of Massachusetts in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY
December 1974
Polymer Science and Engineering
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THERMODYNAMIC AND THERMAL
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ROY PICKARD McKNIGHT

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OCTOBER 1974
ACKNOWLEDGEMENTS

The author wishes to thank the University of Massachusetts and the Polymer Science and Engineering Program for the opportunity to obtain this degree. Special thanks are due to Dr. Frank Karasz for his many hours of patience and his guidance of the work, and to Dr. Judith Simon, Dr. Ottfried Heybey, Dr. Don C. S. Lu, and Kenneth Roskuszka for their many helpful suggestions and encouragement during the course of the work. Special thanks also goes to Mr. Joseph DeCaro for his technical expertise and help in construction of the Calvet Cold Box. Finally, I gratefully acknowledge the assistance of Mrs. Barbara Ferris in the preparation of many of the figures for publication.
Thermodynamic and Thermal Analysis of a Synthetic Protein Analog (December 1974)

Roy P. McKnight, B.S., Carnegie-Mellon University
M.S., University of Massachusetts
Directed by: Dr. Frank E. Karasz

The synthetic polypeptide poly-β-benzyl-γ-aspartate was studied in the mixed solvent system dichloroacetic acid (DCA) - tetrachloroethane (TCE) using polarimetric and calorimetric methods.

The polarimetric results are analyzed using the thermodynamic treatment of Karasz and Cajnos to give the intrinsic stabilization parameters of the PBA helix and the binding parameters of the DCA to the PBA coil in TCE. The parameters so derived were then used to predict the helix-coil phase transition lines in the temperature regions outside the experimentally accessible region.

A special cell was developed for use in the Tian-Calvet microcalorimeter which could be used with the highly corrosive solvent mixture employed. After developing procedures for determining the magnitudes of side reactions and the corrections for these, the calorimetric heats of transition of the PBA were measured over the temperature range 270°K to 320°K. The experimental values were found to agree within experimental error with the predictions of the Karasz-Cajnos treatment.
using the parameters derived by polarimetry.

In addition, some work was done with the natural enzyme lysozyme (muramidase) in an attempt to determine the relative importance of hydrogen bonding in the stabilization of the native conformation. No differences were found beyond those induced by the decreased ionization of the Brönsted acid groups in heavy water.
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INTRODUCTION

Order-disorder transitions of polymers in solution have been of interest for some time. The denaturation of protein molecules is only one example of such transitions which has biological significance. These transitions have been found to depend on many factors, among them pH, ionic strength, solvent composition, pressure, and of course temperature. It has long been recognized that these transitions in proteins are of a very complex nature.

Proteins, however, do have simpler synthetic analogs which have been investigated, these being the homopolypeptides, polymers of the \( \alpha \)-amino acids. While some of these polymers are soluble in water, the majority are either insoluble or only sparingly soluble. It has thus been found judicious to study the transition of polypeptides in mixed organic solvents so that we may gain a clearer understanding of the transition process itself.

This transition can, to a first approximation, be described in terms of only two steps: the disruption of the intramolecular hydrogen bonds stabilizing the ordered conformation, and the preferential binding of the coil residues by the 'active' solvent in the solvent mixture. Such a model has been used since the earliest consider-
ations of the phenomenon\(^2-5\) and leads, for certain cases, to the prediction of two thermal transitions. The 'phase diagrams' for these transitions require only four basic parameters: the enthalpy and entropy of each of the two steps described above.\(^6\)

The purpose of the present study was to obtain original data on a polymer-solvent system that the Karasz-Gajnos treatment predicted, from previous data on the enthalpies and entropies, would exhibit the double thermal transition, and to obtain refined values for the principle parameters which could then be used to investigate the theoretical predictions in greater detail, particularly such values as the transition width (in both the isothermal and thermally-induced planes) and the cooperativity parameter, \(\sigma\).

To achieve these aims, it was decided that two instruments present in the departmental facilities should be used. Because of the disymmetry of the ordered state (an \(\alpha\)-helix for polypeptides) the thermally-induced transition studies can be followed using a polarimeter, in our case the Perkin-Elmer Model 141MC. Due to the sharpness of the transition, the temperature control was judged to be critical, so a Haake Model FK constant temperature circulating bath was purchased to supply the jacketed polarimeter cell. No other modification of this instrument was found necessary for
satisfactory results.

A Tian-Calvet microcalorimeter was also available for the isothermal studies. As expected, the design of the cells for this instrument was a critical phase in obtaining satisfactory results, and several designs were tried and rejected before settling on a final design. The various designs and their flaws are described briefly below, as well as a detailed description of the final cell and the procedures used.

The principle system for this study was poly-ß-benzyl-ß-aspartate (PBA) in a mixture of 1,1,2,2-tetrachloroethane (TCE) and dichloroacetic acid (DCA). This system was chosen because of the weak helix in the PBA and the strong interaction of DCA with peptides, the conditions necessary for the occurrence of the double thermal transition in an accessible temperature range. Some work was also done with lysozyme (muramidase) in an attempt to determine the relative importance of hydrogen-bond strength to the order-disorder transition in protein molecules.
THEORETICAL CONSIDERATIONS

It is well known that the dissymmetry of the $\alpha$-helical conformation in macromolecules is responsible for a portion of the optical rotatory power of the polymer solutions. The rotation from the asymmetric centers in the backbone chain should be independent of the conformation of the chain, and can thus be regarded as a constant background rotation to which the rotation due to the ordering is added. In fact, one may analyse the optical rototory dispersion curves using the Moffitt equation\(^7\) to yield a parameter \((b_0)\) which is proportional to the helical content of the polymer.\(^8\)

A good review of this technique has been given by Yang\(^9\), and only a brief summary will be given here. The general form of the Moffitt equation is

$$\left[\frac{m}{\lambda}\right] = \frac{a}{2} \frac{\lambda^2}{\lambda^2 - \lambda_o^2} + \frac{b}{2} \frac{\lambda^4}{(\lambda^2 - \lambda_o^2)^2}$$

where $\lambda$ is the wavelength at which the measurement is made and $\left[\frac{m}{\lambda}\right]$ is the reduced mean residue rotation defined by

$$\left[\frac{m}{\lambda}\right] = \frac{3\alpha}{n_\lambda^2 + 2} \frac{M}{C}$$

Here $n_\lambda$ is the solvent refractive index at wavelength $\lambda$, \(\alpha\) the measured rotation in degrees, \(M\) the residue molecular weight (equal to the molecular weight of the $\alpha$-amino acid
minus 18 for the water lost on polymerization), and c is the concentration in grams per deciliter.

The Moffitt equation contains three adjustable parameters, \(a_o\), \(b_o\), and \(\lambda_o\). Since this equation is a phenomenological one the parameters are all derived from experimental data. The equation is usually rearranged into the form

\[
\frac{\lambda}{\lambda_o^3} \left( \frac{\lambda^2 - \lambda_o^2}{\lambda_o^2} \right) = a_o + b_o \left( \frac{\lambda^2 - \lambda_o^2}{\lambda_o^2} \right)^{-1}
\]

and solved graphically by plotting the left side against \((\frac{\lambda^2 - \lambda_o^2}{\lambda_o^2})^{-1}\). The parameter \(\lambda_o\) is found by adjusting its value until the data fall on a straight line. Much work (reviewed in References 9 and 10) has shown that in general a good fit is obtained using \(\lambda_o = 212\text{nm}\), and that the value of \(b_o\) for right-handed helical polypeptides is about \(-630^\circ\), although this value still depends somewhat on the solvent used.9

Several studies on poly-\(\beta\)-benzyl-\(\beta\)-aspartate of high molecular weight have given values of about \(+660^\circ\) for the helical form (positive because of the left-handed helical sense) and \(-270^\circ\) for the random coil form.11,12 We have found that the \(b_o\) values correlate well with the specific rotation at 436 nm (Figure 1) and have thus used the rotation values as indicators of helical content for most of our work, since the points on the curve are found to be in-
dependent of both temperature and solvent composition within the ranges studied. The fractional helical contents calculated in this way are found to be precise to about 2.5 percent helix.

For a thermally-induced transition, one can obtain an apparent van't Hoff heat at the midpoint of the transition from the equation

\[
\frac{d(\ln K)}{d(1/t)} = \frac{\Delta H_{\text{VH}}}{R}
\]

(1)

where \( K \) is defined as the ratio of the fraction of peptide residues in the disordered form to the fraction in the ordered (helical) form. The heat is, by definition, the enthalpy of the transition per mole of cooperative units, and can be compared to the heat determined calorimetrically on a residue mole basis to give the number of residues in a cooperative unit.\(^\text{13}\)

\[
\sigma^{\frac{1}{2}} = \frac{\Delta H_{\text{VH}}}{\Delta H_{\text{cal}}}
\]

(2)

For a polypeptide with complete cooperativity, this should be the degree of polymerization of the polymer, since then the entire molecule would be a single cooperative unit. Typical values of \( \sigma \) for polymers similar to PBA have been found to be about \( 10^{-4} \), giving a cooperative length of about 100 residues in polymers with a degree of polymerization of about 1000. Such evidence suggests that the coop-
erativity of the helix-coil transition is less than perfect, and that once started, the helix may not unwind completely, but rather remain in an equilibrium state with the coil residues. This in fact substantiates the assumption of an equilibrium in the development of the theory.\(^3\)

The midpoint of the thermal transition determined polarimetrically is found to be a function of the solvent composition.\(^{13-15}\) In a helix-to-coil transition, increasing the amount of hydrogen-bonding solvent in the solvent mixture will decrease the transition temperature (50% helix point) from that in the pure helicogenic solvent. This lowering of the transition temperature can be considered analogous to a melting-point depression in simple fluids, and we may define a 'compositional enthalpy' from this effect of the active solvent on the transition temperature:

\[
\Delta H_{\text{comp}} = -R \left( \frac{\delta \ln X_A}{\delta 1/T} \right) T = T_c \tag{3}
\]

Since the compositional enthalpy is defined on a per mole active solvent basis, and the calorimetrically determined enthalpy is reduced to a residue mole basis, one may find the fraction of residues bound to solvent from the ratio of the two

\[
P_c = \frac{\Delta H_{\text{cal}}}{\Delta H_{\text{comp}}} \leq 1 \tag{4}
\]

One would expect this ratio to be less than one for all cases in which an equilibrium exists between solvent and
unbound residues, and would only equal one if every coil residue were bound to an active solvent molecule. A more rigorous mathematical derivation of Equation 4 may be found in the recent paper by Karasz and Gajnos.\textsuperscript{6}

The treatment given below is a condensed version of that given in Reference 6. The statistical-mechanical basis of this treatment was proposed by Schellman\textsuperscript{16} and was developed further by Gibbs and DiMarzio\textsuperscript{17} and by Lifson and Roig.\textsuperscript{18} The concept of a transition induced by preferential binding of an 'active' solvent to the coil residues was first presented by Peller\textsuperscript{19} in 1959, and developed into practical use by Bixon and Lifson.\textsuperscript{20} Although the Zimm-Bragg\textsuperscript{3} treatment appeared shortly after Gibbs and DiMarzio's, the former has received the most acclaim in the literature, and the Zimm-Bragg notation is used in all of the recent extentions of the theory developed originally by Schellman. For this reason, the Karasz-Gajnos treatment has been cast in the Zimm-Bragg notation.

In this treatment of the helix-coil transition in mixed organic solvents, one may think of the molecule as existing in two separate states. In the helical state, all residues (except the first two in the helix) are hydrogen-bonded to the third proceeding and to the third following residues, this compact molecule then being dispersed in an homogeneous mixture of the organic solvents. In the random-coil state,
the molecule assumes a random configuration in the solvent, and all peptide residues are free to bond with the active component of the solvent mixture. Since the reaction is not completely cooperative, it becomes necessary to define a helical residue as one which is not bonded to the remainder of the polymer chain. We may now view the transition as occurring in two steps: the breaking of the hydrogen bond to the helix

\[
\frac{1}{K_1} \quad \text{HHHC} \xrightarrow{\text{HHCC}}
\]

and the reversible binding of active solvent to the coil residues

\[
A + (CA)_m C_n \xrightarrow{1/K_2} (CA)_{m+1} C_{n-1}
\]

In order to be consistent with the Zimm-Bragg treatment of the transition, and since both reactions are reversible, we shall define the equilibrium constants for the formation of helix from coil. Therefore

\[
K_1 = \frac{X_H}{X_C} \quad \text{and} \quad K_2 = \frac{X_C X_A}{X_{CA}} \quad (5)
\]

where \(X\) is used to indicate mole fractions, which are identical to activities if we assume ideal solution behavior.

The equilibrium constant for the addition of a peptide residue to an existing helical sequence is then given by

\[
s = \frac{X_H}{X_C + X_{CA}}
\]
which, on substitution with Eqns. 5 becomes

$$s = \frac{K_1 K_2}{K_2 + X_A}$$  \hspace{1cm} (6)

By definition, the midpoint of the transition is the point where $X_R = X_C + X_{CA}$, and therefore $s = 1$. Using this fact, we find at the midpoint of the transition

$$K_1 = \frac{K_2 + X_A}{K_2} = 1 + \frac{X_A}{K_2}$$  \hspace{1cm} (7)

which is the equation relating the two equilibrium constants.

Consider also the definition of the fraction of coil residues bound

$$F_c = \frac{X_{CA}}{X_C + X_{CA}}$$  \hspace{1cm} (8)

Multiplying numerator and denominator by $X_A/X_{CA}$

$$F_c = \frac{X_A}{X_A X_C + X_A} = \frac{X_A}{K_2 + X_A}$$  \hspace{1cm} (9)

by Eq. 5B, which in turn yields

$$K_2 = X_A \left( \frac{1 - F_c}{F_c} \right)$$  \hspace{1cm} (10)

Recalling that $K_1$ and $K_2$ are equilibrium constants, we may reasonably assume

$$\ln K_1 = -\frac{4H_1}{RT} + \frac{4S_1}{R}$$  \hspace{1cm} (11a)

and

$$\ln K_2 = -\frac{4H_2}{RT} + \frac{4S_2}{R}$$  \hspace{1cm} (11b)

Thus, if we can find $F_c$ as a function of transition temper-
ature, we can get $K_2(T_c)$ from Eq. 10 and $K_1(T_c)$ from Eq. 7 and furthermore the parameters $\Delta H_1$, $\Delta S_1$, $\Delta H_2$, $\Delta S_2$ from plots of $\ln K_1$ vs. $1/T$ and $\ln K_2$ vs. $1/T$ as indicated by Eqs. 11. In turn, Eq. 4 will give us $F_c(T_c)$ if we know $\Delta H_{\text{cal}}(T_c)$ and $\Delta H_{\text{comp}}(T_c)$.

Further we find from Eq. 6 that
\[ \frac{1}{s} = \frac{1}{K_1} + \frac{X_A}{K_1K_2} \quad (12) \]

Which allows determination of $K_1$ and $K_2$ from the slope and intercept of an $X_A$ vs. $1/s$ plot at a given temperature. Repeating this procedure for several temperatures, we can find $K_1(T)$ and $K_2(T)$ from the optical data alone, and determine the parameters as outlined above.

It also follows from Eq. 6 that
\[
RT \ln s = -(\Delta H_1 - T\Delta S_1) - RT \ln \left(1 + X_A \exp \left[\frac{(\Delta H_2 - T\Delta S_2)}{RT}\right]\right) \quad (13)
\]

From Applequist's treatment of the transition we know that the calorimetric heat is given by the equation
\[
\Delta H_{\text{cal}} = -R \left(\frac{\Delta \ln s}{\Delta 1/T}\right) \quad T = T_c \quad (14)
\]

and therefore, from Eq. 13 and 9
\[
\Delta H_{\text{cal}} = \Delta H_1 + F_c\Delta H_2 \quad (15)
\]

since $s = 1$ at $T = T_c$. Also from Eqs. 3 and 9
\[
\Delta H_{\text{comp}} = \frac{\Delta H_1}{F_c} + \Delta H_2 \quad (16)
\]

If we know $F_c$ as a function of temperature, and the heats
as functions of temperature, plots of $\Delta H_{\text{cal}}$ vs. $F_c$ and of $\Delta H_{\text{comp}}$ vs. $1/F_c$ (with temperature the implicit variable) should give straight lines whose slopes and intercepts yield $\Delta H_1$ and $\Delta H_2$. We therefore have three ways of determining these parameters, in addition to the direct fitting of the optical data to Eq. 13, and any parameters so determined must be consistent with all the plots in the various methods. Such overdetermination of the variables allows more precise values to be found for them than a single set of data and a single method could provide.

Although many workers in the field of polypeptide transitions have recognized the value of direct calorimetric measurement of the enthalpy of the helix-coil transition, very little work has been done in this field. In fact, until very recently, all of the work in the literature has come from only four laboratories. Ackermann and co-workers\textsuperscript{22-24} at the University of Münster and Karasz and O'Reilly\textsuperscript{13,15,25} at General Electric, Schenectady, dominate the early literature, each using his own individual version of the precision adiabatic calorimeter. In 1966, there is a single paper by Hermans and Rialdi\textsuperscript{26} using a solution technique and a heat-flow calorimeter similar to the one we use in our studies here. Two years later, two papers by Kagemoto and Fujishiro\textsuperscript{27,28} appeared in which a custom-designed solution calorimeter of the isothermal type was employed. A paper
has recently appeared by Scheraga, et. al.\textsuperscript{29} in which a twin calorimeter of the DTA type is employed with polypeptide solutions. A very recent entrant to the field is the paper by the workers at Osaka University with their precision heat-capacity calorimeter.\textsuperscript{30} In almost every case, the polymer studied is poly(benzyl glutamate) so that this polymer has been very well characterized in organic solvents while data on most other polypeptides are lacking. Small heats of transition and the need to work in dilute solution are cited as the reasons for the scarcity of work in the area.

Some interesting ways of sidestepping the problems have been devised, such as the heat of solution technique used by Giacometti, et. al.\textsuperscript{31} in which amorphous films are dissolved in helicogenic and helix-breaking solvent mixtures, and the difference in solution heats used for the transition enthalpy. However, the most recent development in the field is the use of scanning calorimetry.\textsuperscript{32,33} This method is faster than the old adiabatic calorimeters, and has now been developed to the point where it is sensitive enough for use with dilute polymer solutions.

Despite these new developments, the most promising method for study of solution properties of peptides is the Calvet calorimeter, due to its very high sensitivity and the need for only moderate amounts of sample. The real promise of
work with this instrument was shown by Choquette\textsuperscript{3/4} in his work at the University of Montreal. For these reasons we chose the Calvet calorimeter for the work reported here.
## TABLE A

**Summary of Literature Data on PBA**

### Optical Rotatory Dispersion Data

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$b_0$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform</td>
<td>411</td>
<td>Blout (1958A)</td>
</tr>
<tr>
<td></td>
<td>631</td>
<td>Blout (1958B)</td>
</tr>
<tr>
<td></td>
<td>665</td>
<td>Bradbury (1959)</td>
</tr>
<tr>
<td></td>
<td>665</td>
<td>Karlson (1960)</td>
</tr>
<tr>
<td></td>
<td>720</td>
<td>Bradbury (1960)</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>Goodman (1962)</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>Goodman (1963)</td>
</tr>
<tr>
<td></td>
<td>659</td>
<td>Hashimoto (1966A)</td>
</tr>
<tr>
<td>Dichloroacetic Acid (DCA)</td>
<td>-250</td>
<td>Bradbury (1959)</td>
</tr>
<tr>
<td></td>
<td>-220</td>
<td>Bradbury (1960)</td>
</tr>
<tr>
<td>m-Cresol</td>
<td>534</td>
<td>Bradbury (1959)</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>Bradbury (1960)</td>
</tr>
<tr>
<td>Dimethylformamide</td>
<td>-315</td>
<td>Hashimoto (1966B)</td>
</tr>
<tr>
<td>Dioxane</td>
<td>630</td>
<td>Dubin (1972)</td>
</tr>
<tr>
<td>Dimethylsulfoxide</td>
<td>-250</td>
<td>Dubin (1972)</td>
</tr>
</tbody>
</table>
TABLE A (CONT.)

Derived Parameters

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$\sigma$</th>
<th>$\Delta H_{\text{cal}}$</th>
<th>$\Delta H_1$</th>
<th>$\Delta H_2$</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform-DCA</td>
<td>$0.6 \times 10^{-4}$</td>
<td>260 cal/mole</td>
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<td></td>
<td>1</td>
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<tr>
<td>m-Cresol</td>
<td>$1.6 \times 10^{-4}$</td>
<td>-450</td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>6.7%DCA/DCE</td>
<td>$1.0 \times 10^{-4}$</td>
<td>270</td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>7.0%DCA/DCE</td>
<td>$1.1 \times 10^{-4}$</td>
<td>230</td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>7.3%DCA/DCE</td>
<td>$1.1 \times 10^{-4}$</td>
<td>175</td>
<td></td>
<td></td>
<td>2</td>
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<tr>
<td>DCA/DCE</td>
<td></td>
<td>-560</td>
<td>3300</td>
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<td>2</td>
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<tr>
<td>DCA/CCl$_4$</td>
<td>$0.76 \times 10^{-4}$</td>
<td>-650</td>
<td>5200</td>
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<tr>
<td>DCA/CHCl$_3$</td>
<td></td>
<td>-390</td>
<td>3030</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Dioxane/DMSO</td>
<td></td>
<td>-390</td>
<td>800</td>
<td></td>
<td>4</td>
</tr>
</tbody>
</table>

1 Hayashi (1969)
2 Sayama (1972)
3 Norisuye (1973)
4 Karasz (1973)
REFERENCES FOR TABLE A

Polarimetry. All data reported here were taken using the Perkin-Elmer Polarimeter and the standard one-decimeter cells provided with it. Two cells were used during the course of the work: a microcell holding 0.6 ml of polymer solution (#141-0024) was used for the first part of the work; later a macrocell holding 6.0 ml of solution (#141-0023) was substituted since it allowed a wider beam to pass through the cell to the detector and thus more precise readings to be obtained. These cells are made of two concentric cylinders; a central sample tube which is surrounded by a large jacket through which thermostatted water or other fluid can be circulated for good control of the sample temperature over a wide range.

In a polarimeter run, the polymer was dissolved in the appropriate solvent mixture, and the solution placed in the polarimeter cell. The polarimeter was set to zero with the sample chamber empty, and the cell then placed in the chamber. Next, the hoses to the Haake bath were attached to the cell, and the previously equilibrated bath liquid circulated through the cell jacket. After about about a five minute equilibrium period, the reading was checked, and any drift in the reading over a one to two minute period was taken as an indication that the sample temperature was not yet uniform and equal to that of the bath. When the readings showed no further drift, the magnitude of the ro-
tation was recorded, and the bath raised to the next temperature where the whole procedure was repeated. The changes in bath temperature were kept quite small near the transition temperature (one-half to one degree increments) and the increments increased to five or ten degrees at the extremes of the transition curve to get an initial and final slope of the curve with temperature.

After a run was completed, the cell was cleaned by repeated washing and flushing with solvent, and thoroughly dried with dry, filtered air. In the rare cases where washing caused precipitation of the polymer onto the cell windows, the cells were cleaned with chromic acid cleaning solution or, in later runs, with concentrated DCA solution in chloroform.

The Model 141MC polarimeter provides a digital readout of the rotation in degrees. This value $\alpha$ is then reduced to the specific rotation using the equation

$$[\alpha]_\lambda = \frac{1000\alpha}{c \lambda}$$

where $c$ is the polymer concentration in grams/liter and $\lambda$ is the path length in decimeters.

As mentioned in the theory above, we have used the specific rotation at 436 nm as an indication of helical content. Moffitt plots were done on polymer solutions in which the transition temperature occurred at temperatures within the -20° to 55°C. range, and the values of specific
rotation plotted against the Moffitt $b_0$. These studies were later extended to $85^\circ\text{C}$ using tetrachloroethane (TCE) in place of chloroform. In all cases studied, the values of specific rotation and $b_0$ fell on the same straight line (Fig. 1). For subsequent analyses, a single value of the rotation was determined, and the helical content estimated by interpolation between the known values for 100% and 0% helix (see Theoretical above).

**Calorimetry.** A description of the basic Calvet instrument can be found in the recent paper by Maron and Filisko\textsuperscript{36} and will not be reported here in detail. The instrument in our laboratory was used as supplied by IMass, Inc. and included the basic Tian-Calvet calorimeter and, in a separate housing, the Setaram controller, a Bristol Model 552 Recorder, a Keithly Model 148 Nanovoltmeter-amplifier, and an IMass calibration circuit.

The calorimeter was calibrated using the calibration cells provided with the instrument, and the calibration circuit provided by IMass. The calibration cell contains a central ceramic cylinder, on which 1000 ohms of resistance wire is wound. The outer cell is a stainless steel cylinder with a teflon cap, similar to the reaction cells, except that only the leads to the resistance coil run through the teflon cap. A steel rod is attached to the top of the cap for the purpose of lifting the cell in and out of the
calorimeter block, and to provide a conduit for the heater leads.

To calibrate the instrument, these special cells are placed in the instrument and allowed to equilibrate. A current is then run through the resistance coil and measured on the appropriate meter of the calibration panel. The current is adjusted to the desired magnitude by adjusting the voltage applied, and can be varied from one microwatt to one milliwatt. The power dissipated by the resistor can be determined from the measured current and known resistance of the coil:

\[ P = I^2R \]

It was found through several calibration runs that the peak areas for identical runs agreed to within \( \frac{1}{2} \% \), so the limiting factor of the electrical calibration runs was the setting of the current on the meters. In practice, this limits the accuracy of the calibration to about two per cent. Five separate calibrations were made during the course of the work, and the values so determined were averaged for the final value used. The results of one of these runs is shown in Table 1 and Figure 2. Differences in the instrument constant at the various temperatures all fell within the two percent range of repeatability.

It is the task of the experimenter to design the cells in which the reaction takes place, and the design of these
TABLE 1
Electrical Calibration

<table>
<thead>
<tr>
<th>Current (mA)</th>
<th>Time (min.)</th>
<th>Energy (mcal)</th>
<th>Area (in$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>.030</td>
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<td>.194</td>
<td>.007</td>
</tr>
<tr>
<td>.050</td>
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<td>.037</td>
</tr>
<tr>
<td>.100</td>
<td>15</td>
<td>2.15</td>
<td>.064</td>
</tr>
<tr>
<td>.100</td>
<td>15</td>
<td>2.15</td>
<td>.067</td>
</tr>
<tr>
<td>.100</td>
<td>29.5</td>
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<td>.129</td>
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<td>.200</td>
<td>14.5</td>
<td>8.30</td>
<td>.262</td>
</tr>
<tr>
<td>.200</td>
<td>15.25</td>
<td>8.73</td>
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<td>19.31</td>
<td>.591</td>
</tr>
<tr>
<td>.400</td>
<td>15</td>
<td>34.34</td>
<td>1.058</td>
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<td>.400</td>
<td>15</td>
<td>34.34</td>
<td>1.054</td>
</tr>
<tr>
<td>.600</td>
<td>15</td>
<td>77.25</td>
<td>2.417</td>
</tr>
<tr>
<td>.800</td>
<td>14</td>
<td>128.18</td>
<td>3.995</td>
</tr>
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<td>.800</td>
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<td>4.238</td>
</tr>
<tr>
<td>1.000</td>
<td>15</td>
<td>214.59</td>
<td>6.834</td>
</tr>
</tbody>
</table>

Total: 672.70 | 21.037

Instrument Constant = 32.00 ± 0.5 mcal/in$^2$
cells proved to be the most time-consuming part of the experimental procedure. For the PEA system, the requirements for the reaction cells are quite restrictive. The dichloro-acetic acid is a very corrosive solvent, and the inclusion of any materials within the cell except teflon and glass results in a reaction large enough for the instrument to detect. The original cells were made of grade 303 stainless steel, but the reaction of the DCA with even this material caused a large baseline shift upon opening, and a glass liner had to be added to separate the reacting materials from the metal cell. We found the glass liners quite sufficient for the purposes of these experiments; others have reported the successful use of grade 304 stainless with DCA solutions.\textsuperscript{31,34}

Another constraint of the cell is imposed by the extreme sensitivity of the instrument. The Calvet is reportedly able to detect as little as one milicalorie per hour in heat flux\textsuperscript{37}; the experiments reported here produce about 200 mcal of heat, or roughly the equivalent of ten micrograms of solvent evaporating. Thus an appreciable change in vapor space will cause sufficient vaporization to completely swamp out the effect we wish to observe, and any flow of gas through the cell is completely prohibited.

In addition, the temperature at which the transition occurs is high enough to preclude use of the fluorocarbon
greases as sealing materials, so the cell design must include a glass-to-glass or glass-to-teflon seal, and in addition, provide for accurate measurement of the denaturant mass or volume. Many cell designs were tried and rejected for one or more of the above reasons.

In his thesis Filisko relies heavily on the cell design depicted in Figure 3. This design employs a mercury seal around the inner cell (cup) and a teflon cap. This design allows for complete isolation of the inner and outer solutions before mixing, opening without change in the vapor space, and unlimited, reproducible mixing. Unfortunately, we were never able to construct a cap which could be opened reliably without sticking and yet be of sufficient tightness to keep the mercury from displacing some of the inner fluid. Thus reliability suffered from the leakage of an unknown and irreproducible amount of denaturant.

We also tried the cell design of Hermans and Rialdi in which the inner cell is suspended from the inner tube (to the outside) and sealed with mercury or petroleum jelly. The contents are forced out using a syringe above the cap. This design was found to be totally unsuitable for our experiments, as the change in vapor space and height of the solution in the cell causes large heat effects due to solvent evaporation and condensation. In addition, mixing is inefficient and incomplete, and every movement of the sy-
Figure 3
ringe plunger during mixing causes heat effects similar to those at opening.

Our first attempt at original cell design is shown in Figure 4, a version of the 'trap door' design mentioned in Filisko's thesis. This cell has the advantages of easy communication of vapor spaces in the inner and outer cells to prevent evaporation upon opening, and unlimited, reproducible mixing. The problem lies in the sealing of the 'door' to the inner cell. As mentioned above, the denaturant is a very reactive organic acid, and the upper temperatures at which we worked were sufficiently close to the softening point of the fluorocarbon greases available that they were unusable. Parafilm®, Saran Wrap®, press-fit teflon and ground glass joints were all tried for the seal, but none proved successful in preventing leakage of the inner cell during the equilibration period.

The final design we used to gather all of the data presented here is shown in Figure 5. This design provides for absolute separation of outer solution and denaturant during equilibration, repeatable mixing, minimal change in vapor pressure upon opening, and conservation of total vapor space. There remains some problem with the level of the liquid changing upon opening and with redistribution of vapor space, but these can be minimized by filling the inner bulb as completely as possible with denaturant. A
Figure 4

- Teflon cap
- Teflon washer
- Glass bell
- Glass or Teflon "door"
- Denaturant solution
- Polymer solution
Figure 5
brief description of the procedure for making a calorimeter run follows: a more detailed description of the operation of the Calvet may be found in Appendix I.

Once the Calvet has stabilized at the desired temperature one is ready to make experimental runs. The first step is the manufacture of the inner bulbs to hold the denaturant. These are hand-blown from normal-wall Pyrex tubing, and must be blown to as nearly the volume required to hold the denaturant for that individual run as is possible, but must not exceed fourteen millimeters in diameter. The bulbs must be thick enough to withstand handling during the filling and sealing procedure, yet thin enough to shatter when pressed against the tungsten point in the bottom of the cell. Although pin-hole leaks can and do occur, these show up in the sealing procedure and, while the bulbs containing them must be replaced and the run delayed, they will not otherwise adversely affect the run.

The inner cells (bulbs) are then filled with denaturant, the teflon ring stirrer placed on the stem, and the stem sealed to the glass rod extending to the outside. The outer cells may then be filled with polymer solution and the cells assembled. Care should be taken to insure that the stirrer disk is below the liquid surface of the assembled cell, and that it will remain below even with any loss of volume on breaking of the bulb. Such positioning will assure temperature equilibration of both inner and outer cells,
and will minimize solvent evaporation effects due to exposure of surface area when the bulb is broken.

Here, then, is a step-by-step guide to filling the cells:

1. Manufacture the inner bulbs.
2. Weigh the denaturant into the inner cells.
3. Secure the stirring disk to the inner cell capillary.
4. Seal the completed inner cell assembly to the rod and heat the bulb capillary to expel any denaturant drawn into this space.
5. Adjust the height of the inner cell so the stirring disk will be below the liquid surface in the completed cell assembly.
6. Weigh the polymer solution into the outer cell.
7. Thread the outer cell assembly onto the rest of the cell and tighten.

The cells are now ready to be placed in the calorimeter for equilibration. Both cells should be introduced into the calorimeter at the same time to minimize equilibration time. Over the temperature range used here, the cells should equilibrate to the point where the baseline is stable in about two hours.

The procedure for starting and recording the reaction is as follows:
1. After the baseline has stabilized, turn the amplifier on and to the appropriate scale for the expected magnitude of the reaction.

2. Select the polarity of the recorder - endothermic in the right cell or endothermic in the left. (Since the two cells are connected in series opposition, an exotherm in the right cell is equivalent to an endotherm in the left, and vice versa)

3. After recording the baseline for ten to fifteen minutes, lower the inner bulb far enough to pierce it on the tungsten needle and continue to press until the bulb has shattered on the bottom.

4. Raise and lower the rod four more times to mix the solutions, then return it to its original position.

5. When the trace has returned to the baseline, repeat the mixing operation. This must be done at least twice, and usually three times to allow for correction of the peak area for incomplete mixing and mechanical mixing heat.

6. When the final mixing peak has returned to the baseline, repeat steps 2 - 5 with the second cell.

7. When both cells have been run and recorded, turn off the recorder and the amplifier and remove the cells for cleaning and reuse.

To clean the cells, we relied on repeated rinsing with
solvent, followed by a thorough drying of the outer cell. Generally this clean-up and the attachment of new inner cells required an hour and a half to two hours, the equilibration period about two hours, and an additional two hours for the run in each cell. It is thus theoretically possible to squeeze three runs into a twenty-four hour period, if all necessary samples and solutions have been previously prepared. In practice, an experienced and dedicated operator can manage two runs a day, including sample preparation. If data analysis is to be done concurrently, along with the checking of all polymer solutions polarimetrically before and after each run, an upper limit of six complete runs (two samples per run) each week is sustainable over a long period of time.

The peak areas were determined using either a Gelman Compensating Planimeter or a cut-and-weigh technique. The planimeter measures directly in square inches to a precision of 0.01 square inch. Areas found by this technique were repeatable to about ½%; in all cases the area recorded was an average of at least three and usually five determinations. For the cut-and-weigh technique, the peak was traced onto Keuffel and Esser Albanene Tracing Paper which had been aged for 24 hours in air, and this copy was cut out and weighed on our semi-micro balance. The precision of this technique was at least as good as the planimeter results,
and the accuracy was found to be superior when measuring small areas. The cut-and-weigh technique was therefore used exclusively throughout the latter part of the data analysis.

The above cell design and experimental techniques proved to be satisfactory for the systems we studied. At each temperature, five runs were made at different polymer concentration and the results averaged by taking the slope of a heat vs. sample weight plot (see below) as the value for that temperature. Accuracy of the results so obtained is estimated at around ten percent, including the possible two percent inaccuracy in calibration.

The Calvet as supplied is capable of operating from about ten degrees above ambient to $570^\circ$K. In our experiments, it rapidly became apparent that the most useful results lay in the subambient region. To allow operation in this region, a cold box was constructed around the Calvet, the details of which may be found in Appendix II. This cold box reduced the ambient temperature seen by the Calvet to $250^\circ$K., allowing measurements to be made over the entire range of $270^\circ$ to $310^\circ$K. Calorimetric measurements above $310^\circ$K were impractical due to the small magnitude of heat evolved (approaching zero at $325^\circ$) and polymer degradation at temperatures higher than $350^\circ$K.
Materials. The poly-β-benzyl-α-aspartate (PBA) for the optical work was purchased from Sigma Chemical Corporation (#P-9378 Type I) lots #90C-0560 and #30C-3200, viscosity average molecular weight 230,000 and 200,000 respectively. The PBA used in the calorimetric studies was purchased from Biopolymers Corporation, lot #100, viscosity average molecular weight 360,000. This material as received proved identical to the Sigma Chemical batches in optical parameters, and all polymer samples were used without further purification.

The 1,1,2,2-tetrachloroethane (TCE) used in this study was purchased from Eastman Organic Chemicals (#241) and from Fisher Chemical Corporation (#A-31). No differences were found between the performance of the two batches, and both were reagent grade. The chloroform used in the optical studies as solvent was Eastman Organic SpectroACS grade (#13056) and that used for general cleaning, reagent grade. The dichloroacetic acid (DCA) was purchased as technical grade material, and was purified by distillation at 75°C under 6mm (absolute) pressure. The distillate was then frozen and stored at 0°C until shortly before use to retard degradation.
RESULTS AND DISCUSSION

**Polarimetry.** For the PBA, percent helix was calculated using the empirical equation for the rotations

\[
f_H = -\frac{[\alpha]}{272} + \frac{20}{272}
\]

(18)
as calibrated using the Moffitt \( b_0 \) values (see above).

Typical runs at various solvent compositions are shown in Tables 2 and 3 and Figure 6.

The temperature at which the polymer in the various solvent mixtures attain given values of helical content are cross-plotted against the solvent composition to give 'phase diagrams' of the isohelical contents in Figure 7. Paths across these curves in the vertical and horizontal directions represent, respectively, the thermally-induced and isothermal transitions.

Values of the compositional heat can be determined directly from the curve representing \( f_H = 0.5 \) through an equation derived from Equation 3:

\[
\Delta H_{\text{comp}} = \frac{RT_c^2}{X_{A,c}} \left( \frac{\partial X_A}{\partial T} \right) T = T_c
\]

(19)

Likewise, the variation of \( s \) with either temperature or solvent composition may be calculated from the plot using Applequist's equation 5 for \( s \) in terms of \( f_H \):

\[
f_H = \frac{1}{2} + \frac{(s - 1)}{2 \left\{ (1 - s)^2 + 4s \right\}^\frac{1}{2}}
\]

(20)
### TABLE 2

**Temperature vs. Rotation**

5.20 mole % DCA

2.87 mg/ml PBA

<table>
<thead>
<tr>
<th>T(°C)</th>
<th>α</th>
<th>(-[\alpha]_{436})</th>
</tr>
</thead>
<tbody>
<tr>
<td>-13.5°</td>
<td>99.905</td>
<td>33.10</td>
</tr>
<tr>
<td>-12°</td>
<td>99.888</td>
<td>39.02</td>
</tr>
<tr>
<td>-8°</td>
<td>99.750</td>
<td>87.11</td>
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<tr>
<td>-5°</td>
<td>99.587</td>
<td>143.90</td>
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<td>99.415</td>
<td>203.83</td>
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<td>1.5°</td>
<td>99.306</td>
<td>241.81</td>
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<td>5°</td>
<td>99.244</td>
<td>263.41</td>
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<tr>
<td>10°</td>
<td>99.201</td>
<td>278.40</td>
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<td>15°</td>
<td>99.186</td>
<td>283.62</td>
</tr>
<tr>
<td>20°</td>
<td>99.173</td>
<td>288.15</td>
</tr>
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<td>25°</td>
<td>99.174</td>
<td>287.80</td>
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<td>99.178</td>
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<td>47°</td>
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<td>81°</td>
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<td>empty</td>
<td>00.007</td>
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</table>
TABLE 3

Temperature vs. Rotation

6.58 mole % DCA

3.12 mg/ml PBA

<table>
<thead>
<tr>
<th>T(°C)</th>
<th>α</th>
<th>$-\left[\alpha\right]_{436}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>-11°</td>
<td>99.937</td>
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<tr>
<td>(9°)</td>
<td>99.933</td>
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<td>(1°)</td>
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<td>99.361</td>
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<td>99.366</td>
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<td>59°</td>
<td>99.383</td>
<td>197.76</td>
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<td>80°</td>
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<td>99.381</td>
<td>198.40</td>
</tr>
<tr>
<td>22°</td>
<td>99.647</td>
<td>113.14</td>
</tr>
</tbody>
</table>
Figure ?
In order to apply Equation 20 to the experimental data, it is necessary to have a value for $\sigma$, the initiation parameter of the Zimm-Bragg theory.\(^3\) This parameter is accessible through a combination of data from the optical work with the calorimetrically determined heat. For the denaturing reaction, we may define an equilibrium constant

$$K = \frac{X_D}{X_H} = \frac{f_D}{f_H}.$$  \hspace{1cm} (21)

Using this constant, we may further define an apparent heat of transition using the van't Hoff equation:

$$\Delta H_{VH} = R \left( \frac{d \ln K}{d \frac{1}{T}} \right)$$  \hspace{1cm} (22)

The parameter $\sigma$ is then related to the ratio of the van't Hoff heat to the calorimetric heat.\(^13\)

$$\sigma^{-\frac{1}{2}} = \frac{\Delta H_{VH}}{\Delta H_{\text{cal}}}$$  \hspace{1cm} (23)

Figure 8 shows the plot of $\ln K$ vs. $1/T$ for a series of solvent compositions which give $K = 1$ close to the temperatures at which the calorimetric heats were measured (see below). A summary of the van't Hoff heats at $K = 1$ for these plots is given in Table 4, along with the measured calorimetric heats and calculated values for $\sigma$.

Plots of $1/s$ vs. $X_A$ are shown in Figure 9. A weighted least squares fit was applied to the curve for each tem-
Figure 8
TABLE 4

Calculation of $\sigma$

<table>
<thead>
<tr>
<th>Mole % DCA</th>
<th>$\Delta H_{VH}$ cal/mole res.</th>
<th>$\Delta H_{VH}$ (interpolated cal/mole res.)</th>
<th>$\Delta H_{cal}$ cal/mole res.</th>
<th>$\sigma$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.02</td>
<td>40.36</td>
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<tr>
<td>5.36</td>
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<td>34.89</td>
<td>357.9</td>
<td>1.052 x 10^{-4}</td>
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<tr>
<td>5.50</td>
<td>32.64</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>5.64</td>
<td></td>
<td>32.11</td>
<td>334.5</td>
<td>1.085 x 10^{-4}</td>
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<td>5.77</td>
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</tr>
<tr>
<td>6.03</td>
<td>28.05</td>
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</tr>
<tr>
<td>6.15</td>
<td></td>
<td>28.24</td>
<td>298.0</td>
<td>1.114 x 10^{-4}</td>
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<td>6.31</td>
<td>28.49</td>
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<tr>
<td>6.44</td>
<td>25.14</td>
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<tr>
<td>6.52</td>
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<td>25.36</td>
<td>229.4</td>
<td>0.818 x 10^{-4}</td>
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<tr>
<td>6.58</td>
<td>25.53</td>
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Average $\sigma = 1.005 (\pm .12) \times 10^{-4}$
perature, and the values of $K_1$ and $K_2$ determined from the intercept and the slope according to Equation 12. The values of $\ln K_1$ and $\ln K_2$ were then plotted against $1/T$ (Fig. 10) and values for the enthalpies and entropies calculated. The values determined for $K_1$, $K_2$, $\Delta H_1$, $\Delta H_2$, $\Delta S_1$, and $\Delta S_2$ are shown in Table 5.

These values for the parameters were then inserted into Equation 13 with $s = 1$ ($f_H = \frac{1}{2}$, $T = T_c$) and adjusted within the experimental uncertainty to generate the phase diagram shown in Figure 11. As can be seen from the figure, the line so determined is a very good fit to the data actually gathered polarimetrically. In fact, the fitting of the data by small variations in the parameters would seem to provide a rather precise refining of the values. Figure 12, A through D, shows the change of the fit of the calculated values with a five percent change in the individual values of $\Delta H_1$, $\Delta H_2$, $\Delta S_1$, $\Delta S_2$ respectively. It is seen that the effect of changing $\Delta S_2$ is simply a shift in the entire curve along the $X_A$ axis with only minimal change in the curve shape. Thus $\Delta S_2$ may be used as a 'fitting parameter' in this dimension when the other parameters are changed. In fact, the changes induced by each of the parameters can be made to compensate for one another, as is shown in Figure 13 where all of the parameters have been changed substantially, yet the fit to the data is still good.

To get the best fit of the data, shown in Figure 11
TABLE 5
Calculated Constants

<table>
<thead>
<tr>
<th>T(°C)</th>
<th>$K_1$</th>
<th>$\ln K_1$</th>
<th>$K_2$</th>
<th>$\ln K_2$</th>
</tr>
</thead>
<tbody>
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$\Delta H_1 = 481 \pm 140$

$\Delta S_1 = 1.13 \pm 0.35$

$\Delta H_2 = 3226 \pm 330$

$\Delta S_2 = 7.93 \pm 0.8$
Figure 11
above, we used a computer program which displayed the goodness of fit (sum of the squares of the errors) of the calculated line to the measured points at the various values of the parameters. The program stepped through three of the parameters incrementally, while calculating the fourth parameter to make the curve include one of the points. Since the curve is most sensitive to variations where it rounds the 'nose', the experimental point just below the 'nose' was chosen as the point through which all calculated curves would pass.

This data fitting method is consistent with the experimental data because of the large errors introduced into the analysis by the long extrapolations necessary to determine the parameters. It should be noted that the error in the values of \( \ln K_1 \) are of the order of 30% for this reason. However, the values found for \( \ln K_2 \) are precise to about 7%.* These numbers, in turn, lead to the error limits shown in Table 5 above.

Using the final values of these parameters and Equations 20 and 13 we may generate the phase boundaries for isohelical contents other than 0.5. The results of such calculations are shown in Figure 14 along with the experimental data.

*A good treatment for the estimation and propagation of errors in experimental data may be found in Reference 39, and the analyses outlined in Chapter 6 of this book are used for error analysis throughout this thesis.
Figure 14
It is seen that the fit is quite good along the lines representing helical fractions from 0.3 to 0.8. The polarimetric data therefore support the theoretical predictions for transition width. While it is obvious that the good agreement between the data and the calculated results is due in part to the use of the data in the analysis, the self-consistency of the results is encouraging. The independent check on the data will be the agreement of the calorimetric values measured with those predicted by the theory. These results are reported below.

We have assumed throughout the analysis that the activity coefficient of the active solvent (DCA) is one. In several recent papers, the workers at Osaka University argue for the existence of the DCA in dimer form. A reanalysis of our data assuming that the DCA reacts in dimerized form leads to the results shown in Table 6 and Figure 15. Since the same data is used in the two analyses, both that assuming monomer and dimer, if compared on a weight fraction DCA basis, the numerical differences in the values of Table 5 and Table 6 are mathematically significant, and show a difference in all of the parameters except $\Delta H_2$. From an experimental standpoint, it is obvious that the change in $\Delta H_1$ and $\Delta S_1$ are well within the experimental uncertainties. This is probably due to the very small DCA content of our solvents, and the differences between monomeric and
TABLE 6
Calculated Constants
DCA Dimers

<table>
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<tr>
<th>T(°C)</th>
<th>K₁</th>
<th>ln K₁</th>
<th>K₂</th>
<th>ln K₂</th>
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<td>.145111</td>
<td>-1.930259</td>
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<tr>
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</table>

\( \Delta H_1 = 464.5 \pm 140 \)
\( \Delta S_1 = 1.095 \pm .35 \)
\( \Delta H_2 = 3228 \pm 330 \)
\( \Delta S_2 = 6.70 \pm 0.8 \)
dimeric DCA would probably be outside the experimental uncertainty if the solvent contained more DCA, as for example with the PBG system.\textsuperscript{6,13,32}

The value of $4S_2$ does change significantly depending on the mode of action of the DCA. However, as was noted above, the effect of changing this parameter is simple translation along the $X_A$ axis, so it may be regarded as an activity coefficient. In summation, the experimental polarimetric data fit the predictions with either assumption about the action of DCA, and are not precise enough to distinguish between them.

**Calorimetry.** The first runs done with our experimental cells were made using aqueous HCl and NaOH solutions in an attempt to check the electrical calibration. The problems encountered using organic systems are magnified considerably when aqueous systems are used due to the five-fold increase in the heat of vaporization of the solvent, and even when the corrections for all known sources of error (such as dilution heats for acid and base, heat of formation and heat capacity of the salt formed, concentration effects, and all mechanical heats from opening and stirring) were included in the analysis, the instrument constant calculated from the acid-base reaction was still ten percent higher than that determined electrically. It has been argued\textsuperscript{43} that some differences may be expected between elec-
trical and chemical calibrations since the chemical reactions are uniform throughout the cell, while the electric heater is a point source. Such an argument assumes perfect and instantaneous mixing of the reactants in the cell during the chemical calibration, which is probably a weak assumption, and in any case the effect would not be expected to amount to ten percent. Such large differences are probably due to side-reactions such as the reaction of the base with the cell walls during the equilibration period. Unfortunately, these chemical calibrations could not be repeated with the final cell design used in the polymer work because of the obvious corrosion of the tungsten needle by the hydroxide solution. The electrical calibration is straightforward, precise, and easy to accomplish, and there is no evidence that the accuracy of this method is outside the two percent repeatability of the calibration runs, so we decided to abandon the attempts to check the calibration with aqueous systems and proceeded to use the instrument constant derived from the electrical calibration data.

An example of a recorder trace used as raw data for the calorimetric analysis is shown in Figure 16. One of the auxiliary stirring peaks is shown following the main transition peak. In general, three stirring peaks after the initial opening and stirring peak were recorded to help correct for incomplete mixing and non-equilibrium effects.
In each case, the areas indicated by the last two stirrings were taken to be the mechanical heat of stirring plus the decay of the (small) non-equilibrium effects induced by the opening procedure. From these two peaks, the expected areas of the stirring peaks at opening and at the first stirring after opening were determined by extrapolation and these areas used as corrections to the measured areas.

The Calvet calorimeter is a twin-type instrument, and may be used to measure two similar reactions during each run, as described by Filisko.\textsuperscript{37} For most runs, we used identical solvent mixtures and denaturant in both sides of the instrument, except that the solution in the left cell included PBA while that in the right cell did not. Since determination of the (normalized) heat at each temperature required five different polymer concentrations, we recorded during the same runs five 'blank' or 'tare' reactions. The tare reactions were then normalized to unit weight of denaturant and averaged to give a conversion constant which was used to determine the area which would have been expected had the polymer not been present in the solution. The results of the tare runs at 38°C. are shown in Table 7. Previous runs had shown that the heat of dilution of the polymer from the addition of solvent was negligible compared to the recorded heat. The difference between the recorded area and the expected area therefore represents the heat
### TABLE 7

Summary of Data

Gathered Calorimetrically

Tare runs at 28.8°C.

<table>
<thead>
<tr>
<th>Date</th>
<th>Weight Denaturant (g)</th>
<th>Area (in²)</th>
<th>Area/(g_{\text{den}})</th>
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<td>.62937</td>
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<tr>
<td>7/11</td>
<td>.63730</td>
<td>4.005</td>
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</table>

Overall average Area/\(g\) = 7.059 ± 0.535 rms
of transition of the polymer from the helix to the coil state.

Because the transition of the polymer is smeared out over a wide range in active-solvent composition, some correction for incomplete conversion must be made. We therefore checked the polymer solutions using the polarimeter both before and after each run. The change in fractional helical content indicated by the polarimetric rotations at the known temperature of the calorimetric transition averaged about 90%, and the figure for each run was used to correct the calculated heat for that run to 100% conversion. We assumed a linear relationship between heat output and conversion, an assumption which is reasonable based on theoretical considerations.\(^44^4\) The corrected areas were then converted to heats using the instrument constant derived from the electrical calibration (see Experimental above).

For determining the final value of the transition heat, the five values at different concentrations were plotted against the weight of polymer in the respective solutions. The slope of the line through the origin is taken as the normalized value of the transition heat at that temperature.

Table 8 shows the entire scheme for evaluation of the calorimetric data. Fig. 17 shows the heat vs. mass polymer plot for 29°C., and the final heats determined for all temperatures are presented in Table 9 and Figure 18. The
<table>
<thead>
<tr>
<th>Date</th>
<th>conc.</th>
<th>wt.</th>
<th>Area</th>
<th>Projected</th>
<th>ΔA</th>
<th>Δf</th>
<th>ΔA</th>
<th>Sample wt (mg)</th>
<th>Heat (mcal)</th>
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38.0°C  
30.515 mcal/in²

<table>
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<th>Area</th>
<th>Projected</th>
<th>ΔA</th>
<th>Δf</th>
<th>ΔA</th>
<th>Sample wt (mg)</th>
<th>Heat (mcal)</th>
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<tr>
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<td>Tare</td>
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<td></td>
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<tr>
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### Table 9

Summary of Data Gathered Calorimetrically

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<th>$T(^\circ\text{C})$</th>
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<th>$\Delta H_{\text{cal}}$</th>
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</table>
estimated precision of the data, as indicated by the error bars on the plot, is about ten percent of the measured values. Also shown on the plot in Fig. 18 are the calorimetric heats calculated from Equation 15, which lie quite close to the experimental values. The fit of these values is a true test of the theoretical predictions since the calorimetric data have not been used in calculating the parameters, and therefore constitute a truly independent check of the theory's validity. In these calculations, $F_c$ was figured using Equation 9 and did not involve the use of the experimental calorimetric heats. It is thus shown that the theoretical predictions are born out by the experimental data.
SUMMARY AND CONCLUSIONS

The Calvet calorimeter, with the reaction cells developed in the course of this work, proved to be quite servicable in the direct determination of the transition heats of polymeric systems, despite the rigid and constrictive requirements imposed by working with dilute solutions of these substances in corrosive solvents. For reactions in which the heat evolved is of the order of one calorie per gram of polymer, we were able to obtain a precision of about ten percent in our measurements. As is true for most experimental techniques, the precision is expected to increase with increasing heat of the reaction, but especially so in this case where differences between measurable 'tare' heats and experimental values must be used for the evaluations. The precision of our data was found to increase with decreasing temperature, not only because the reaction heat increased, but also because of the decrease in the measured tare heats. Thus at about 0°C., the experimental data became a sum of two smaller peaks rather than the difference between two large peaks, as originally found at 30°C.

The studies here encompass only one polymer-solvent system. Because of the interaction of polymer and solvent, one would expect different magnitudes of reaction heat in different solvents or with different polymers. Since it is
known that the transition heat of poly(benzyl glutamate) in our solvent system is about four times the heat per gram of the aspartate studied here, a study of this polymer would be of interest both in terms of increasing the precision of the technique, and as a more critical test of the predictions of theory. With more precise data it would be possible to calculate $F_C$ directly from the calorimetric heats and derive $K_1$ and $K_2$ from the temperature dependence of $F_C$. Such calculations were precluded in the present study by the imprecision of the data.

Another improvement in the experimental technique which should be explored is the possibility of a completely closed cell, which would avoid all of the problems of solvent evaporation and condensation encountered in this study. The principle hurdle in this suggestion is the possible pressure effects encountered in heating or cooling a closed system containing a volitile liquid. It might be possible, however, to load and seal such a cell at approximately the same temperature at which the experiment is to be run. The advantages of a closed system seem large enough to justify exploration of this possibility.

The optical rotatory dispersion measurements on poly (benzyl aspartate) made here are sufficiently precise to allow determination of the intrinsic polymer enthalpy and entropy of the helix-coil transition in the mixed solvent
system. Although the extrapolations needed in the data analysis limit the precision of the data to about 30%, the parameters so determined agree quite well (to about 5%) with those determined by a computer-fitting of the data to the equation derived from the Karasz-Gajnos treatment. In addition, the calorimetric heats determined with the Calvet agree with the predictions of the theory to within the uncertainties in the measurements. It is thus shown that one can obtain quite reasonable estimates of the calorimetric heat from non-calorimetric data and the presently-available theoretical treatments relating these physical constants. Special note should be made, however, of the need to correct for incomplete conversion in the calorimetric work.

Also of particular interest is the confirmation of the double thermal transition in the PBA which was predicted from the theory. Recent results, as yet unpublished, by coworkers in this lab have shown that the thermal decomposition of the polymer depends in part on the solvent system employed in the study. Thus we may reasonably expect to observe the double transition in other polymers as well by using a solvent system of suitable active-solvent strength and one which protects the polymer against thermal decomposition.

Finally, the question of the mode of action of the DCA remains unresolved, although the actual calculations
for the PBA system show the general problems in distinguishing between monomer and dimer modes require very precise determinations of the parameters (of the order of 2% for this system). The differences in effect will be enhanced by increasing the mole fraction of DCA, and again the PBG system is suggested because of the high DCA content at the transition point (about 80% at 25°C).

These are a sampling of the possible extensions of the work reported here which might be expected to be fruitful. The techniques have been proven, and now need additional refinement to answer these and similar questions.
EFFECT OF DEUTERIUM EXCHANGE ON THE STABILITY OF LYSOZYME IN SOLUTION

Introduction. For polypeptides, the nature of the helix-coil transition has enabled several authors to develop thermodynamic treatments of experimental data which allow the evaluation of intrinsic stabilization energies for the helical forms of these polymers. In the case of proteins, the situation is much more complex and is not yet amenable to such detailed theoretical treatment. It is therefore necessary to use more indirect methods for evaluating the relative significance of certain factors in stabilizing the protein conformation. It is generally supposed that one large factor in this stabilization energy is that of the hydrogen bonding in the helical portions of the proteins. Some idea of the importance of the hydrogen bonds can be deduced from the change in stability of the protein when labile hydrogens are replaced by deuterium atoms.

Several biological molecules have been studied using this principle, with varying results. Maybury and Katz found an increased stability for ovalbumin in D$_2$O as compared to H$_2$O, as was also found by Hermans and Scheraga for ribonuclease. However, the thorough treatment by Tomita, et al. shows that the effects of replacing hydrogens with deuterium atoms can be either stabilizing or disruptive.
These predictions were confirmed by the work of Hattori, et al.\textsuperscript{48} who found that deuteration could cause an increase in denaturation temperature of phycocyanin, or a decrease in this transition point, depending on the placement of the substitutions.

For this study, we chose the protein lysozyme (muramidase) whose structure has been well characterized.\textsuperscript{49-52} Our results on the normal lysozyme confirm the findings of Sophianopolis and Weiss\textsuperscript{51} and have been reported elsewhere.\textsuperscript{53} The effect of the deuteration is reported here.

**Materials.** The lysozyme used for all experiments reported here was purchased from Gallard-Schlesinger (Miles-Seravac \#36-323 Egg White Lysozyme Chloride, batch 110) as a 3X crystalized powder and was used without further purification. Acid solutions were made up from distilled water and Fisher Reagent Grade Hydrochloric Acid. Base solutions were made using Reagent Grade Sodium Hydroxide and distilled water.

Buffer solutions used in adjusting the pH meter were Fisher Certified (pH 1.0 and 2.0) and Radiometer (pH 6.50). Deuterium Oxide was purchased from Stohler Isotope (99.8\% D) and deuterium chloride (20\% in D\textsubscript{2}O) from Diaprep.

**Instrumentation.** Optical rotations were measured with a Perkin-Elmer Model 141MC spectropolarimeter.
ments were made at 436 nm using a mercury lamp, and temper-
ure control was provided by a Haake Model FK circulating
constant temperature bath. The Perkin-Elmer polarimeter
has a digital readout, and both temperature and rotation
were recorded by hand for later analysis.

The pH or pD of all solutions was measured using a
Radiometer Model pH26 meter, equipped with a semi-micro
porous pin electrode (#GK2321). The pD values for samples
in D₂O were calculated by adding 0.4 to the apparent pH.54

The calorimetric data for this paper were gathered
using a modified Perkin-Elmer DSC Model 1B. The sensitiv-
ity was improved by three times using specially-made seal-
able sample pans which allowed the use of three times the
volume of sample as the commercial sealable pans. These
pans were a scaled-up version of the Perkin-Elmer volatile
sample pan, and were made by deep-drawing an aluminum blank
of 5 mil thickness. A complete description of these sample
pans is given in a recent paper by Simon and Karasz.32 An
example of the DSC traces which were evaluated is given in
Reference 53.

Deuteration of lysozyme. Since the denaturation of
lysozyme is not completely reversible,55 it is not possible
to denature the protein to assure complete exchange of the
protons with deuterium. We relied on the slow exchange of
protons over a twelve hour period for this study. Solutions
of lysozyme were made up by dissolving the lysozyme in H₂O
(D₂O) solution of HCl (DCl) or NaOH (NaOD) of the proper pH or pD which were then allowed to stand overnight before use. This procedure assured us of at least 85% exchange before use. No attempt was made to buffer the solutions, and the salt content from adjustment of the pH (pD) never exceeded 0.1 N. Concentrations of lysozyme were kept at 3% w/v.

Results and discussion. A typical polarimetric run showing reduced rotation as a function of temperature is shown in Figure 19. The plot is of the usual sigmoidal shape typical of cooperative transitions. The temperature of the transition, Tₑ, is taken as that temperature at which the transition curve reaches half-height. The van't Hoff enthalpy was figured at the midpoint of the transition in the usual way, and the results are shown in Fig. 20. Because of the extreme sharpness of the transition, the error in the reported van't Hoff heats is large, and any difference in the heats between the normal and deuterated forms is well within the experimental errors. We cannot therefore distinguish between the normal and deuterated forms on the basis of van't Hoff heats. However, as illustrated in Figure 21, the transition temperature of the deuterated molecule has shifted to a lower temperature at the pD corresponding to the pH. A plot of transition temperature versus pH (pD) is shown in Figure 21, and shows a shift of the
Figure 20

$\Delta H_{vH}$ (Kcal Mole) vs $pH (pD)$

- $H_2O/HCl$
- $D_2O/DCl$
transition to the alkaline by about 0.4 units. We could have anticipated this result from the work done by Appel and Brown\textsuperscript{56} whose work with myoglobin showed the same shift in pH (pD) for both acid and alkaline denaturation. Their explanation for this apparent decrease in stability toward alkaline denaturation and increase toward acid denaturation with deuteration is the decreased ionization of the Brønsted acid groups on the protein chain in heavy water.\textsuperscript{57,58}

The data derived from the DSC runs shows more promise in distinguishing between normal and deuterated forms of lysozyme. As shown in Figure 22, the heat of transition measured calorimetrically for the deuterated samples fall considerably below those for the normal samples. The scatter in the data is admittedly quite large, but it must be remembered that we are working on the very edge of the instrument's sensitivity. Nonetheless, the trends in the data for the normal and deuterated samples seem to be quite real, with the deuterated samples giving about half the heat of the normal samples.

Many explanations for the difference in stability of proteins in heavy water have been proposed by various authors.\textsuperscript{45,46,48,56} Several of these explanations could lead to a measurable difference in transition enthalpy, notably the hypothesis that heavy water has a different and more ordered structure around the protein molecule than does
light water. Thus when the protein denatures, more water "freezes" around the newly exposed surface of the protein, and the measured enthalpy of the denaturation is thereby lessened. In any case, the effect of changing the solvent cannot be separated from the intrinsic effects of deuterium substitution for labile hydrogens unless an aprotic solvent is used. There is to date only one study done with aprotic solvents, and it showed no effect with deuterium substitution. Combining these results with the lack of effect found in the van't Hoff data reported above, it seems quite reasonable to assume that the difference in measured calorimetric heat is due to a solvent effect rather than to the effect of isotope substitution in the protein.

Conclusions. The optical and scanning calorimetric studies on lysozyme reported here show no indication, outside of experimental error, of a change in stability of this protein due to substitution of deuterium for the labile hydrogens. The observed difference in the optical results is easily explained by the weakened ionization of the protein's ionizable groups, and the observed change in calorimetric heat is more likely attributable to solvent effects than to intrinsic protein stability. These conclusions are in agreement with the presently available literature.
REFERENCES

24. T. Ackerman and E. Newmann, Biopolymers 5:649 (1967).


APPENDIX I

A Practical Manual for Operation of the Tian-Calvet Microcalorimeter

Provided with the Tian-Calvet microcalorimeter and the measuring circuitry is a set of six different instruction manuals to guide the user in the operation of the instrument. For practical purposes, only one of these manuals is really useful in providing the user with a knowledge of the essentials of the instrument operation, and this is somewhat incomplete in its description on the assumption that the other manuals provide the necessary missing information. While this is true, it takes a great deal of time and effort to separate the wheat from the chaff in these manuals, and it is therefore the purpose of this composition to bring together all of the procedures needed to run the Calvet on a day-to-day basis. When modifying the read-out system, more specific instructions and circuit diagrams may be found in the operating manuals of the individual components.

Immediately after unpacking the instrument parts, there are many procedures which must be performed for set-up and operation of the instrument. Most of these (such as aligning and plumbing the inner block) should be performed by an agent of the importer (IMass) and need be done only once.
We are not concerned here with these procedures. However, it is occasionally necessary to move the instrument from one laboratory to another, and the set-up procedures for this will therefore be included.

Preparing the Instrument for Moving. If the instrument is to be moved, all cables between the control console and the calorimeter block should be disconnected. All cables have uniquely coded plugs and sockets except the inputs to the Keithly. Careful note should therefore be made as to which lead from the Keithly is connected to each color lead in the wire from the calorimeter box. Other than these two leads, the cables cannot be interchanged. Also, the caps at the top of the entrance tubes in the Calvet should be unscrewed, and the glass tube liners replaced with bakelite tubes. The calorimeter is now ready to be moved, and should be able to withstand accidental shocks in transit if handled with reasonable care. If the unit is to be shipped by commercial carrier, consult the importer (IMass) for instructions and further precautions.

Setting the Instrument Up in the New Location. The calorimeter block assembly should be set up on a sturdy base away from large heat sources (or sinks), and in an area as free from drafts as possible. Experience has shown that setting the instrument on a solid base (up off its rubber
feet) is preferable to simply allowing it to stand as designed, since the minor vibrations from the floor are not as large as those induced by the operator in starting a run when it is mounted on a shock-absorbing base. The base should be level, and the control console must be placed within reach of the cables (about four feet). Reinstall the glass tube liners in the entrance tubes.

Reconnect the cables to the temperature regulator and reattach the input cables to the Keithly input leads, being sure the polarity is correct.

**Grounding cables.** There is provision for an external grounding cable on the case of the temperature regulator unit and on the Keithly nanovoltmeter-amplifier. The nanovoltmeter ground should be connected to the calorimeter shell, and the shell grounded to a good earth ground through the same point. All ground wires should be woven copper grounding straps or copper wire of .050 in. diameter (16 gauge) or larger.

The control console is provided with a three-wire (grounded) plug for use in grounded outlets. To prevent significant line noise in the calorimeter circuitry, an isolation transformer is provided with the unit, but this has only a two-wire plug and socket. We designed a special patch-cord connector to isolate the console from line noise using the transformer and to jumper the grounding wire around
the transformer. This unit has an exposed alligator clip in the center of the jumper wire, and it is to this clip that the ground of the temperature regulator should be connected, not to the common ground point on the calorimeter shell. Connection of the grounding wires in this way prevents the noise from the temperature regulator from feeding back into the Keithly circuitry.

When all cables have been connected and the grounding wires connected, check to see that main switch (Power) on the control console is off, and plug the panel into a 115V power source. If a filtered, instrument power circuit is available, this is ideal. Otherwise normal laboratory power may be used, although the noise level in the instrument will then be slightly higher.

**Initial Equilibration.** Check to see that all components are off at their respective power switches, then switch the main power on. The pilot light above the power switch should light to indicate that power is flowing to the instrument.

The temperature regulator has five small dials on the lower panel. The left-most dial is for manual regulation, and is rarely used (explanation below). The second dial is for setting the probe resistance scale, and for most normal measurements is set to the 100-150 scale. For temperatures in the 130° to 200°C range, the 100-200 scale
is used; for temperatures below $0\,^\circ C$, the 10-260 scale is used. The remaining dials are for setting the actions of the controller; proportional, integral, and differential respectively. The manual for the RT3000 regulator has an elaborate procedure for setting these controls to minimize hunting and maximize sensitivity and control accuracy. Experience has shown that only three settings need ever be used, at least for the temperature range of $-10^\circ$ to $60^\circ$C.

For initial equilibration and for temperature changing, the P/I/D settings should be 25/1/0. After the temperature is reached and the regulator is in control, switching to a setting of 15/2/1 gives excellent temperature control over the entire range given above.

The large dial is used to set the resistance in the comparison arm of the control bridge, of which the control temperature probe is a part. The proper setting for a given temperature is given in Table A-1. For initial equilibration this dial should be set to about 23 ($30^\circ$C.) with the 100-150 ohms scale. To set, the small knob marked 'blockage' (lock) must be loosened and the setting adjusted (use the small 'consigne' (set) knob for the fine adjustment) and the lock retightened.

**Summary of the settings for initial equilibration:**

- Probe resistance: 100-150
- Proportional: 25
<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Resistance (ohms)</th>
<th>Divisions on Dial</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>100-150</td>
</tr>
<tr>
<td>-30</td>
<td>88.26</td>
<td>---</td>
</tr>
<tr>
<td>-20</td>
<td>92.18</td>
<td>---</td>
</tr>
<tr>
<td>-10</td>
<td>96.10</td>
<td>---</td>
</tr>
<tr>
<td>0</td>
<td>100.00</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>103.90</td>
<td>7.80</td>
</tr>
<tr>
<td>20</td>
<td>107.79</td>
<td>15.58</td>
</tr>
<tr>
<td>30</td>
<td>111.67</td>
<td>23.34</td>
</tr>
<tr>
<td>40</td>
<td>115.54</td>
<td>31.08</td>
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<tr>
<td>50</td>
<td>119.40</td>
<td>38.80</td>
</tr>
<tr>
<td>60</td>
<td>123.24</td>
<td>46.48</td>
</tr>
<tr>
<td>70</td>
<td>127.07</td>
<td>54.14</td>
</tr>
<tr>
<td>80</td>
<td>130.89</td>
<td>61.78</td>
</tr>
<tr>
<td>90</td>
<td>134.70</td>
<td>69.40</td>
</tr>
<tr>
<td>100</td>
<td>138.50</td>
<td>77.00</td>
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<td>110</td>
<td>142.28</td>
<td>84.56</td>
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<td>99.64</td>
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<td>140</td>
<td>153.57</td>
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</tr>
<tr>
<td>150</td>
<td>147.32</td>
<td>---</td>
</tr>
<tr>
<td>160</td>
<td>161.05</td>
<td>---</td>
</tr>
<tr>
<td>170</td>
<td>164.76</td>
<td>---</td>
</tr>
<tr>
<td>180</td>
<td>168.47</td>
<td>---</td>
</tr>
<tr>
<td>190</td>
<td>172.16</td>
<td>---</td>
</tr>
<tr>
<td>200</td>
<td>175.84</td>
<td>---</td>
</tr>
</tbody>
</table>

--- DO NOT EXCEED THESE SETTINGS ---
Integral: 1
Derivative: 0
Temperature set: 23

Now switch the regulator on ('marche'). The meter in the upper left portion of the controller will read full power (10) until the block temperature reaches the set point temperature. With the above settings, this will probably take about three to four hours, and the power input will vary between 0 and 10 for another six hours, gradually settling to a reading of about 1. Full warm-up and equilibration of the block will take 24 to 72 hours, and a full warm-up of three days is recommended before proceeding.

Measuring the temperature of the block. During a heating or cooling period, a rough estimate of the temperature of the block may be found by adjusting the P/I/D controls to 0/0/10 and turning the large temperature set dial until the heaters just go on (power indicated on the meter). The temperature corresponding to this dial setting is the approximate temperature of the block.

For a more accurate measurement of the block temperature, a copper-constantan thermocouple pair has been provided. The three small red wires coming from the box on the calorimeter shell are the leads from this set of junctions. One junction is imbedded in the calorimeter block, and the reference junction is in a small metal block in the box on the
front of the instrument. The reference block has a hole drilled into it for a thermometer so the reference temperature can be easily determined. A millivolt potentiometer should be used to measure the differential voltage, and the block temperature can be calculated from Table A-2. Two illustrations follow.

Suppose the measured EMF is 7.350 mV and the reference block measured 22°C. Then from Table A-2, the reference voltage is 0.868 mV:

<table>
<thead>
<tr>
<th>EMF read</th>
<th>7.350 mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMF reference</td>
<td>0.868 mV</td>
</tr>
<tr>
<td>Total EMF</td>
<td>8.218 mV</td>
</tr>
</tbody>
</table>

and again from Table A-2 the temperature is calculated to be 179.9°C. by interpolation.

As a second example, suppose the voltage read is 0.520 mV, and the known reference is -16°C. Then,

<table>
<thead>
<tr>
<th>EMF read</th>
<th>0.520</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMF reference</td>
<td>-0.602</td>
</tr>
<tr>
<td>Total EMF</td>
<td>-0.078</td>
</tr>
</tbody>
</table>

and the block temperature is calculated as -3°C. The results using this method should be checked using a calibrated thermometer in a reaction cell filled with fluid of high heat capacity (such as a water/glycol mixture) and any necessary corrections in the thermocouple calibration be made. Once calibrated, this thermocouple output is quite
TABLE A-2

Temperature - EMF Values for Copper-Constantan Thermocouples
(reference junction 0°C)

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>EMF (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-30</td>
<td>-1.11</td>
</tr>
<tr>
<td>-20</td>
<td>-0.75</td>
</tr>
<tr>
<td>-10</td>
<td>-0.38</td>
</tr>
<tr>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>10</td>
<td>0.39</td>
</tr>
<tr>
<td>20</td>
<td>0.79</td>
</tr>
<tr>
<td>30</td>
<td>1.19</td>
</tr>
<tr>
<td>40</td>
<td>1.61</td>
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<td>50</td>
<td>2.03</td>
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<tr>
<td>60</td>
<td>2.47</td>
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<tr>
<td>70</td>
<td>2.91</td>
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<td>80</td>
<td>3.36</td>
</tr>
<tr>
<td>90</td>
<td>3.81</td>
</tr>
<tr>
<td>100</td>
<td>4.28</td>
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<tr>
<td>110</td>
<td>4.75</td>
</tr>
<tr>
<td>120</td>
<td>5.23</td>
</tr>
<tr>
<td>130</td>
<td>5.71</td>
</tr>
<tr>
<td>140</td>
<td>6.20</td>
</tr>
<tr>
<td>150</td>
<td>6.70</td>
</tr>
<tr>
<td>160</td>
<td>7.21</td>
</tr>
<tr>
<td>170</td>
<td>7.72</td>
</tr>
<tr>
<td>180</td>
<td>8.23</td>
</tr>
<tr>
<td>190</td>
<td>8.76</td>
</tr>
<tr>
<td>200</td>
<td>9.29</td>
</tr>
</tbody>
</table>

reliable as an indicator of block temperature.

**Zeroing the Recorder.** If the calorimeter block has stabilized enough for an experimental run to begin, the meter on the Keithly should read zero without zero suppression down to about the ten microvolt scale. With no cells in the calorimeter, it should be possible to get a good trace on the recorder using the one microvolt scale of the Keithly. Acceptable noise level under these conditions is given in the manual as 40 nanovolts peak-to-peak, or about half an inch deflection of the pen peak-to-peak on the one microvolt scale.

The zero to one hundred scale on the recorder corresponds to the zero to full-scale readings of the Keithly, regardless of the range on which the Keithly is set. The switch marked 'endotherm' on the recorder controls the polarity of the recorder. When set to 'right', the recorder pen moves in the same direction as the needle on the Keithly meter; in 'left,' it moves in the opposite direction. The zero suppress can be used as a zero adjust to place the pen zero at any desired point on the recorder scale.

The procedure for turning the instrument on to record is as follows:

1. Set the Keithly range switch to the appropriate recording range for the reaction to be observed.
2. Turn the Keithly on by moving the power switch to
AC (for normal work) or to Battery (for high temperature work). Battery operation completely isolates the amplifier from the power line, but cannot be used for more than eight consecutive hours, after which fourteen hours must be allowed to recharge the batteries.

3. When the beginning transient is past (about two seconds at most) adjust the needle to the desired zero point using the zero surpress controls.

4. Turn the recorder to the proper polarity (left or right endo.)

5. Turn the power switch to the recorder on.

6. Turn the chart on.

When the recording of a run is finished, the instrument must be turned off before removing the reaction cells. Steps for this procedure are

1. Turn the recorder chart off.

2. Turn the Keithly amplifier off.

3. When the pen reaches zero, turn the recorder power off.

The sample cells may now be taken out for cleaning or exchange without risk of damage to the readout instrumentation.

When setting up the instrument for the first time in a new location, it is recommended that the baseline be re-
corded for an hour or more to check the noise level and drift of the system. If three-days warm-up has been allowed, the instrument should give no trouble at this point. This completes the set-up of the calorimeter after a move, and the remainder of this section will be devoted to everyday operating procedures for the Calvet.

Changing the temperature of the block. If it is desired to run the experimental reactions at a different temperature than the 30°C. set above, the temperature controller should be set to the appropriate probe resistance scale and temperature set dial reading, and the P/I/D set to 25/1/0. The calorimeter as manufactured has heaters imbedded in the block, but has no provision for cooling below ambient, and is therefore useable only within the range of ten degrees above ambient to 200°C. It is possible to work at sub-ambient temperatures if the calorimeter block assembly is placed in a cold-box similar to that described in Appendix II, in which case the 'ambient' seen by the instrument is that inside the cold box, and the range of the instrument is extended to ten degrees above this temperature. The calorimeter cannot be used above 200°C.

If the temperature set is within the range described above, the instrument will reach its new operating temperature within four days at most. If the new temperature is above the previous one, the indication given when the
proper temperature is reached is a fall of the indicated power input to zero. Fluctuations between zero and ten will occur and finally the power input will settle to a new level which maintains the new temperature. Equilibration can be speeded if the following procedure is observed as soon as the power level rises after falling to zero.

Allow the power level to rise to about five on the scale, then press the 'effacement' (erasure) button on the controller. This will reset the controller to the power level needed to maintain the present temperature of the block. By switching the 'mesure' switch alternately from automatic to manual and back, use the manual regulation dial to set the power delivered on manual to match the automatic input (the needle doesn't move when switched). Now turn both meter and control circuitry ('commande') to manual operation and allow the block to equilibrate for eight to twelve hours. Then switch both back to automatic control, push the erasure button again, and allow the controller to make the necessary final adjustment to the set temperature.

If the calorimeter is to be cooled instead of heated, the above procedure becomes more tedious and less effective, and it is generally better to leave the controller in automatic operation. In either case, an equilibration period of twelve to twenty-four hours after the needle appears stabilized is required for accurate measurements and a stable
baseline. P/I/D should be set to 15/2/1 for this period.

**Electrical Calibration.** As was mentioned in the text above, the electrical calibration was not found to be detectably temperature dependent. However, two points should be considered before accepting our instrument constant as 'universal' for this instrument. First, if measurements are made at temperatures significantly outside our measurement range (say, for example, 100° to 200°C.) a temperature dependence of the instrument constant might become measurable. Second, the stability of the baseline using the calibration cells and the reproducibility of the curves makes an electrical 'calibration' run a good indicator of thermal equilibrium after a change in temperature. A significant change in baseline or a discrepancy in determined area for a given power level indicates either a lack of uniformity in the block temperature or some experimental error which must be identified. For these reasons, the instrument constant should be checked at each temperature before beginning the experimental runs.

To make a calibration run, the electrical calibration cells are placed in the instrument and allowed to equilibrate for two hours. The leads from the appropriate cell are then connected to the binding posts of the calibration panel. Special leads and DPDT switch have been added to one of the cells to allow approximate adjustment of the
current before application to the resistance winding in the calibration cell. Placing this cell in the side of the calorimeter which is to be calibrated allows calibration and comparison of the results from the two calorimeter elements with the fewest uncontrolled variables. These special leads are equipped with banana plugs to facilitate connection to the calibration panel.

The final connection in the calibration circuit is made with a link between two of the three binding posts located in the center of the calibration panel. The choice of binding posts allows choice in the range of power. For power up to ten microwatts the right binding post and right meter ($100 \mu A$ full scale) are used; for power between 10 microwatts and one milliwatt the left binding post and left meter (1 mA full scale) are used. When the calibration panel is first switched on, the link should be free, connected to only the Common post. The link is then used to complete the circuit.

The DPDT switch has a resistor of approximately 1000 ohms at one end. The switch should be in the position toward this end when the calibration circuit is first completed. Adjustment of the current to the desired value is then made using the potentiometers, and the switch turned to the resistance coil connections when the timing is started. Because of some mismatching of the dummy resistor and the
resistance coil, a slight adjustment on the potentiometers is necessary at high currents. After the desired time interval, the switch is changed to the dummy setting and the link disconnected to stop the current flow. The area of the resulting peak can then be measured, the power calculated from the equation
\[ P = I^2 R \]
and an instrument constant determined in whatever units are most convenient. Since our planimeter measured directly in square inches, we calculated our constants in terms of mcal/in\(^2\), but some may prefer units of mcal/cm\(^2\) or \(\mu W/in^2\). Conversion to such units involves only multiplication by a numerical constant and is easily accomplished if desired.

The step-by-step procedure for recording a calibration run is given below:

1. Put the calibration cells into the instrument and allow to equilibrate for two hours.
2. Check to see that the connecting link on the calibration panel is connected to only the common terminal, then switch power to the calibration panel on.
3. Select the proper range on the Keithly to maximize the area without causing the pen to run off scale.
4. Turn the Keithly on.
5. Select the proper polarity of the recorder.
6. Turn the recorder power on.
7. Turn the recorder chart on and record the baseline for 10 to 15 minutes.

8. Check to see that the switch on the heater leads is in the dummy position, then complete the circuit to the meter with the connecting link.

9. Using the potentiometers, set the current through the resistance to the desired level.

10. Turn the switch to the resistance coil position and start the timer.

11. Make adjustments to the current due to the unmatched resistance of the dummy and coil, if necessary.

12. When the desired time at the selected power level has elapsed, turn the switch to the dummy position and turn off the timer.

13. Break the circuit by disconnecting the link.

14. Allow the trace to return to the baseline and repeat steps 3 through 13 until all the runs at different power levels and/or times necessary for the calibration plot have been made.

**Experimental runs.** The exact procedure for experimental runs will depend on the cell designs employed and the type of experiment being run. For the solvent-induced transitions reported above and the cell design we used, the exact procedure may be found in the Experimental section above. The procedures necessary to record the run
are reported immediately above under 'Zeroing the recorder' along with the description of the shut-down procedure.

Changing the instrument sensitivity. One further note about the instrument concerns the sensitivity and the use of the Peltier effect for cooling. The detecting thermopiles, as used in these experiments, consist of 453 thermocouples each which are further divided into two piles of 329 and 124 couples. Either of the latter thermopiles may be used separately for detection, thus decreasing the instrument sensitivity, or they may be connected in series for maximum sensitivity as was done here. The terminal block to which the four ends of the two thermopiles are connected is located in the metal box on the front of the calorimeter. The connections within this terminal block which give the three sensitivities are shown in Fig. A-1. If one of the smaller thermopiles is used for sensing, the other may be used for Peltier cooling. A discussion of the use of Peltier cooling may be found in the SETARAM manual supplied with the calorimeter.
Wires from Thermopiles

<table>
<thead>
<tr>
<th>NOT USED</th>
<th>e₁</th>
<th>e₂</th>
<th>E₁</th>
<th>₁₁</th>
<th>₁₂</th>
<th>E₂</th>
<th>( \frac{I_1}{I_2} )</th>
</tr>
</thead>
</table>

External Wiring

Low Sensitivity

<table>
<thead>
<tr>
<th>e₁</th>
<th>e₂</th>
<th>E₁</th>
<th>₁₁</th>
<th>₁₂</th>
<th>E₂</th>
<th>( \frac{I_1}{I_2} )</th>
</tr>
</thead>
</table>

Keithly Inputs  \( P_1 \)  Copper Jumper  \( P_2 \)  \(-P\)

Medium Sensitivity

<table>
<thead>
<tr>
<th>e₁</th>
<th>e₂</th>
<th>E₁</th>
<th>₁₁</th>
<th>₁₂</th>
<th>E₂</th>
<th>( \frac{I_1}{I_2} )</th>
</tr>
</thead>
</table>

 Keithly Inputs  \( P_1 \)  \( P_2 \)  \(-P\)

High Sensitivity

<table>
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<tr>
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Keithly Inputs  Copper Jumpers

Figure A1
APPENDIX II

Construction Detail of the Calvet Cold Box

Adapted from the plans in Gerald J. Stout's Book,
The Home Freezer Handbook, D. van Nostrand Co., Inc.,

Materials Required:

5 sheets 3/4" plywood, 4' x 8'
5 sheets 1/2" plywood, 4' x 8'
1 sheet 3/8" plywood, 4' x 8'
1 shipping pallet, approx. 3' x 4'
9 2" x 4" x 4' #2 pine
6 1" x 4" x 6' #2 pine
1 2" x 6" x 6' #2 pine
12 sheets styrofoam insulation, 2" x 2' x 8'
2 sheets styrofoam insulation, 1" x 2' x 8'
5 cubic feet styrofoam packing chips
1 1/2-hp Freon-12 condensing unit, air cooled
1 thermostat, double-contact
1 thermometer, remote sensing bulb
2 thermostatic expansion valves, 1/2-ton
1 dehydrator-strainer combination
1 sight glass
250 ft. 1/2" refrigeration tubing (copper)
10 ft. 1/4" copper refrigeration tubing
Misc. nails, screws, lag bolts, fittings, wiring, etc.
Safety cutoff box and fuses
VERTICAL CROSS-SECTION
CALVET COLD BOX

POLYSTYRENE CHIPS

RIGID POLYSTYRENE FOAM

COIL SUPPORTS

APPROX. SCALE 1" = 10"

Figure E-1
Horizontal Cross-section

Base for Cold Box

Approx. scale 1" = 10"

Figure B-2
Detail of the Top of
The Calvet Cold Box
View from Beneath

Approx. scale 1" = 10"

Figure B-3
Scale 1" = 2"

Inner wall

\(\frac{1}{4}\)" shim (1\(\frac{1}{2}\)" deep)

2" x 4" frame

Front wall

Outer wall

Figure B-4