BIOCHEMICAL THERMOCHEMISTRY

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ABSTRACT

Biothermochemical investigations are performed on systems made up of purified biochemical compounds but also on simple model compounds. Calorimetric work on the biological level has, however, its main interest in analytical experiments. Developments and current trends in biothermochemistry are briefly discussed and are illustrated by examples from a few recent studies.

INTRODUCTION

Like many other experimental techniques in the life sciences, calorimetric experiments are performed on different levels: model work, work on purified biochemical compounds and work on very complex or crude systems which have more or less of the biological organization and life functions retained (*Figure 1*).

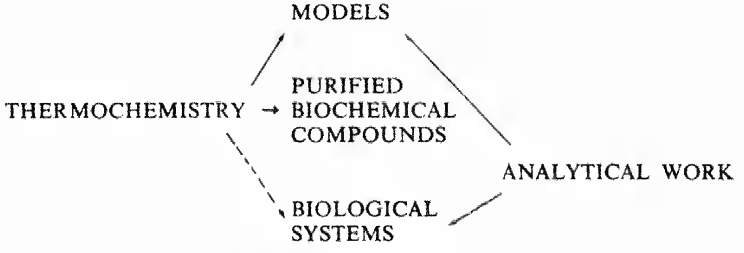

Figure 1. The field of biocalorimetry.

Biothermochemical work concentrates on models and on systems made up from purified biochemical compounds. Biological systems and crude mixtures like homogenates or impure biochemical preparations are poorly defined from a thermodynamic point of view. Calorimetric investigations on such systems will therefore not provide thermodynamic results which can be discussed on a molecular level in the same sense as those determined for purified systems. Among biologically oriented calorimetrists there are at present several groups who do perform thermodynamic experiments with living materials or with poorly defined systems of biological origin. Results from such studies are hardly useful in discussions of molecular properties.

but are mainly undertaken to determine energy balances for organisms and ecosystems.

Calorimetric studies on biological systems and on impure biochemical preparations have their main interest as analytical experiments, and they therefore fall outside the scope of the present paper. Such applications are at the present time being developed into important experimental techniques in basic biology as well as in applied areas like the clinical sciences, biotechnology and for environmental research. It is likely that among the future biocalorimetrists, those primarily interested in thermodynamics will form only a small minority.

In the present paper the field of biothermochemistry will be discussed rather generally. Examples of recent work are discussed but no attempt is made to review the field, which recently was done elsewhere[1]. Due to the general thermochemical interest in calorimetric work on biochemical models a particular emphasis has been given to this area.

WORK ON BIOCHEMICAL MODELS

It is rarely an easy task to interpret the thermodynamic values arrived at in a study on a biochemical system even for the case where the system may be considered to be well characterized. One obvious approach towards a deeper understanding of biothermochemical data is to study biochemical model systems. Within a series of model compounds the structure can be varied systematically and correlations can be made between thermodynamic data and structural features. It may not be possible gradually to increase the complexity of a model with the intention finally to reach the level of an intricate biochemical system. However, at the present time there are still large areas on the most basic model level which remain to be covered.

We need data from model studies for discussions of results from biothermochemical work, but, as important, they are needed for the sound formulation and discussion of new theories and hypotheses related to the biochemical systems. In addition, thermochemical studies on biochemical models can usually be looked upon as part of the general field of thermochemistry. As such the results will have their full value even if it will turn out that a particular study will not be of any immediate importance for the biochemical field. It is then required, however, that the work is done systematically and that the data are determined with adequate accuracy. It is therefore desirable that studies on simple model compounds are made with the highest possible accuracy even if it is not judged to be necessary for the biochemical problem which may have been the reason for initiating the model study.

An area of particular importance in current model studies is the one dealing with interactions between water and other solvents and solutes, including the thermodynamics of transfer of compounds and groups between different media. Such transfer processes contribute very substantially to the overall thermodynamic picture of most biochemical processes. As a characteristic example we may look upon a simple enzyme–inhibitor reaction

$$(E + I \rightleftharpoons E\text{–}I)_{aq} \qquad (1)$$

At the binding process the inhibitor molecule is transferred from bulk water to the active site of the enzyme, which often seems to have the nature of a cleft in the protein molecule. At the binding site more or less specific non-covalent bonds are formed between the protein and the inhibitor and in addition the process may be accompanied by a conformational change of the protein. Prior to the reaction the inhibitor molecule is solvated by bulk water and we have reason to believe that the properties of the water surrounding the inhibitor molecule are greatly affected by the properties of the inhibitor. Before the binding reaction the active site of the enzyme may accommodate a certain number of water molecules which probably do not have the properties of bulk water. As a result of the binding process we may expect that (part of) the water shell initially surrounding the inhibitor molecule will be transformed to bulk water and that the water molecules in the active site cavity will be expelled and thus transformed to bulk water. In addition a conformational change of the protein may cause a substantial change in the contacts between groups in the protein and the water.

Other typical biochemical processes where medium effects are believed to play a dominant role are protein unfolding processes. For such processes groups from the interior of the folded protein (not the least, hydrophobic groups) will be brought in contact with the water and as a result the thermodynamic properties of the system will be greatly affected.

Model studies undertaken to give an insight into problems of this sort are from an experimental point of view quite simple. They mainly consist of solution calorimetric work and of determinations of enthalpies of vaporization and heat capacities. Measurements are preferably made on comparatively large samples and are mainly performed in a convenient temperature range. Still, it may be concluded that until recently this type of work has been much neglected and a substantial quantity of theoretical work in the area has been based on very meagre experimental data.

Among recent aqueous solution studies we may note work on simple alkyl compounds: carboxylic acids, amines, amides, ethers, alcohols (cf. ref. 1). Work on a number of phenyl group compounds has recently been completed[2–5] and the aqueous solution properties of tetra-alkylammonium compounds have been given much attention[1]. Important groups of compounds for which aqueous solution properties are poorly known include hydrocarbons, thiols and disulphides, organic phosphates, indoles, imidazoles, purines and pyrimidines. Much calorimetric work on aqueous solutions of amino acids and simple peptides remains to be done, in particular heat capacity determinations.

Quite generally one would wish that these kinds of solution studies were extended to larger molecules but here solution problems will in many cases soon provide a limiting factor. Further, it would be most valuable if measurements were made both at low and at high concentrations of the solutes and if possible heat capacity data should be determined for a range of temperatures.

In the thermochemistry of water–solute interactions the main interest is at present focusing on heat capacity data. As an example of a recent aqueous solution study the results in *Figure 2* are shown, where $\bar{C}p_2^0$ values for straight chain alcohols, carboxylic acids, amines and *N*-substituted amides are plotted versus number of alkyl carbon atoms[6].

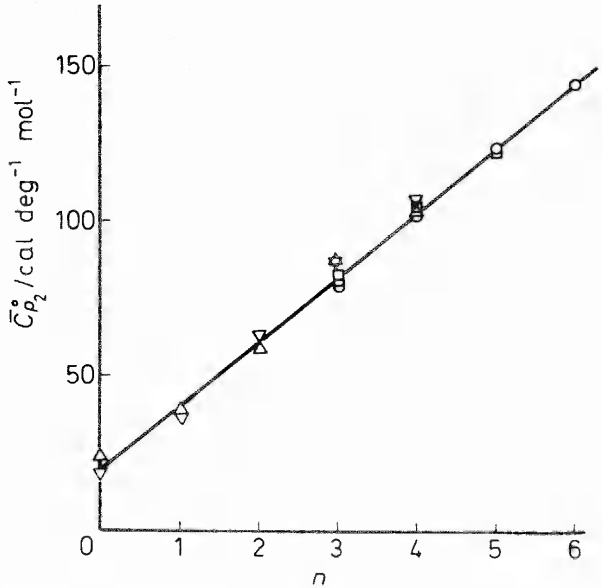

Figure 2. Partial molar heat capacities for some straight chain alcohols (▽), carboxylic acids (△), amines (○) and *N*-substituted amides (□) versus total number of carbon atoms in the alkyl groups, *n* (ref. 6; reproduced by permission).

It is seen that the CH_2-increments are large, *ca.* 90 $J\,K^{-1}\,mol^{-1}$, i.e. about three times larger than the value for a CH_2-group in an organic solvent. This type of effect is usually believed to be due to the interaction between hydrophobic groups and water, which phenomenon has been given names like 'water structure formation' or 'iceberg formation'. As a molecular interpretation of the large $\bar{C}p_2^{\circ}$ values the solute–water system is currently thought of as a partially structured system which will gradually melt with increasing temperature. With this interpretation a substantial part of the $\bar{C}p_2^{\circ}$ values (and the ΔCp values for transfer processes) should be considered as 'transition enthalpy values'. From *Figure 2* we further note that the CH_2-increments are essentially constant. Thus, the value is not affected by the nature or the position of the hydrophilic group. This may suggest that the water structure around the hydrophobic chains is only slightly affected by the nature of the biochemically important groups like —OH, —COOH, —NH_2 and —CO—NH—. $\bar{C}p_2^{\circ}$ values are usually best arrived at from results of enthalpy of solution measurements performed at different concentrations and temperatures combined with the heat capacity for the pure compound (Cp°):

$$\bar{C}p_2^{\circ} = Cp^{\circ} + \Delta Cp_2^{\circ} \qquad (2)$$

In this connection we may note that there is a remarkable lack of high quality Cp° values for many of our most simple and most important organic compounds.

A direct determination of $\bar{C}p_2^{\circ}$ values for dilute solutions requires a very high precision and few of the calorimeters which are available today are actually useful for such work.

STUDIES ON PURIFIED BIOCHEMICAL COMPOUNDS

Binding reactions—Among the biochemical processes given most attention at the present time are different kinds of specific binding and association reactions, e.g. enzyme–inhibitor reactions and association reactions between proteins. For studies of specific binding of low molecular compounds to proteins is an area of particular activity, see, e.g., ref. 1. For these systems the reacting molecules as well as the reaction products have in many cases been well characterized and the binding processes are frequently related to the biological function of the protein. For enzyme–inhibitor reactions or, e.g., for antigen–hapten reactions the binding site is usually believed to be identical with the biologically active site.

In many studies both K and ΔH values for the binding processes have been worked out from the calorimetric results and frequently information about the stoichiometry is obtained. Several studies have been extended over a large enough temperature range in order to give precise ΔCp values.

Figure 3 summarizes results from such a study where the substrate analogous inhibitor D-hexitol-1,6-diphosphate is bound to rabbit muscle aldolase[7]. It is very striking that ΔG values are essentially constant over the investigated temperature range whereas enthalpies and entropies show a very marked temperature dependence. The picture illustrates clearly the fact that ΔG values alone are not well suited for discussions of energy–structure relationships in biochemical systems. We further note the strong enthalpy–entropy compensation which is common for biochemical processes.

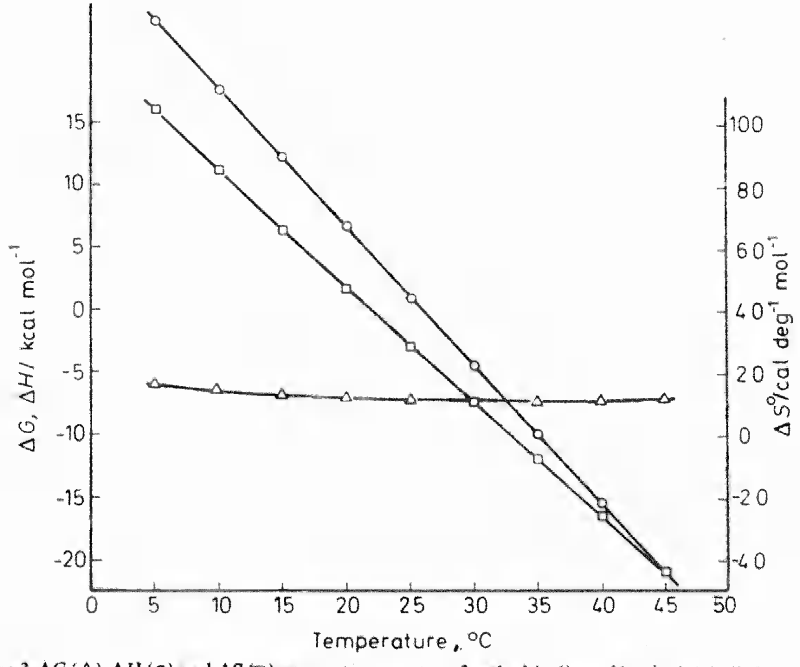

Figure 3. ΔG (△), ΔH (○) and ΔS (□) versus temperature for the binding of hexitol-1,6-diphosphate to rabbit muscle aldolase at pH 7.5 (ref. 7; reproduced by permission. Copyright by the American Chemical Society).

This phenomenon is in fact being thought of as being related to solute–water interactions (cf. ref. 4).

It is thus important to keep in mind that ΔH and ΔS values frequently vary substantially with the temperature. For some studies performed at one temperature only, it is not unlikely that arguments used at the discussion of the derived thermodynamic data will be contradicted if the measurements are extended over a range of temperatures.

The essentially linear relationship of ΔH with temperature leads to a constant, strongly negative ΔCp value, $-1.7\ \mathrm{kJ\ K^{-1}\ mol^{-1}}$ inhibitor. ΔCp values of this magnitude are common for this kind of processes but low values have also been reported (*Table 1*). In line with results from model compound work the large negative ΔCp values are currently usually interpreted in terms of a decreased exposure of hydrophobic groups to bulk water.

Table 1. ΔCp values for some protein binding reactions (*ca.* 25°C)

Protein complex	ΔCp kJ K^{-1} (mol of sites)$^{-1}$	Ref.
Aldolase, D-hexitol-1,6-diphosphate	−1.7	7
Lysozyme, GlcNac; $(GlcNac)_2$	±0	9
Glycerylaldehyde phosphate dehydrogenase, NAD	−2.2	10
Carbanhydrase (Human C), sulphonamides	±0	11
Biotin, avidin	−0.97	12
Antibodies, ε-DNP lysine	−0.9– −6.2	13, 14
Protein A, Fc fragment of IgG	−3.9	15
Haemoglobin, haptoglobin complexes	−1.7– −4.5*	16

* The values refer to mole of haemoglobin dimer. Cf. the strongly negative ΔCp value reported in ref. 17, where, however, less well defined material was used.

Other experimental variables than the temperature should if possible also be varied, e.g. pH, type and concentration of buffer substance and of salts. In cases where protons may be released or bound it is very useful to perform the reaction in two buffers with different enthalpy of ionization values, in which cases the number of protons involved per mol of bound inhibitor can be calculated. This procedure naturally requires that the buffer substances do not interfere with the binding process.

Unfolding reactions—Another field which at present attracts a great deal of interest is the one dealing with unfolding reactions of proteins, nucleic acids and lipid materials. Such processes are induced either chemically or thermally.

In the former case a solution of the macromolecule is mixed with a denaturant solution such as urea or guanidine hydrochloride or the pH of the solutions may be changed, sometimes with a strong denaturant present. Such studies are usually but not necessarily performed in micro-reaction calorimeters. The interpretation of results from such systems may be difficult, not least because of the unknown quantities of denaturant molecules bound to the protein before and after the unfolding process. Thermally induced unfolding processes are therefore considered as simpler processes but such studies require more sophisticated calorimetric equipment. They are

performed as heat capacity measurements in temperature scanning calorimeters (cf. below).

ACCURACY AND PRECISION IN BIOTHERMOCHEMICAL WORK

In model compound studies and in work on simple purified biochemical compounds such as amino acids, simple peptides and sugars, etc., precision and accuracy should be kept at the same level as in general thermochemistry.

In experiments with purified but complex biochemical compounds, such as proteins and nucleic acids, it is rarely possible to define the state of the sample to better than a few per cent. These systems are thus poorly defined compared with current standards in general thermochemistry and it may be meaningless to perform anything but low-precision calorimetric measurements on such systems. In some other cases, however, it is possible to arrange the experiments so that the rather poorly defined sample of the biochemical compound serves as an 'internal reference'. One may, e.g., let a sample of a biomacromolecule take part in highly specific reactions with a series of well-defined low molecular compounds. In such studies the absolute values determined may not be very accurate but it can still be meaningful to make a comparison between precisely determined values within such a series of compounds. Furthermore, such experiments can often be performed as 'titration experiments', which may lead to thermodynamic values referring to a calorimetrically determined purity or activity value for the sample.

In other kinds of experiments it may be necessary to perform the calorimetric measurements on a moderately well defined system with a very high precision in order to obtain any useful information. Prime examples are provided by heat capacity measurements on biochemical compounds in dilute solutions and by determination of ΔCp values for biochemical processes. Let us as an example consider a one per cent aqueous protein solution (which often is considered as a rather concentrated solution). The heat capacity of the water will amount to about 99.6 per cent of the total heat capacity of the system, the calorimetric vessel not included. If we want to determine the apparent heat capacity of the protein with a modest accuracy of five per cent it is thus necessary to perform the heat capacity determination with a precision better than 0.02 per cent (which for economic reasons normally must be performed on ml quantities of solution).

As was stressed above it is important to determine ΔCp values for biochemical reactions. These values are normally obtained from reaction calorimetric measurements performed at different temperatures and such determinations always call for precise measurements. Because of a limited temperature stability for the biochemical sample, it is in practice often necessary to work within a limited temperature range which thus will further increase the precision requirements.

CALORIMETERS FOR BIOTHERMOCHEMICAL WORK

From a survey of the literature published during the last few years[1] it is apparent that the activity in the field of biothermochemistry is rapidly

increasing. One major reason for this is undoubtedly associated with the instrumental developments and perhaps in particular with the fact that some of the designs have been made commercially available. Today a large majority of the biothermochemical work reported is produced by use of commercial equipment.

In experiments with macromolecules the heat quantity actually measured per mass unit of the compound is usually small. Further, biochemical compounds are often expensive. In reaction calorimetric experiments on biochemical compounds typical sample quantities are in the range of one micromole or less. Corresponding heat evolution is then usually of the order of less than 50 mJ. In steady state processes (flow experiments) the measured heat effects may be in the general range of 50–100 μW. It is thus clear that 'microcalorimeters' usually have to be employed in thermochemical work on biochemical systems. Developments in microcalorimetric instrumentation suitable for biochemical work were reviewed elsewhere[18] and will only be briefly commented upon here.

Model compound work is normally performed on samples of inexpensive, low molecular compounds. The measurements have to a very large extent the character of solution experiments and for these studies precise 'macro' solution calorimeters are usually the most adequate instruments. For dilution experiments 'micro flow calorimeters' ought to be very useful but so far few experiments of this nature have been reported. Recently an interesting flow microcalorimetric method for a direct determination of transfer enthalpies was developed[4].

Two major categories of microcalorimeters are in use in biothermochemistry: reaction calorimeters and temperature scanning (heat capacity) calorimeters. It is noteworthy that it is the interest in biocalorimetric studies which has been the major incentive for recent developments in microcalorimetry, both in the field of various kinds of reaction calorimeters and in heat capacity instruments.

Reaction calorimeters used are of the batch type or designed for continuous or stopped flow operations. Most, by far, of the currently used designs are based on the thermopile conduction principle and the instruments are usually of the twin type. Characteristic properties of such calorimeters are a simple design, easiness of operation and high sensitivity, and they are furthermore suitable for studies of very slow processes. It should be stressed, however, that other calorimetric principles are by no means without interest for this field.

As indicated earlier studies of thermal transitions of macromolecules form one of the most active working areas of biothermochemistry. The most successful instruments for such studies are of the automatic temperature scanning type utilizing the adiabatic shield principle.

Prestanda for a recent model of this type[19] has been reported as follows: the calorimetric vessel was charged with 1.3 ml of 0.1–0.5 per cent protein solution. The temperature scanning rate was 1 K min^{-1} for the temperature interval 20–90°C. The partial specific heat of the protein could be determined with a precision of three per cent.

A typical record from such work[19] is shown in *Figure 4*. The change in heat capacity at the protein denaturation, ΔC_d, is obtained by linear extra-

polation of the *Cp* values before and after the denaturation process. From the peak area above the extrapolated lines the enthalpy of transition is obtained. (There has been some controversy about the interpretation of such heat absorption curves[1].)

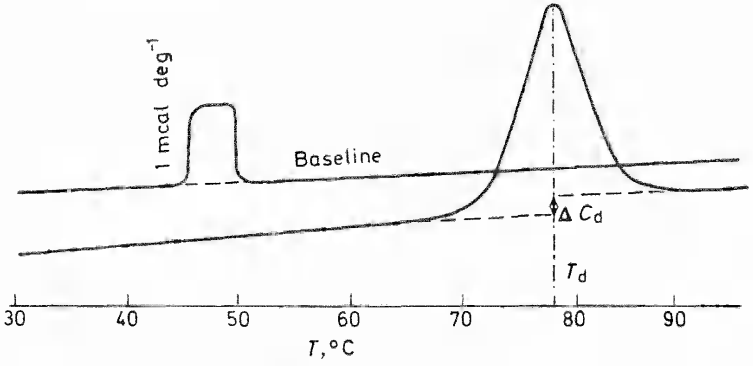

Figure 4. Microcalorimetric recording of heated lysozyme solution. The concentration of the solution was 1.6 gl^{-1} (ref. 19).

For heat capacity measurements on small solid or liquid samples at fixed temperatures a recent microdrop calorimetric method has been reported[20]. The specific heat for 0.7 ml of aqueous solution can be determined to 0.01 per cent or better. This calorimeter utilizes a twin heat conduction calorimeter as a receiver.

ON THE NATURE OF CURRENT WORK IN BIOTHERMOCHEMISTRY

The field of biothermochemistry has attracted a fair number of people who, whilst possessing a thorough biochemical background, lack previous thermochemical experience. This may have led to publication of reports where the authors have not been fully aware of all the possibilities of systematic errors in calorimetric experiments, or where the documentation of the experimental work does not meet the traditional standard in thermochemistry. But this new group of thermochemists have specific biochemical problems which they wish to tackle by a calorimetric technique and they usually do that in close contact with other kinds of experimental techniques. I believe that it is this kind of close integration between biochemistry and thermochemistry which at present leads to the most interesting projects in biothermochemistry. It may then be noted that in such 'problem-oriented' work it is not very often that highly accurate thermodynamic results are needed—more often very precise measurements are required. Many of the most significant results reached do have the character of 'general analytical information' rather than actual numbers for the derived thermodynamic quantities.

It was pointed out earlier that it is difficult to discuss biothermochemical results with the intention to 'explain' the derived values in the same sense as may be possible for systems with simple compounds. In fact it is not very often that any use is made of the absolute number of, e.g., an enthalpy change

of a biochemical reaction. Derived data are often discussed in terms of honoured concepts like 'hydrogen bonds', 'hydrophobic interactions', 'conformational changes' or 'steric effects', etc. However, it seems that many current 'explanations' of this type to a large extent are only speculations. They may not be harmful but it would probably be healthier if in general our present limitations were more clearly spelled out. One day we may be able to make a thorough analysis of the determined values but before that much systematic work remains to be done, not least on the level of model compound investigations.

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