BIOCHEMICAL CALORIMETRY

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INTRODUCTION

The borderlines of biochemistry are not very well defined and it is therefore not always possible to state whether a certain piece of work should be considered as biochemistry or not. It is, however, customary to quote that about 50 per cent of all chemical research activities is devoted to biochemistry. Too much confidence should not be given to this figure, but one would perhaps expect that biochemical calorimetry would constitute a very considerable part of the thermochemical field of research. But this is not the case.

For instance, among the articles listed under *Thermochemistry* in Current Chemical Papers 1962, there is only about 3 per cent which seems to be of a primary biochemical interest. This symposium has attracted only four papers—that is 6 per cent of the total—to the biochemical session, and that despite the fact that workers in the field were encouraged by being given a special session. Why, then, does this situation exist? Are biochemical systems not particularly suitable for calorimetric studies, or are thermochemical results of little interest in biochemistry? I will not try to answer these questions directly but I would rather like to have them as a background when, in the following short survey of biochemical calorimetry, I bring up a few general aspects for discussion.

CALORIMETRY ON INTACT BIOLOGICAL SYSTEMS

Starting from the biological side, we have, first, investigations on physiological processes performed on intact biological systems such as animals, plants, micro-organisms or isolated tissues. In these experiments—which in general are related much more to biology than to chemistry—the calorimeter is merely used as an analytical instrument for detection of, and preferably also for kinetic studies of, various biological processes. Information is mainly of a very general nature: heat effects are recorded and these heat effects are indications of processes which have to be identified by other types of observations.

Animal calorimetry is a very old science—it was started around 1780 by Crawford in England and by Lavoisier and Laplace in France[1]. Leaving out the stages of progress in the field, I ought to mention the recent advancements made in human calorimetry[2] by the Benzinger group at U.S. Naval Medical Research Institute in Bethesda, Md., which has constructed a "gradient calorimeter", where heat flow is recorded by measuring the temperature gradient produced in the calorimeter wall. The construction is designed for human objects and is used in combination with other methods for the study of various physiological processes, such as heat effects following drug injection and heat loss during exercise.

Another line of development within the calorimetry of biological processes is

represented by the work in France and Canada of Calvet and Prat and their co-workers[3–5]. They make use of Calvet's well-known types of micro-calorimeter in their investigations of heat evolution in seeds, plants and micro-organisms, as well as in small animals. We can immediately see that calorimetry of this type not only provides a powerful analytical tool as a pure research instrument, but that it should also be possible to apply the calorimetric technique in, for instance, biotechnical process control and in pharmacological investigations.

In one of the papers presented at this symposium, Boivinet and Calvet have reported investigations on enthalpy changes associated with bacterial growths. Data obtained permitted them to study kinetics of growths as affected by nutritional requirements and also—in favourable cases—to relate the heat effects to the combustion value for glucose.

I would also like to mention the work on isolated muscles and nerves by Hill and co-workers at University College, London. Although their experimental device is usually not called a calorimeter, I think it is very appropriate to remember it here, especially as it seems to have been omitted in recent thermochemical review articles. Their apparatus[6] can be characterized as a temperature-sensitive disc made up of a number of isolated thermocouple junctions. Temperature effects are recorded by a very rapidly-acting galvanometer fitted with a phototube amplifier. The apparatus is used for studies of phenomena which are fast enough to be recorded under virtually adiabatic conditions. With this device they discovered[7], for instance, that an earlier observed gross value for the heat evolution in a stimulated crab nerve could be resolved in a very short exothermal effect (0·08 sec) and a slower endothermal effect (0·3 sec). The gross heat effect was only 2·0 μcal evolved per g of nerve tissue, the exothermal effect being 8·8 μcal and the endothermal 6·8 μcal.

CALORIMETRY ON REACTIONS IN TISSUE FRACTIONS

Intact biological material is usually too complex to allow interesting correlations between observed heat quantities and well-defined chemical reactions. If the biological structure is broken down to give cell particle fractions (such as mitochondria and microsomes) the complexity is decreased and observable heat effects could possibly be identified with certain dominating chemical reactions or chains of reactions. To my knowledge, no such experiments have been carried out and it seems at present doubtful if any valuable interpretations of a physico-chemical nature can be made in this way. But, again, the possible use of calorimeters as general analytical instruments in biochemistry should be kept in mind.

CALORIMETRY ON ISOLATED BIOLOGICALLY IMPORTANT COMPOUNDS

The next step towards the pure physico-chemical experiment is where the biological structure of the investigated object is completely abandoned and the chemical constituents are isolated and purified. Even here, however, it is generally impossible to identify unambiguously enthalpy changes as being due to well-defined chemical or physical processes. This is, for instance, usually

true for the important group of experiments concerned with reactions of high-molecular weight compounds such as proteins and nucleic acids. Nevertheless, calorimetric experiments have contributed considerably to our understanding of the chemistry of very complex structures. Such compounds have molecular weights of the order of 10^4–10^6, experiments are always performed on dilute solutions and heat effects investigated are often of the order of only a few kcal/mole. It is thus necessary to use very sensitive calorimeters, operating in the microcalorie range. At present there are three major types of constructions in use: Calvet types of calorimeter[4, 5], Sturtevant's adiabatic shield calorimeter[8] and Benzinger–Kitzinger's so-called "heat burst" calorimeter[9, 10].

There have recently been several calorimetric studies on these biologically important, high molecular weight compounds involving denaturation, polymerization and ionization processes. Antigen–antibody reactions as well as other protein–protein interactions and enzyme–inhibitor coupling have also been studied (see recent review article[11]). A great number of these experiments is of the type where the heat of an assumed process is measured and consideration is then given as to whether or not the accompanying enthalpy change is reasonable. An example of this type of work is provided by the paper given at this symposium by Evans and Carney of the Seed Protein Pioneering Research Laboratory in New Orleans, La., where the authors discuss heat effects associated with a pH-lowering of a haemoglobin solution. Their results are in agreement with titration data, thus strengthening the view that most imidazole units present in haemoglobin are unmasked and accessible to titration in the pH-range 8·6–5·3. As pointed out by the authors, however, the observed heat effects may not be attributable solely to simple ionization reactions, the system being probably considerably more complex. It is obvious that results from experiments of this type—although very valuable—must be interpreted with care and used merely as indications and never as proof. It is also clear that in such cases it is usually not worth-while aiming at very high precision.

It is obvious that for complex and largely obscure reactions it is very desirable to have values for several of the thermodynamic functions in order to get a clearer picture of the underlying process and to decrease the risk of misinterpretations. This approach in calorimetry of complex reactions has extensively been made by Sturtevant and co-workers at Yale University on denaturation reactions for proteins and nucleic acids[11].

Calorimetric experiments on processes where equilibrium constants—and thus the standard free energy change—are known, will immediately give the entropy change as well. It is still usually not possible to draw any detailed conclusion from thermal data on these complex changes but, especially where there is a dramatic change in configurational structure, enthalpy and entropy values together may give fairly definite answers where equilibrium data alone give no useful information, *e.g.*, where the standard free energy change is near zero, but where enthalpy and entropy changes—working in opposite directions—are large.

If in calorimetric experiments pure reactants are allowed to attain equilibrium from both directions, enthalpy values can be used to calculate the equilibrium constant for the reaction. This method was first demonstrated by Sturtevant[12] on the mutarotation of glucose. Recently Benzinger and his co-workers

have thoroughly discussed the principles and in several cases demonstrated the usefulness of the method[9, 10].

If heat effects are studied at different temperatures, changes in apparent heat capacities can be calculated and may give important information on configurational changes and solvation processes.

Biologically important compounds are not necessarily of high molecular weight, and there is also a large variety of medium and low molecular weight compounds with well-known chemical structures. A number of calorimetric studies involving these compounds have been made[9], *e.g.* determination of heats of hydrolysis of phosphates, peptides and amides, further mutarotations for sugars, and methyl-transfer reactions and heats of ionization for a number of simple compounds. These latter reactions are of a very great importance as most biochemical compounds take part in ionization reactions within physiological pH-ranges. As an illustration the heats of hydrolysis of peptides may be quoted:

$$\begin{array}{lcll} (\mathrm{RCONHR} + \mathrm{H_2O} \rightarrow \mathrm{RCOOH} & + & \mathrm{RNH_2})_{aq} & \Delta H \approx +8 \text{ kcal/mole} \\ \quad \Big\downarrow \Delta H_i \approx +1 \text{ kcal/mole} & & \Big\downarrow \mathrm{H^+}\ -\Delta H_i \approx -11 \text{ kcal/mole} & \\ \mathrm{RCOO^-} + \mathrm{H^+} & & + \mathrm{RNH_3^+} & \\ (\mathrm{RCONHR} + \mathrm{H_2O} \rightarrow \mathrm{RCOO^-} + & & \mathrm{RNH_3^+})_{aq} & \Delta H \approx -2 \text{ kcal/mole} \end{array}$$

For the (hypothetical) case where products are un-ionized, ΔH for the reaction in aqueous solution is of the order of +8 kcal/mole. Heats of ionization of carboxylic groups are about +1 kcal/mole, whereas the heat of protonation of the amino groups is here of the order of −11 kcal/mole. The heat of reaction will thus be very different at different pH-values, being about −2 kcal/mole at pH values where reaction products are completely ionized. It should also be noted that most biochemical experiments are performed in buffer solutions and it is therefore necessary to know also heats of ionization of these buffers in order to evaluate the desired enthalpy changes. Ionization reactions should be especially remembered when the magnitudes of biochemical energy data are discussed. There has been much confusion in the biochemical literature with regard to expressions of the type "peptide bond energy", "phosphate bond energy" and "thiol ester bond energy". In addition to the bond breaking reaction there are also ionization reactions which often account for a very considerable part of the energy change discussed.

Many of the simple biologically important compounds are stable enough and can be purified to a degree where it is meaningful to determine their heats of combustion. From heats of combustion together with heats of solution desired heats of reaction in aqueous medium can be calculated. In one of the papers at this meeting Wilhoit and Amador at New Mexico Highland University describe the design of a solution calorimeter which may be used for this purpose.

Among recent calorimetric combustion determinations of biochemical importance can be mentioned the work by Ponomarev and co-workers at Moscow State University. They have for instance burned several peptides[13] in an aneroid microbomb requiring only about 20 mg of substance. Precision seems to be

surprisingly high, and reported uncertainties are frequently only of the order of 2 parts in 10,000.

With the development of suitable reaction calorimeters the importance of the calorimetric approach to combustion processes has decreased as reaction calorimetric technique usually provides more accurate results with less effort. However, combustion calorimetry on very simple biologically important compounds is also of great value for general thermochemistry in that heat of formation data for simple organic compounds are increased. An example of this type of work is presented at this symposium by Wilhoit and Shiao who have determined heats of combustion for many of the compounds in the Krebs cycle.

CALORIMETRY ON BIOCHEMICAL MODEL SUBSTANCES

Speaking in very general terms it is rare in biochemical calorimetry to discuss enthalpy values in terms of molecular structures, and it is further safe to state that the precision of results from current biochemical calorimetry is rarely fully used. The prime reason for this is, I think, as follows. In general calorimetry one is usually investigating series of related compounds and discussions of the obtained results are based on the observed differences in their thermal data. Biochemical compounds do not usually appear in homologous series and thermal data therefore tend to be "isolated", as there are frequently no data which are closely comparable.

An obvious approach to a better understanding of calorimetric results on biochemical systems is to study also "non-biochemical" compounds which are similar in structure but preferably simpler than the biochemical prototypes. These compounds are usually called model-compounds. Within a series of model compounds, the structure can be varied systematically and the effects on the thermodynamic properties can be investigated. Models can often be designed to be volatile enough for vaporization calorimetry or vapour pressure measurements. Data from such gas phase measurements are essential for evaluation of medium effects which is a very important sector of biochemical energetics. There is one other important feature of biochemical model reactions, and this is that results from these experiments are of general value in thermochemistry as well as in theoretical chemistry.

So far only a few biochemical model reactions have been investigated calorimetrically. As an example can be mentioned the determinations of heats of hydrolysis of a number of peptides and amides by the Sturtevant group[14], where some correlations can be found between structure and enthalpy change. Still simpler models have recently been investigated here in Lund[15] and data have for example been obtained for esters, thiol esters, and acylated amines (the latter are models for peptides) in aqueous solution and in the gas phase. With these very simple models it is possible to analyse the relationships between enthalpy values and molecular structure. It should be noted that the highest possible precision can be obtained and, furthermore, is essential.

Earlier, the importance of interactions between the reacting compounds and the medium was mentioned. I will now discuss this subject a little further. In all biochemical calorimetric experiments on isolated substances, water is always the reaction medium. It seems to me, however, that biochemists often look

upon water as nothing but a good solvent with certain peculiar macroscopic properties. When interpreting experimental results it is often forgotten that water is a compound with a very pronounced structure and that there is reason to believe that water interacts in a specific manner with most solutes with concomitant change of structure. I believe that much would be gained if in biochemical energetics more interest was focussed on the molecular properties of water and on the interactions between water and solutes.

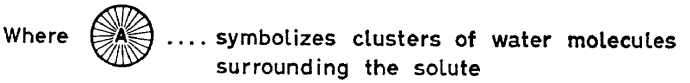
When experiments carried out in aqueous solution are discussed one should never forget that the equation:

$$A + B \rightarrow C + D$$

implies

$$A(aq.) + B(aq.) \rightarrow C(aq.) + D(aq.)$$

or, more accurately,



There is increasing evidence that the structure of the water molecules grouped around a solvated molecule is a function of the properties of the solute[16–18].

Water is indeed a very important biochemical compound but it alone does not form the medium for biochemical reactions. Even in cases where water is very abundant at the site of reaction there will also be present proteins (usually as enzymes), nucleic acids, lipids and metal ions, and their presence will affect the energetics of the various reaction steps by interactions as hydrogen bonds, complex ions, further electrostatic bonds and van der Waals' forces.

As examples where these considerations are of crucial importance can be mentioned the stability of native proteins and denaturation processes. It is, for example, assumed that denaturation of a globular protein involves unfolding on exposure to water of parts of the macromolecule which were previously embedded in a non-aqueous environment, where they were stabilized by hydrogen bonds and hydrophobic bonds. An important question in this connection is the relationship between hydrophobic bonds and water structure, which has recently attracted considerable interest[17–21]. However, no recent calorimetric experiments seem to have been carried out in this field.

Energetics of biochemical medium effects are to a large extent obscure but I believe that much clarification could be effected by calorimetric experiments on model compounds. It should also be stressed that enthalpy measurements should, where possible, be supplemented with free energy data and thus entropy data. Furthermore, heat capacity data may give very valuable information.

I have tried in this lecture to emphasize the physico-chemical side of the subject. I am aware that other aspects are also very important, for example,

purely analytical uses of the calorimetric equipment (see *e.g.* recent articles by Calvet and Prat[5] and by Benzinger and Kitzinger[9, 10]), but I feel that we should not be satisfied simply to record processes and energy changes. We should also try to use the thermodynamic results to get a further insight into the nature of the studied compound or the studied process. But in order to get out more information from usually very complex biochemical systems we need more basic knowledge, preferably obtained from experiments on simple and well-defined processes.

References

[1] See *e.g.* M. Kleiber. *The Fire of Life*, p. 116, John Wiley, New York (1961).
[2] T. H. Benzinger, R. G. Huebscher, D. Minard, and C. Kitzinger. *J. Appl. Physiol.* **12**, Suppl. 1 (1958).
[3] H. Prat. In *Experimental Thermochemistry* (Ed. H. A. Skinner), Vol. 2, Chapter 18, Interscience, London (1962).
[4] E. Calvet. In *Experimental Thermochemistry* (Ed. H. A. Skinner), Vol. 2, Chapter 17, Interscience, London (1962).
[5] E. Calvet and H. Prat. *Recent Progress in Microcalorimetry* (Ed. and transl. H. A. Skinner), Pergamon Press, Oxford (1963).
[6] B. C. Abbot, X. M. Aubert, and A. V. Hill. *Proc. Roy. Soc.* (*London*) **B139**, 86 (1951).
[7] B. C. Abbot, A. V. Hill, and J. V. Howart. *Proc. Roy. Soc.* (*London*) **B148**, 149 (1958).
[8] A. Buzzell and J. M. Sturtevant. *J. Am. Chem. Soc.* **73**, 2454 (1951).
[9] C. Kitzinger and T. H. Benzinger. In *Methods of Biochemical Analysis*, Vol. VIII, Chapter 8, Interscience, New York (1960).
[10] T. H. Benzinger and C. Kitzinger. *Temperature—Its Measurements and Control in Science and Industry*, Vol. 3, Part 3, Reinhold, New York (1963).
[11] J. M. Sturtevant. In *Experimental Thermochemistry* (Ed. H. A. Skinner), Vol. 2, Chapter 19, Interscience, London (1962).
[12] J. M. Sturtevant. *J. Phys. Chem.* **45**, 127 (1941).
[13] V. V. Ponomarev, T. A. Alekseeva, and L. N. Akimova. *Zh. Fiz. Khim.* **36**, 872 (1962).
[14] M. Rawitscher, I. Wadsö, and J. M. Sturtevant. *J. Am. Chem. Soc.* **83**, 3180 (1961).
[15] I. Wadsö. *Svensk Kem. Tidskr.* **74**, 121 (1962).
[16] H. S. Frank and W. Y. Wen. *Discussions Faraday Soc.* **24**, 133 (1957).
[17] G. Némethy and H. A. Scheraga. *J. Chem. Phys.* **36**, 3382, 3401 (1962).
[18] I. M. Klotz. In *Horizons in Biochemistry* (Eds. M. Kasha and B. Pullman), Academic Press, New York (1962).
[19] W. Kauzman. *Advan. Protein Chem.* **14**, 1 (1959).
[20] J. M. Klotz and J. S. Franzen. *J. Am. Chem. Soc.* **84**, 3461 (1962).
[21] C. Tanford. *J. Am. Chem. Soc.* **84**, 4240 (1962).