A Review of Calorimetric Investigations of the Thermodynamics of Actomyosin ATPase

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INTRODUCTION

In muscle the two proteins, actin and myosin, are separated in the filament structure, but able to interact with each other. This interaction is held responsible for the development of tension and the performance of mechanical work by the muscle. The cyclic interaction process in which these mechanical phenomena are manifest is driven by the splitting of ATP. This ATPase activity can also be studied *in vitro* using purified actin and myosin (commonly the proteolytic fragments of myosin, HMM and S-1 are used, as they are soluble at low ionic strength). Such studies, both kinetic and thermodynamic, are likely to throw light on the mechanical and energetic behaviour of the intact muscle. In this article we review calorimetric work on this system and discuss the requirements for further progress in this area.

Kinetic studies have shown that actin (A) and myosin (M) split ATP by a process which will be simplified here as the sequence of reactions shown in Fig. 1.

Myosin alone splits ATP (reactions 1, 2, 3, and 4), but only slowly, because reactions 3 and 4 are slow. When actin is present the complex A·M·ADP·Pi is formed and dissociation of products occurs by reactions 7 and 8. ATP then binds to the A·M complex and rapidly dissociates it (reactions 5 and 10) before being split by reaction 2. The mechanism of ATP splitting by actin and myosin together (the path shown by the heavy arrows) is thus characterized by the dissociation and reassociation of actin and myosin during each cycle. The ATP splitting occurs when the two proteins are dissociated. If, as seems likely, the same mecha-

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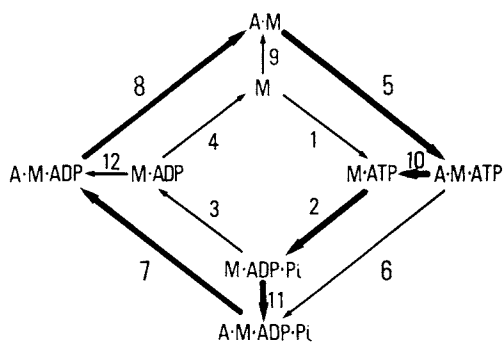** Department of Pharmacology, Juntendo University School of Medicine, Tokyo, Japan.

Fig. 1. A simplified scheme of the sequence of reactions by which myosin and actomyosin split ATP. M stands for a myosin active site and A for an actin monomer. The heavy arrows show the route of actomyosin ATPase.

nism operates in intact muscle the performance of mechanical work, which requires an association of actin and myosin, cannot be directly coupled to ATP splitting, but must be indirectly coupled. The reaction to which it is coupled is probably that of Pi release (reaction 7)[1]. Thus ATP splitting can be regarded as "charging up" the myosin molecule to a state in which it is able later to do work on the actin. This *in vitro* system is obviously an experimental model of the mechanical transduction process which is of considerable intrinsic interest, quite apart from the implication of the study for understanding muscle contraction. In this article we review experiments in which calorimetry has been used to study this system. For many of the reactions, free energy changes are available from kinetic measurements. These measurements are not generally precise enough for the determination of reaction heats however.

EXPERIMENTS WITH MYOSIN ALONE

The pioneering calorimetric work on myosin

ATPase was reported by Yamada, Shimizu and Suga[2]. They mixed stoichiometric amounts of HMM with ATP (or ADP) in an LKB precision calorimeter and were able to follow the subsequent heat production with a time resolution of about 10 seconds. When ADP was added a rapid heat production was seen. As ADP binds tightly to HMM this heat presumably comes largely from the reverse of reaction 4, however it should be noted that the observations were not controlled for heat of mixing. When ATP is used (see Fig. 2) a very similar rapid heat production is seen, followed by a slower exponential heat production which has the same time constant as the release of Pi from the myosin product complex (reaction 3). This seems to show that reaction 3 is exothermic ($\Delta H \simeq -80$ kJ mol^{-1}). The rapid heat production comes presumably from reactions 1 and 2 together with the heat of mixing. As ATP binding to HMM is known to be very strong the observation that excess ATP (upper line in Fig. 2) gives more heat in the rapid phase than does a saturating amount (middle line) suggests that the heat of mixing is not negligible. It is thus hard to obtain quantitative information about the heats of reactions 1 and 2 or reaction 4 from these results. Although Yamada et al. analyze their results in a quite different way
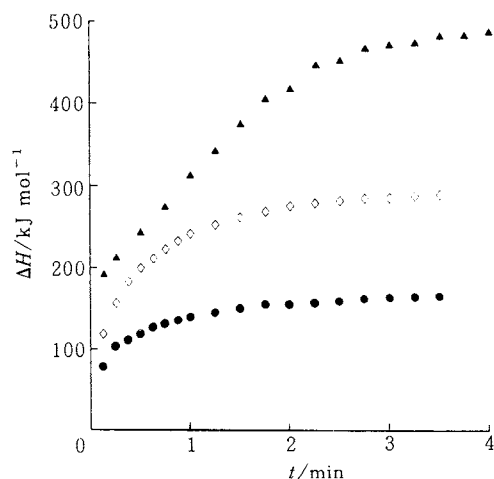
(with which the present authors do not sympathise) based on the subtraction of results with ADP from those with ATP, their main conclusion that the dissociation of Pi from myosin is strongly exothermic can be accepted.

The implication from the results of Yamada et al. that ADP binding is strongly exothermic ($\Delta H \simeq -100$ kJ mol^{-1}) was challenged by Goodno and Swenson[3] in a detailed calorimetric study. They reported that the heat of this reaction was small, between -4 and -12 kJ mol^{-1}. The question was reexamined by Kodama and Woledge[4] using a calorimetric titration technique with a modified LKB batch microcalorimeter. This technique, though lacking in time resolution, had sufficient sensitivity to allow study of the process of saturation of myosin with ADP in a number of steps. The technique was later further improved and the study was extended to HMM and S-1[5]. An example of the results obtained is shown in Fig. 3, from which it can be seen that acceptable binding isotherms can be obtained in this way for which values of both ΔH and the binding constant K can be obtained. As the figure illustrates the heat of reaction turned out to be definitely greater at 12°C than that at 0°C and a value of ΔC_p was obtained. However, preliminary observations at 25°C suggest that the value does not continue to increase. Reliable results were hard to obtain at 25°C probably because of instability of the protein at
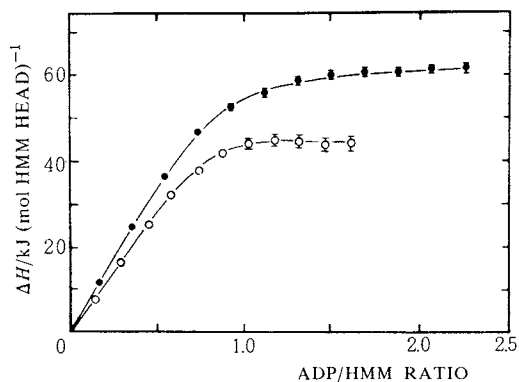

Fig. 2. Heat produced when ATP and myosin (HMM) are mixed. Results redrawn from Yamada et al.[2] The three sets of points are for 0.5 ATP per active site (●), 1 ATP per active site (◇) and 2 ATP per active site (▲). The heat is expressed per mol of HMM, that is per 2 mol of active site.

Fig. 3. Heat produced by binding of ADP to HMM. Redrawn from the Results of Kodama and Woledge[4] and Kodama et al.[5] Means and standard errors (S.E.) are shown except where the S.E. is too small. Open symbols are for myosin at 0°C and the filled symbols for HMM at 12°C.

this temperature. This may be connected with the small heat of reaction reported by Goodno and Swenson whose experiments were at 25 °C although this does not seem to be a complete explanation. However the detailed observations of Kodama et al. seem to leave little doubt that, at least at low temperatures, the reverse of reaction 4 produced 60 to 90 kJ mol^{-1}. Thus the forward reaction is endothermic by this amount.

We have also performed experiments on the addition of ATP to myosin[6,7]. These were done initially in a Calvet calorimeter, giving a time resolution of about 20 seconds. More recently we have used a more rapid calorimeter which will be described below. We find that most of the heat observed when ATP is mixed with myosin is produced in a single exponential phase amounting to 125 kJ mol^{-1} (see Fig. 4). The rate of this
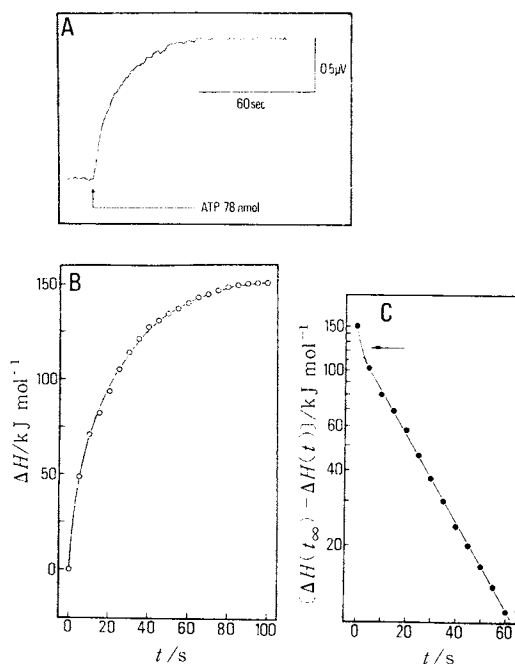

Fig. 4. Heat produced when ATP is mixed with excess myosin. Heat is expressed per mol of ATP. A: experimental record from the calorimeter. B: after correction for instrumental distortion. C: plotted semilogarithmically. The dotted line in C is an extrapolation of the linear part of the plot. The arrow shows the intercept of this extrapolation on the heat axis. Full details of these experiments will be published elsewhere[7].
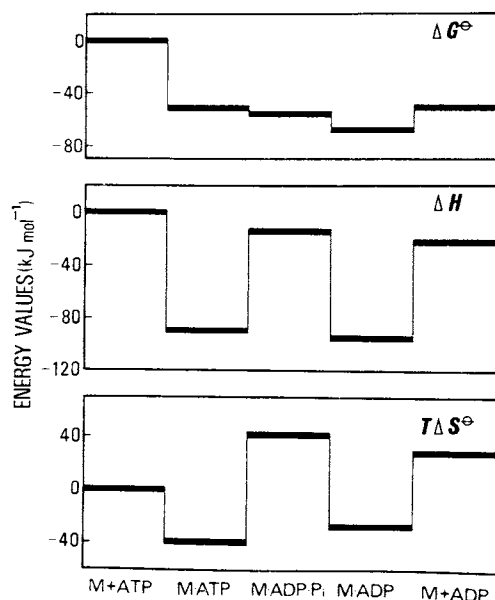
phase agrees with that for the dissociation of Pi and thus this result confirms that of Yamada et al. Also in agreement with their observations we find a rapid phase of heat production preceding the slow exponential phase. The amount of heat we find in this phase (27 kJ mol^{-1}) is however less than that they report. As already mentioned, their result may contain some heat of mixing whereas in our experiments this artifact is negligible.

This rapid heat production must correspond to the heat from reactions 1 and 2 together, as these are both rapid processes on this time scale. One way of obtaining an estimate of the heat of reaction 1 is by using, instead of ATP, an analogue of ATP which is split more slowly. We have used ATPγS and AMP-PNP. In both cases the heat of binding to myosin is large, -110 and -90 kJ mol^{-1} respectively. Thus we estimate ΔH_1 as -100 kJ mol^{-1}. Having a value of -27 kJ mol^{-1} for $\Delta H_1 + \Delta H_2$ we can now estimate ΔH_2 as $+73$ kJ mol^{-1}. The splitting of ATP on myosin is thus endothermic, in contrast to the value for the reaction in free solution which is of course exothermic ($\Delta H =$

Fig. 5. Energy profile of the 4 steps of myosin ATPase. The $\Delta H^{\ominus}$ values are from our observations, of which the example is in Fig. 4. $\Delta G^{\ominus}$ values are calculated from Table 2 of Trentham et al.[8] using a standard state of 1 mM. $T\Delta S^{\ominus}$ calculated from $\Delta H^{\ominus}$ and $\Delta G^{\ominus}$ values. A brief account is available[6].

-70 kJ mol^{-1}). A similar contrast in the case of the standard free energy of reaction has been demonstrated by Trentham, Eccleston and Bagshaw[8]. As free energy changes are also known for other reactions of the myosin cycle, we can now calculate the entropy changes. The result is shown in Fig. 5. Reaction 2 is characterized by a large increase in entropy and reaction 3 by a large decrease. This must be due to the change in the myosin molecule as it is "charged up" by ATP splitting and the reversal of this change as Pi is released. One interpretation of the entropy increase on ATP splitting would be a loss of ordered water structure as the protein takes up a more compact configuration less exposed to the solvent. An increase in fluorescence also occurs on this reaction[8] and can be interpreted in the same way. If this interpretation is correct we can predict using the ideas of Tanford[9] that there should be a decrease in heat capacity for this reaction. The calorimetric experiments that might show this have not yet been performed.

EXPERIMENTS WITH ACTIN AND MYOSIN

Although as we have explained, a reasonably complete picture is now available of the thermodynamics of the myosin ATPase cycle, only one reaction (reaction 2) is part also of the actomyosin cycle and only relatively little experimental work has been done on the other reactions. The results available to date will now be summarised.

In collaboration with Drs. H. D. White and S. J. Smith calorimetric experiments have been made on the interaction of actin with myosin (reaction 9). The equilibrium of this reaction favours the bound state and thus the calorimetric titration technique can be used. As has already been reported briefly[10] and is shown here in Fig. 6 the reaction is endothermic. At 12°C, when HMM is the form of myosin used, the ΔH value is about $+30$ to $+40$ kJ for each mole of myosin active site attaching to actin. Some data is now available for 0°C (Fig. 6). The reaction heat is clearly much greater at the lower temperature. There is thus substantial decrease in heat capacity for this reaction (several kJ mol^{-1} K^{-1}). This is probably due to exclusion of water from the area where the two proteins approach close to each other.
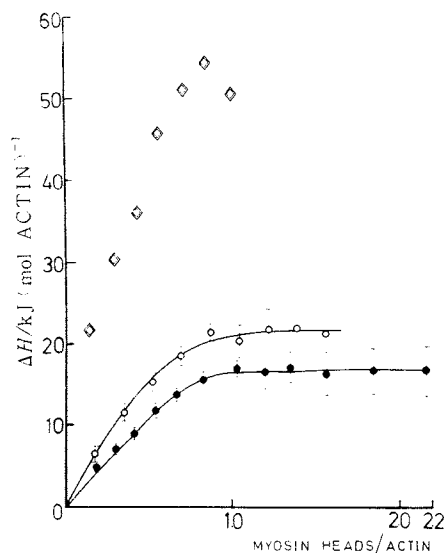

Fig. 6. Heat produced by binding of myosin to actin. Titration experiments were carried out with actin as titrand and HMM or S-1 as titrant. ● : S-1 at 12°C, ○ : HMM at 12°C. ◈ : HMM at 0°C. The heat evolved is expressed per mole of actin monomers. The bars show ± 1 S.E., where these values are available.

Dr. H. D. White (personal communication) has studied the reverse of reaction 8 (ADP binding to A·M) and finds the heat to be small (<10 kJ mol^{-1}) in contrast to the large heat production in reaction 4.

FUTURE EXPERIMENTS

All values of ΔH are liable to depend on the pH and ionic strength, and where protons are involved in the reactions, also on the heat of protonation of the buffer. It is therefore very desirable that observations should be repeated under a range of conditions and buffers having different heats of protonation. This has not so far been done for any of the reactions we are discussing. Of course the observations should also be repeated at a number of different temperatures so that the heat capacity value can be obtained. This has only be done for reactions 4 and 9 so far. The existence of large ΔC_p values in these reactions indicates that there must be other large values in the cycle since the value for the complete cycle is very small. It might be best to use scanning calorimetry to

study the heat capacity changes. This large ΔC_p values might reflect the existence of fairly sharp thermal transitions in the proteins which can be best studied by a scanning technique. The sensitivity of the technique, for instance as it has been developed by Professor Privalov, is now quite adequate for this task.

Beside this detailed work there also remain two reactions of the cycle accessible to calorimetry with existing instrument, but not yet investigated. These are reaction 12 and the reverse of reaction 3. From consideration of the cycle of reactions 4, 9, 8, and 12, for which the overall ΔH is of course zero, the heat of reaction 12 can be calculated. Using the results reported above the value is found to be approximately +100 kJ mol^{-1}. This ought to be easily observable. Even though the value can be found indirectly, the direct experiment would provide a valuable confirmation of the correctness of all the observations or a valuable indication of an error in them! Similar considerations apply to studying the reverse of reaction 3. Although the binding of Pi to M·ADP is weak ($K \simeq 10$ M^{-1}) it should be possible to obtain some calorimetric information from a study of this process.

Improved calorimeters — Further progress will require improvements in instrumentation. For example separation of the heat of reactions 1 and 2 without using ATP analogues could probably be achieved at low temperature if a calorimeter with a resolution of 0.2 s could be made. Of course many calorimetric measurements with this time resolution, or better, have been reported, for example the work of A. V. Hill and W. Roughton. The difficulty is to arrange for a combination of (1) a rapid heat flow from the reaction system to the detector, (2) a high sensitivity, required because of the necessarily low concentration of proteins, and (3) a means of initiating the reaction, for instance by mixing the two reactants together.

We have constructed a very simple calorimeter which is an improvement in these respects over earlier designs and has a response time of about 1 s. The instrument consists of two cells of 2 cm^3 capacity with aluminium jackets. The cells are connected by a silver/constantan thermopile (about 30 junctions). The thermojunctions are on the inner surface of the cells, separated from the cell contents by only a thin teflon/polyimide laminated

film. The reaction mixture is stirred continuously by means of vertical oscillations of a teflon disk at 5 Hz, and the reaction is started by the addition of one reagent in a small volume of buffer. The stirring has two objects: one is to mix the reagents to initiate the reaction; the other is to speed up the heat flow from the contents of the cell to the detector. The importance of stirring from this point of view is illustrated in Fig. 7 which shows the calculated heat flow for a calorimeter with a glass-jacketed cell with and without stirring. Several other design points are illustrated by the calculations shown in this figure: (1) that a metal jacket gives a better response characteristic than a glass jacket; (2) that the jacket does not slow the speed of response if stirring is adequate (the jacket is perhaps required for mechanical strength anyway); and (3) that the calculated speed of response is much better than that actually achieved. This is probably because the stirring we have used is not optimum for heat flow. The rate of stirring however must be limited by considerations of (1) the heat dissipation that it causes, and (2) the effect on the protein in the calorimeter, which can be denatured by too vigorous stirring. The current problem is thus to achieve efficient stirring without either excessive power input or very high local shear force. More study is needed to find the best technique.

An alternative approach, which we have not explored, but regard as promising, is to mix the reagents outside the calorimeter cell, in other words to use a stop-flow system. We are not aware of any successful biochemical applications of stop-flow calorimetry although a number of instruments have been built. Heat flow could be rapid in such a system if the cell diameter is small enough and if the cell has no jacket. This point is illustrated in Fig. 8. No doubt such a calorimeter would have its own special problems. We hope they will soon be investigated.

Looking further into the future, after the soluble system we have discussed has been fully characterized, the next step is to investigate a system capable of carrying out mechanical work so that the coupling phenomena themselves can be investigated. Of course such a system has to have the actin and myosin arranged in a mechanical structure so that the mechanical work can be

TEMPERATURE
100μ INTO GLASS

WATER

GLASS

3·35 mm   1·75 mm

STIRRED

UNSTIRRED

t / s

X   STIRRED WATER   5000μ   ALUMINIUM   2000μ

TEFLON/COUPLES/TEFLON
(3 × 50μ)

TEMPERATURE AT
THERMOCOUPLES

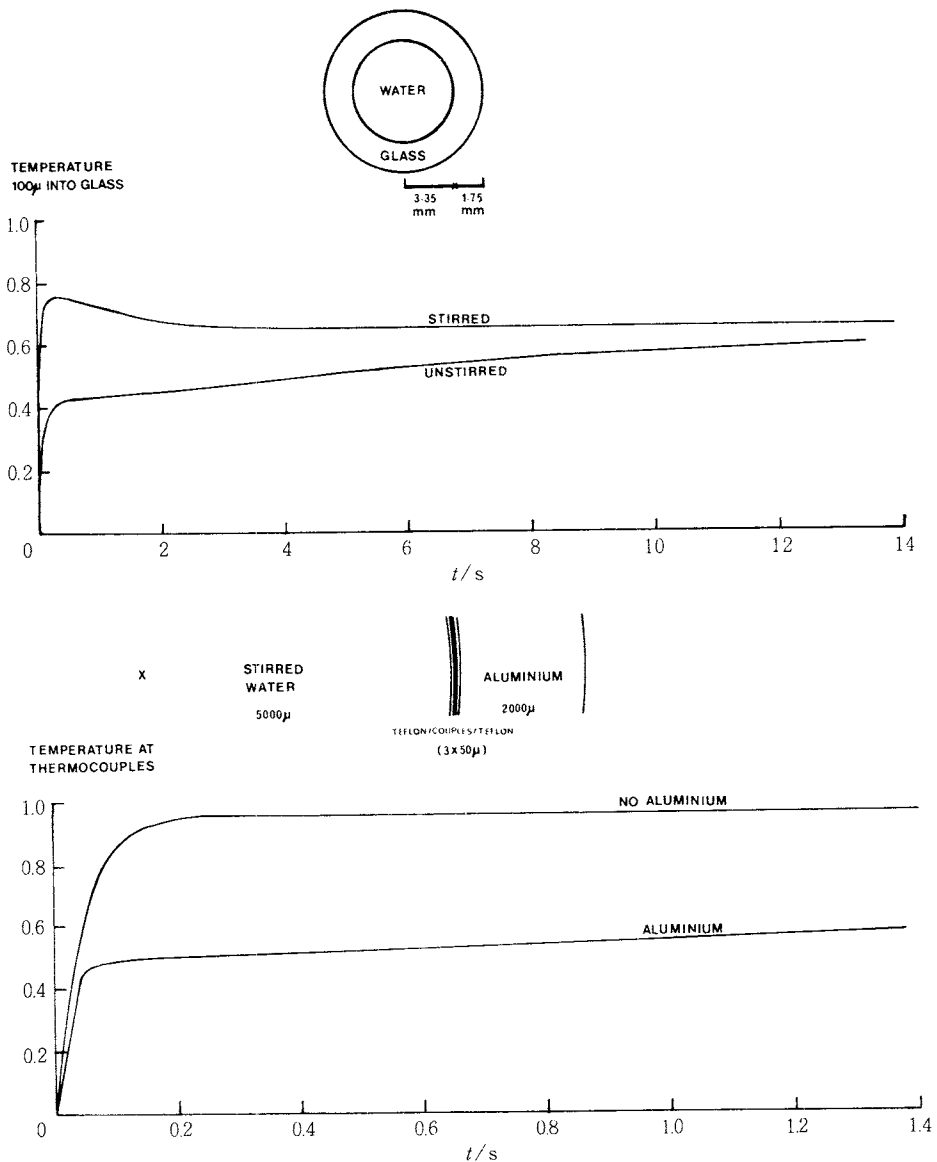NO ALUMINIUM

ALUMINIUM

t / s

Fig. 7. Calculated speed of response of various hypothetical calorimeters. The calculations were performed numerically. They were devised and carried out by Mr. M. Irving. The quantity plotted is the temperature at the thermopiles at various times after the temperature of the cell contents is abruptly increased by 1 unit.

A: for a glass-walled vessel of the dimensions shown in the inset with the cotents either stirred or unstirred. As the heat exchange would be dominated by the thermal resistance of the glass and water the thermocouples have not been explicitly included in the calculations. They are considered to be inserted in the glass 100 μm from the inside surface.

B: for a vessel with a teflon/constantan/teflon wall with or without an aluminium jacket. The dimensions are shown in the inset to the figure. The cell contents are stirred. The constantan layer in the wall represents the thermocouples.
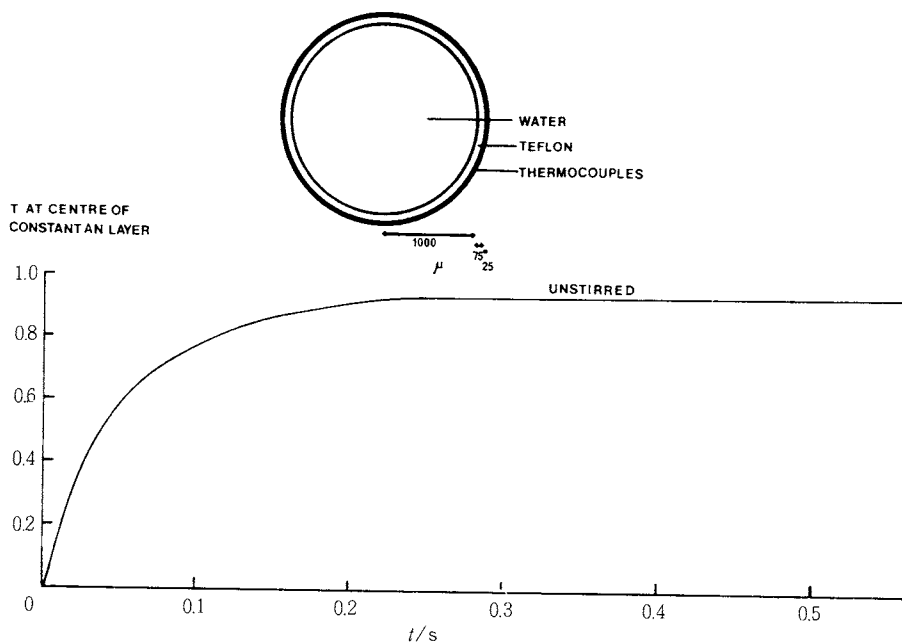
Fig. 8. Calculations similar to those in Fig. 7 for a vessel with a teflon/constantan wall, with the cell contents unstirred. Dimensions shown in the inset. The constantan layer represents the thermocouples.

collected. One might either use the natural structure from the muscle. or an artificially reconstituted structure. In either case stirring will be impossible; diffusion must be used to carry the nucleotides to the proteins. The system must therefore be very small and will present a new set of interesting challenges to the calorimetrist.

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