Calorimetric Studies of Adenosine 5′-Triphosphate Hydrolysis by Heavy Meromyosin[†]

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ABSTRACT: The heat production for the reaction steps of ATP hydrolysis by heavy meromyosin has been reexamined by using a reaction-type calorimeter. So far, the values reported for the heat production have varied substantially among research groups. The present results obtained in 0.3 M KCl, 10 mM $MgCl_2$, and 20 mM Tris-HCl at pH 8.0 and 20 °C show that (1) the binding and splitting of ATP on the myosin head are moderately exothermic ($\Delta H = -23$ kJ mol^{-1}), (2) the decomposition of the M·ADP·P_i intermediate complex to M·ADP + P_i is strongly exothermic ($\Delta H = -66$ kJ mol^{-1}), and (3) the dissociation of ADP from the myosin head is strongly endothermic ($\Delta H = +60$ kJ mol^{-1}). These results agree with our previous findings that the decomposition of M·ADP·P_i as well as the binding of ADP to heavy meromyosin is strongly exothermic [Yamada, T., Shimizu, H., & Suga, H. (1973) *Biochim. Biophys. Acta 305*, 642–653].

Muscle contraction takes place by the mutual sliding of actin and myosin filaments utilizing the chemical energy of ATP hydrolysis. Recently, the reaction schemes of myosin ATPase as well as those of actomyosin ATPase have been well clarified (Inoue et al., 1973; Taylor, 1979). Calorimetric studies of myosin and actomyosin systems are expected to provide us with useful information on thermodynamic aspects of the chemomechanical process of the energy conversion of muscle contraction.

We have performed kinetic studies of the heat production associated with the mixing of ATP and ADP with heavy meromyosin (HMM)[1] prepared from rabbit skeletal muscle (Yamada et al., 1973). A large amount of heat ($\Delta H = -70$ to -90 kJ mol^{-1})[2] was found to be produced on the decomposition of the M·ADP·P_i intermediate complex to M·ADP + P_i as well as on the binding of ADP with HMM. Later, Goodno & Swenson (1975) repeated our calorimetric experiments on the binding of HMM and ADP. Unfortunately, they could not reproduce our results: they observed only a small amount of heat ($\Delta H = -10$ kJ mol^{-1}). Soon after their work, Kodama and his associates (Kodama & Woledge, 1976; Kodama et al., 1977) performed calorimetric studies of the binding of ADP with myosin heads. They observed that a large amount of heat ($\Delta H = -90$ kJ mol^{-1}) is produced, which supported our results. Recently, Swenson & Ritchie (1979) again tried calorimetric studies of the reaction of ATP with myosin as well as the binding of ADP with myosin. They observed that a substantial amount of heat is absorbed at the initial rapid phase after the mixing of myosin with ATP, which is strongly contrasted to the data of our previous study; heat is produced, not absorbed. Very recently Kodama & Woledge (1979) observed a slight heat production (-7 kJ mol^{-1}) associated with this initial rapid stage. On the binding of ADP with myosin, Swenson & Ritchie (1979) observed that only a moderate amount of heat ($\Delta H = -29$ kJ mol^{-1}) is produced. However, even this value is far smaller than those reported by Kodama and his associates and by us.

Since the reaction of ATP and the binding of ADP with myosin heads are the simplest systems to be studied calorimetrically in the actomyosin ATPase cycle, the values of the heat production should be settled first. Here we report the results of detailed calorimetric studies of the reaction of ATP as well as the binding of ADP with HMM which were performed as a continuation of our previous calorimetric studies of the interaction of nucleotides with HMM. The obtained values of the enthalpy of the reaction steps of ATP hydrolysis by HMM agreed well with those reported in our previous paper (Yamada et al., 1973).

Materials and Methods

Myosin was isolated from the back and leg skeletal muscles of rabbit by the method of Perry (1955). HMM was prepared by tryptic digestion of myosin by the method of Lowey & Cohen (1962) and by chymotryptic digestion of myosin by the method of Weeds & Taylor (1975). A 1:200 (w/w) trypsin/myosin solution was added to a solution of myosin. The digestion was carried out at 20 °C for 4 min, and it was stopped by adding trypsin inhibitor (trypsin inhibitor/trypsin, 2:1 w/w). Alternatively a 1:200 (w/w) chymotrypsin/myosin solution was added to a solution of myosin in the presence of 2 mM $CaCl_2$. The digestion was carried out at 20 °C for 10 min and was stopped by adding 0.5 mM phenylmethanesulfonyl fluoride. HMM was fractionated by precipitating it in a 45–55% saturated ammonium sulfate solution. Ammonium sulfate was removed by repeated dialysis of the HMM solution against solutions of 30 mM KCl and 5 mM Tris-HCl (pH 7.6). The solution of HMM was then dialyzed several times against solutions with the salt conditions for the present calorimetric experiments. The solution of HMM was finally centrifuged at 100000*g* for 2 h, and the supernatant was used for measurements.

The preparations of HMM had Mg^{2+}, Ca^{2+}, and EDTA ATPase activities in the ranges routinely obtained in our laboratory, which agreed well with the published data.

ADP and ATP were purchased from Boehringer Mannheim Chemical Co. and used without further purification. AMP,

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[1] Abbreviations used: HMM, heavy meromyosin; EDTA, ethylenediaminetetraacetic acid.

[2] In the present paper, the unit kilojoule per mole represents the amount of heat produced per single myosin head.

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ADP, and ATP contained in ADP and ATP preparations were analyzed by using a high-pressure liquid chromatograph, JEOL Uniflow X1.

The concentrations of HMM and nucleotides (AMP, ADP, and ATP) were determined spectrophotometrically with $\Delta\epsilon_{280}^{1\%} = 6.57$ (Young et al., 1964) and $\Delta\epsilon_{260} = 15.4$ mM^{-1} cm^{-1} (Bock et al., 1956), respectively. The amount of inorganic phosphate was determined by the method of Martin & Doty (1949). The molecular weight of HMM was assumed to be 340 000 (Weeds & Pope, 1977).

Calorimetric measurements were made with a precision calorimeter, LKB 8700. The details of the calorimetric measurements were given in our previous paper (Yamada et al., 1973). The reaction vessels of 25 and 100 mL were employed. The time constant of the calorimeter was about 5 s. The net heat loss (the real heat loss plus the heat production due to the stirring of solution) during the experiments was negligibly small and was ignored from the obtained data. In the present study, care was taken to minimize possible denaturation of HMM during thermal equilibration between HMM solution in the reaction vessel and water in the thermobath of the calorimeter. The temperature of the HMM solution in the reaction vessel and that of the nucleotide solution in the glass ampule were adjusted to a temperature slightly lower than that of the thermobath. After the reaction vessel was set to the calorimeter, the temperature of the reaction solutions was precisely adjusted to that of the thermobath by repeatedly supplying small amounts of heat (~0.5–2 J/5–10 s) with a glass-coated heater installed in the reaction vessel. Thus, the thermal equilibrium was established within about 30–40 min after HMM solutions were introduced into the reaction vessel. The ATPase activities of HMM before and after the calorimetric measurements were the same within experimental error.

Heats are artificially produced on the mixing of a nucleotide solution and an HMM solution. This includes (1) the heat produced mechanically by breaking the thin glass walls of the ampule (15 ± 5 mJ), (2) the heat of dilution of the nucleotide solution (within experimental error), which was determined by mixing a nucleotide solution with the buffer solution used for the final dialysis of an HMM solution, and (3) the heat of dilution of HMM (within experimental error), which was determined by mixing the buffer solution for the nucleotide dissolution and an HMM solution. The amounts of these heats were separately measured and accordingly subtracted from the heat produced on the mixing of a nucleotide solution and an HMM solution. Thus, the dominant experimental uncertainty of the present calorimetric experiments was found to be due to the artificially produced heat by reaction 1 above. Moreover, the heat artificially produced by reaction 1 substantially depended on the thickness of the wall of the glass ampule as well as on the size of pressure applied for breaking the ampule. In this study, we therefore used glass ampules with thinner walls of about the same thickness and carefully broke the walls by applying almost the same magnitude of pressure. Thus, the amount of uncertain heat by reaction 1 was found to be ±5 mJ in the present study.

ATP contained AMP (about 1 mol %) and ADP (about 2 mol %) as contaminants. Since the heats produced by the reaction of these contaminants with HMM are small, the observed heat was not corrected for these heats.

ADP used in the present studies contained AMP (about 10 mol %) and ATP (about 3 mol %) as contaminants. The reaction of ATP with HMM produces net heat of about 100–150 kJ/mol of myosin head (Yamada et al., 1973; Kodama & Woledge, 1979; data below). Since the heat produced by this reaction is far smaller than the uncertain heat artificially produced by reaction 1 above, the observed heat was not corrected for these heats. Myokinase contaminating HMM preparations converts ADP to ATP. The heat associated with this reaction as well as that associated with the reaction of the formed ATP with HMM may also produce heat. The amount of inorganic phosphate, which is equal to the ATP formed by the myokinase reaction, was measured after mixing ADP and HMM solutions, and it was found to be negligible during the period of the calorimetric experiments under the present conditions. Therefore, the heat produced by this reaction was ignored.

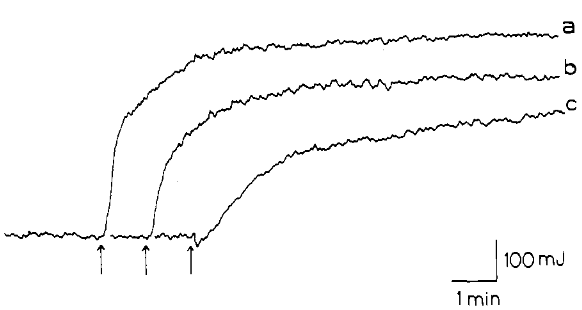

FIGURE 1: Calorimetric traces when a solution of HMM was mixed with a solution of ATP at 20 °C. A reaction vessel contained 100 mL of HMM solution [10.8 mg/mL HMM in 0.3 M KCl, 10 mM MgCl$_2$, and 20 mM Tris-HCl (pH 8.0)] with (a) no ADP, (b) 70 μM ADP, and (c) 200 μM ADP, and a glass ampule contained 0.88 mL of 3.2 mM ATP, 0.3 M KCl, 10 mM MgCl$_2$, and 20 mM Tris-HCl (pH 8.0). After thermal equilibration, the solution of ATP was mixed with the solution of HMM at the times indicated by the arrows by breaking the thin glass walls of the ampule containing the ATP solution. Traces a–c are one sequential experiment.

The heat produced on the association of ADP with HMM was analyzed as shown below. HMM is assumed to have n independent and identical sites for ADP binding. The binding constant K is then given by

$$K = \frac{[\mathrm{M{\cdot}ADP}]}{[\mathrm{M}][\mathrm{ADP}]} = \frac{[\mathrm{M{\cdot}ADP}]}{\{[\mathrm{M}]_0 - [\mathrm{M{\cdot}ADP}]\}\{[\mathrm{ADP}]_0 - [\mathrm{M{\cdot}ADP}]\}}$$

where M represents the myosin head (brackets denote the concentration in molar units) and $[\mathrm{M}]_0$ and $[\mathrm{ADP}]_0$ represent the concentrations of myosin head and ADP added in molar units, respectively. Then the heat produced when $[\mathrm{ADP}]_0$ is mixed with $[\mathrm{M}]_0$ is given by

$$h = -\Delta H n[\mathrm{M{\cdot}ADP}]/2$$

where ΔH represents the enthalpy of ADP binding to the myosin head. This equation was fitted to the obtained calorimetric data by means of an iterative least-squares procedure with assumed K and n values and using an adjustable parameter ΔH. The computation was made with a computer, HITAC 8700/8800.

Results

Interaction of HMM with ATP. When a solution of HMM was mixed with a solution of ATP, a large amount of heat was found to be produced, as shown in Figure 1 (trace a). The observed heat was corrected for artificially produced heat as described under Materials and Methods. The time change of the heat production after mixing of HMM with ATP was determined as shown in Figure 2 (trace a).

Similar measurements were made where various concentrations (70 and 200 μM) of ADP had been added to HMM

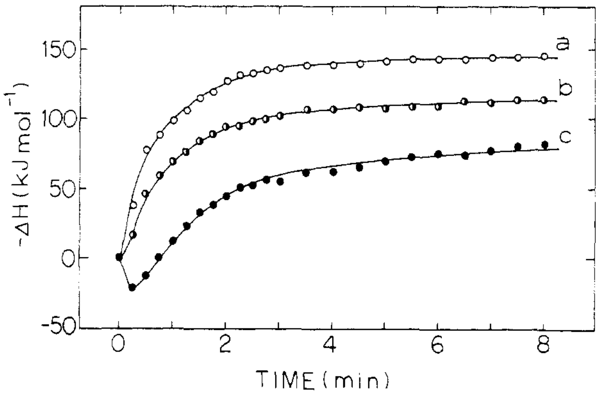

FIGURE 2: Corrected data obtained in Figure 1. Data from traces in Figure 1 were analyzed by correcting for artificially produced heat at the mixing of ATP and HMM solutions.

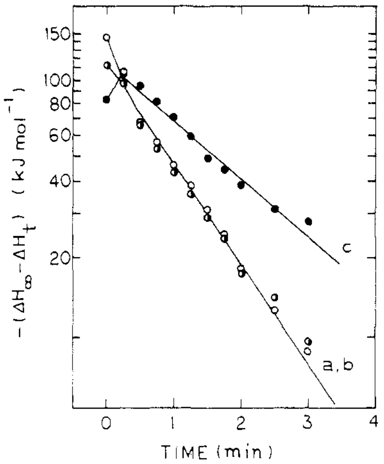

FIGURE 3: Semilogarithmic plots of the data of Figure 2.

solutions before the mixing of HMM with ATP. The results are shown in Figure 1 (traces b and c). Traces b and c in Figure 2 show the data after correcting for the artificially produced heat. In these cases, the total amounts of the heat production are much smaller than that where ADP had not been added to the HMM solution.

A semilogarithmic plot of the heat development shown in Figure 3 (trace a) indicates that the slow heat production after mixing of ATP with HMM is a first-order process. By extrapolating this slow first-order process to zero time, we determined the amount of heat production associated with this slow process to be $\Delta H = -114 \pm 4$ kJ mol^{-1}. This analysis also indicates that there is very rapid heat production ($\Delta H = -33 \pm 5$ kJ mol^{-1}) at the initial stage of the reaction. Similar analyses were performed where various concentrations of ADP had been added to the HMM solution before the mixing with ATP. The slow process shown in trace b of Figure 3 has essentially the same rate constant as the case where ADP had not been added to the HMM solution, while that shown in trace c of Figure 3 has a slightly smaller rate. By extrapolating these slow processes to zero time, we determined the heat production associated with the slow processes to be -114 ± 4 kJ mol^{-1}, which is almost the same amount as that obtained when ADP had not been added to the HMM solution before the mixing with ATP. It is noticed that there is very rapid heat absorption at the initial stage of the reaction when ADP had been added to HMM solutions prior to the mixing with ATP: in trace b of Figure 1, $\Delta H = +1 \pm 5$ kJ mol^{-1}, and in trace c of Figure 2, $\Delta H = +30 \pm 5$ kJ mol^{-1}.

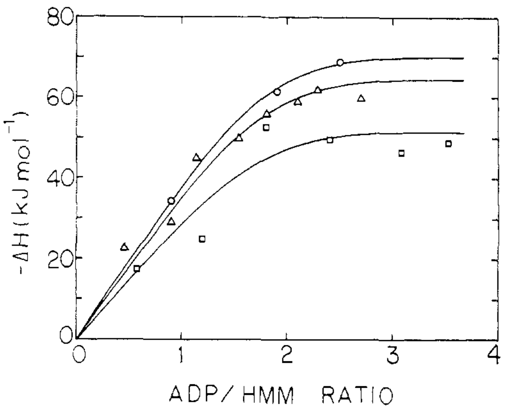

FIGURE 4: Heat produced by the mixing of ADP and HMM. Corrections were made for artificially produced heat. For details, see the text. The points show the experimental results, and the lines were drawn by using the equations shown under Materials and Methods with the values of the adjustable parameters given in the text. The circle, triangle, and square symbols, respectively, represent the data obtained in 0.07, 0.1, and 0.5 M KCl [containing 2 mM $MgCl_2$ and 20 mM Tris-HCl (pH 8.0)] at 20 °C. Each point represents the averaged values from two to three experiments. For clarity, the data in the case of 0.3 M KCl were not included. The uncertainty of the data (±7 kJ mol^{-1}) is dominantly due to an uncertain amount of heat artificially produced by mechanically breaking the thin glass walls of the ampule in order to mix an ADP solution with an HMM solution.

Interaction of HMM with ADP. When a solution of HMM was mixed with a solution of ADP in 0.1 M KCl, 2 mM $MgCl_2$, and 20 mM Tris-HCl (pH 8.0) at 20 °C, a large amount of heat was found to be produced. The observed heat was corrected for artificially produced heat as described under Materials and Methods, and the heat associated with the binding of ADP to HMM was determined. Similar measurements and corrections for artificially produced heat were made by using various concentrations of ADP. The data of the heat of binding of ADP with HMM thus obtained are summarized in Figure 4. Using the equations and analysis described under Materials and Methods, we can estimate the enthalpy of the binding of ADP with HMM by assuming K and n values. Thus, the enthalpy of the binding of ADP with HMM was determined to be $\Delta H = -74 \pm 7$ kJ mol^{-1} for $K = 10^6$ M^{-1} and $n = 1.8$.

Similar experiments were performed for 0.07, 0.3, and 0.5 M KCl in place of 0.1 M KCl, while other conditions were the same as above. The obtained data of the enthalpy of the binding of ADP with HMM are also included in Figure 4. They were estimated to be $\Delta H = -80 \pm 7$, -70 ± 7, and -59 ± 7 kJ mol^{-1}, respectively, for 0.07, 0.3, and 0.5 M KCl by assuming $K = 10^6$ M^{-1} and $n = 1.8$.

Discussion

The present study shows that a large amount of heat is produced when ATP is mixed with HMM. On the basis of the recent ATPase scheme (Inoue et al., 1973; Taylor, 1979), ATP is very rapidly bound and split on the myosin head (M·ADP·P_i intermediate complex is formed almost instantaneously) followed by a slow decomposition of M·ADP·P_i to M·ADP + P_i. Thus, the result shown in trace a of Figure 2 indicates that the initial rapid heat production is associated with the binding and splitting of ATP and is determined to be $\Delta H = -33$ kJ mol^{-1}. The slow first-order heat production corresponds to the decomposition of M·ADP·P_i to M·ADP + P_i and is $\Delta H = -114$ kJ mol^{-1}.

When ATP was mixed with ADP-bound HMM, the heat change observed at the initial rapid stage of the reaction is the sum of the enthalpy changes associated with ADP dissociation, ATP binding, and ATP splitting. This is determined

Table I: Enthalpy Changes, ΔH (kJ mol^{-1}), Associated with ATPase Reaction of the Myosin Head

ATP binding and splitting[a]	M·ADP·P$_i$ decompn[a]	ADP release	total	ref
−62 (−52)[b]	−101 (−53)	+97 (+87)	−66 (−18)	c
−25 (−15)	−128 (−80)	+82 (+72)	−71 (−23)	d
+19 (+21)	exothermic	+31 (+29)		e
−33 (−23)	−114 (−66)	+70 (+60)	−77 (−29)	f

[a] No correction was made for the rapid equilibrium between M·ATP and M·ADP·P$_i$, which slightly contributes to the observed heat (Kodama & Woledge, 1979). [b] The values in parentheses are after correcting for the neutralization heat of the released proton. [c] Yamada et al. (1973): 0.3 M KCl, 2 mM MgCl$_2$, and 20 mM Tris-HCl (pH 8.0) at 20 °C. [d] Kodama & Woledge (1979): 0.1 M KCl, 20 mM MgCl$_2$, and 20 mM Tris-HCl (pH 7.8) at 15 °C. [e] Swenson & Ritchie (1979): 0.5 M KCl, 10 mM MgCl$_2$, and 20 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) buffer (pH 7.8) at 15 °C. [f] Present work: 0.3 M KCl, 10 mM MgCl$_2$, and 20 mM Tris-HCl (pH 8.0) at 20 °C.

to be $\Delta H = +30$ kJ mol^{-1}, as shown in trace c of Figure 2, which agrees well with the calculated value (+70 + −33 = +37) by assuming that the enthalpy change of ADP release from the myosin head is $\Delta H = +70$ kJ mol^{-1} as below. A smaller value of the heat absorption in trace b of Figure 2 may be due to partial conversion of the added ADP to AMP and inorganic phosphate by contaminant myokinase and HMM. The amount of heat production associated with the slow decomposition process is $\Delta H = -114$ kJ mol^{-1} in these cases as shown in traces b and c of Figure 3. This value is almost the same as that in the case shown in trace a of Figure 3 where ADP had not been mixed with HMM before the mixing with ATP. The smaller rate constant of the slow decomposition process in trace c may be partly due to the increase of ionic strength of the solution by the addition of ADP.

Thus, we can determine the enthalpy changes during the ATP splitting cycle of the myosin head, as seen in Table I. It has been reported that a proton is released and absorbed in association with ATPase reaction of the myosin head: 0.2-mol release at the binding of ATP, 0.8-mol release at the decomposition of M·ADP·P$_i$ to M·ADP + P$_i$, and 0.2-mol absorption at the release of ADP (Bagshaw & Trentham, 1974). The neutralization of the released proton with Tris produces heat [$\Delta H = -47.5$ kJ mol^{-1} (Bernhard, 1956)]. We also give the enthalpy changes in Table I after correcting for these neutralization heats. For comparison, the data reported so far are also included. The heat associated with the rapid binding and splitting of ATP agrees well with that recently reported by Kodama & Woledge (1979), while it is fairly smaller than that reported by us previously (Yamada et al., 1973). This may be partly due to improper correction of the artificially produced heat in our previous study (see Materials and Methods). The heat of the decomposition of M·ADP·P$_i$ agrees well with the results of our previous study ($\Delta H = -101$ kJ mol^{-1}). Recently, Kodama & Woledge (1979) reported a much larger value for the heat of the decomposition of M·ADP·P$_i$. This may be due to the difference of the ionic strength of the solution: our previous study showed that the heat of the decomposition of M·ADP·P$_i$ decreases as the ionic strength of the solution is increased.

On the binding of ADP with HMM, the present results clearly demonstrate that a large amount of heat ($\Delta H = -70$ kJ mol^{-1}) is produced in 0.3 M KCl. A proton of about 0.2 mol/mol of bound ADP is released on association with the binding of ADP to HMM (Bagshaw & Trentham, 1974). Correcting for the heat of neutralization of the released proton with Tris in the solution, we can estimate the heat of the binding of ADP with HMM to be $\Delta H = -60$ kJ mol^{-1}.

The present value of the heat of the binding of ADP with HMM agrees well with those reported by Kodama and his associates (Kodama & Woledge, 1976; Kodama et al., 1977) and by us previously (Yamada et al., 1973). As can be seen under Results, the enthalpy of the binding of ADP with HMM decreases as the concentration of KCl in the solvent is increased. This is also consistent with the results reported by Kodama et al. (1977).

On the other hand, Swenson & Ritchie (1979) reported the heat absorption ($\Delta H = 21$ kJ mol^{-1}) at the initial rapid stage of the reaction of myosin with ATP. This large heat absorption shown in Figure 3 of their paper is strongly contrasted to the heat production obtained by Kodama & Woledge (1979) and by us (Yamada et al., 1973; this paper). Their heat trace rather resembles the case where ADP had been added to HMM before the mixing with ATP, as shown in traces b and c in Figure 2.

Furthermore, Swenson and his associates (Goodno & Swenson, 1975; Swenson & Ritchie, 1979) consistently reported much smaller values also for the enthalpy of the binding of ADP with the myosin head: previously, they observed only a small amount of heat production, $\Delta H = -10$ kJ mol^{-1}, and, more recently, a moderate amount of heat production, $\Delta H = -29$ kJ mol^{-1}. They gave no explanation as to why they obtained a larger value by their recent study. However, even their revised value of the heat production is still substantially smaller than those values reported by Kodama and his associates and by us.

In our previous study, we used lyophilized preparations of HMM. In the present study, we used HMM preparations without lyophilization. Thus, the lyophilization process does not induce the observed difference. It has been stated that myokinase contaminating HMM might produce some heat (Goodno & Swenson, 1975). As described under Materials and Methods, the heat to be produced by the myokinase reaction was estimated to be negligibly small in our present as well as in our previous calorimetric studies. Arata et al. (1975) suggested that the high concentrations of HMM employed in our previous studies might bind protons released in association with the binding of ADP with HMM, and it produces artificial heat. In the present studies, we used various concentrations of HMM (including concentrations comparable to and much less than those employed by Swenson and his associates) and obtained almost the same amount of heat for the binding of ADP to HMM. Thus, the difference in the concentration of HMM does not contribute to the observed discrepancy between the values obtained by Swenson and his associates and those obtained by us. Although our calorimetric experiments involve uncertain heat artificially produced by breaking the thin glass walls of the ampule in order to mix nucleotides with HMM, this heat is much smaller than the discrepancy of heat production we are concerned with in the present paper.

Thus, we cannot explain why Swenson and his associates consistently obtained smaller amounts of heat for the reaction of ATP with myosin as well as for the binding of ADP to myosin. The discrepancy might be partly due to the slight difference in the experimental conditions. It should be pointed out, however, that their calorimetric traces rather resemble those obtained when ADP had been mixed with HMM before the HMM solution is mixed with nucleotide solutions. We wonder if they might have used myosin preparations with accidentally bound ADP for their calorimetric experiments.

We conclude that a large amount of heat is produced on the binding of ADP with HMM, which we first observed in

1973. Moreover, since the kinetics of the heat production following the initial transient heat production after the mixing of nucleotide and HMM were very reliable in our experiments, we maintain that our previous conclusion (Yamada et al., 1973) is valid that a large amount of heat is produced on the decomposition of $M\cdot ADP\cdot P_i$ to $M\cdot ADP + P_i$. These conclusions have been well substantiated recently by Kodama and his associates as described above.

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Relationship between Negative Cooperativity and Insulin Action[†]

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ABSTRACT: We have compared the respective abilities of porcine insulin and $[\mathrm{Leu}^{B25}]$insulin to enhance the rate of dissociation of receptor-bound [^{125}I]insulin from IM-9 lymphocytes and isolated rat adipocytes and to increase the rate of adipocyte glucose transport and oxidation. Although porcine insulin (100 ng/mL) greatly enhanced the dissociation rate of previously bound [^{125}I]insulin, $[\mathrm{Leu}^{B25}]$insulin (at a concentration yielding equivalent receptor occupancy) had no effect. Nevertheless, the analogue fully stimulated adipocyte glucose transport and oxidation at concentrations consistent with its reduced (1.7% of normal) intrinsic binding affinity. Activation of glucose transport by the analogue was rapid, and the corresponding rate of activation was indistinguishable from that produced by native insulin. The increased dissociation rate observed with increasing receptor occupancy by native porcine insulin has been interpreted as evidence for negative cooperative site–site interactions between occupied receptors. According to this formulation, $[\mathrm{Leu}^{B25}]$insulin is a "noncooperative" insulin analogue. Since this $[\mathrm{Leu}^{B25}]$insulin retains full biologic activity, the enhancement of the insulin dissociation rate at high levels of receptor occupancy does not reflect a phenomenon inherent in insulin's action to augment glucose metabolism.

Most workers find that when insulin binding to receptors is studied over a wide range of insulin concentrations, Scatchard analysis of the resulting data yields curvilinear plots (DeMeyts et al., 1976a; Ginsberg, 1977). DeMeyts et al. (1973) have also demonstrated that when receptor occupancy is increased with unlabeled insulin the dissociation rate of previously bound [^{125}I]insulin is greatly increased and have suggested that this provides evidence for negatively cooperative receptor interactions. This interpretation could at least partly explain the characteristic curvilinear Scatchard plots of insulin binding. While some workers have failed to detect this phenomenon (Gliemann et al., 1975), and others have questioned its interpretation (Pollet et al., 1977), numerous studies have confirmed the original observations of DeMeyts et al. (1973, 1976a) in a variety of receptor systems (DeMeyts et al., 1976b).

Studies of chemically and enzymatically modified insulins have suggested that residues important for enhancing the rate of receptor-bound [^{125}I]insulin dissociation (notably A21 and

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