Hydrostatic Pressure and the Contractile System*

Patricia Rainford, Hajime Noguchi,† and Manuel Morales‡

ABSTRACT: "Superprecipitation" of myosin B (measured turbidimetrically) on addition of adenosine triphosphate (ATP) and the adenosine triphosphatase (ATPase) activity of myosin B and myosin A have been studied at 1 and 500 atm. Superprecipitation, treated as an irreversible process, appears to be tightly coupled to dephosphorylation by myosin B, and the rates of both processes are approximately halved at 500 atm. Under identical ionic conditions, dephosphorylation by myosin A is unaffected by 500 atm. The "observed" ΔV calculated from $-RT\ (\partial \ln K/\partial p)_T$ is 34–50 ml. A possible "site" of action of the pressure is formation of $(Mg\text{-}ATP)^-$, but the ΔV of this process was measured and found to be too small (21.9 ml/mole). The other likely site is formation of the activated complex, which may involve release of electrostricted water.

Because conventional dilatometry proved unsuitable for studying some of the volume increases which accompany "superprecipitation" of myosin B by ATP[1] (*cf.* Noguchi *et al.*, 1964) we have turned, in the present work, to studying the conjugate phenomena, *i.e.*, the inhibition of certain processes by elevated pressures.

Experimental Section

Sigma Tris and ATP (usually disodium, in a few instances diethanolamine) and reagent grade inorganic chemicals were used throughout the investigation. Cenco high vacuum oil closed the circuit between the pressure generator and the solution under study (*vide infra*). At 0–5° throughout, myosin B was prepared by extracting for 24 hr a fine mince of rabbit back musles, purifying three times (in each purification, at pH 6.8, the supernatant from ionic strength 0.25 and the pellet from ionic strength 0.60 were rejected), and finally centrifuging for 1 hr in the No. 30 rotor of a Spinco Model L-2 ultracentrifuge. When used, myosin A was prepared by a modified Szent-Györgyi procedure. The manner of preparing myosin B solutions for study proved to be important. In all superprecipitation experiments a myosin B concentration of 0.01% was used. This concentration was achieved by first diluting to 0.1% with 0.6 M KCl, and then to 0.01% with 0.0444 M Tris, pH 7.0; the final ionic strength was therefore 0.1 M. The solution was kept at 5° for 12–24 hr before using, as solutions not so kept were far more erratic in response to ATP. Studies using myosin A were made under exactly the same solution conditions as for myosin B. In experiments where a possible magnesium effect was under consideration, attempts were made to eliminate as much trace magnesium from the system as possible; KCl and Tris were recrystallized twice from EDTA, and ATP was used in the diethanolamine form.

ATPase activities were calculated from zero-order plots of orthophosphate concentration *vs.* time. The former was measured by a variation of the Fiske–Subbarow (1925) method which employs 20% perchloric acid as precipitant and $SnCl_2$ as reducing agent; the times which aliquots spend in the various reagents were carefully controlled.

Reactions, whether ATPase activities or "superprecipitations," were carried out in a very heavy walled, pressure-tested, stainless steel container of *ca.* 7-ml capacity, equipped with quartz windows *ca.* 25 mm apart (Figure 1a). The inner surface was coated with "Desicote." The aqueous phase, 7 ml of myosin B, was overlaid with several ml of oil to connect with the pressure system. By using a nylon gasket, the heavy steel cap, fitted with connectors for steel high-pressure tubing, could be screwed on the container so tightly as to defy leakage throughout the pressure range used (up to 7500 psi). The entire "bomb"[2] was arranged so that the optical path of a Zeiss PMQ II spectrophotometer passed normal to the quartz windows and through the aqueous phase in the container. The output of the spectrophotometer was transformed by a Zeiss log converter to log output, and the optical density of the bomb contents, at 550 mμ, was linearly displayed on a Minneapolis-Honeywell recorder ($\tau_{1/2}$ = 0.5 sec) with an accuracy of *ca.* 0.005 OD units. Superprecipitation was followed as OD(t) (Ebashi, 1961). The contents of the bomb was connected *via* oil to an American Instruments Co. hand pressure generator and gauge

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† Senior Trainee, U. S. Public Health Service Training Grant (HTS 5251).

‡ Career Investigator, American Heart Association.

[1] Abbreviations used: ATP, adenosine triphosphate (disodium salt or diethanolamine salt); ATPase, adenosine triphosphatase.

[2] This pressure bomb was manufactured by American Instruments Co., after a design by Dr. Richard Podolsky.

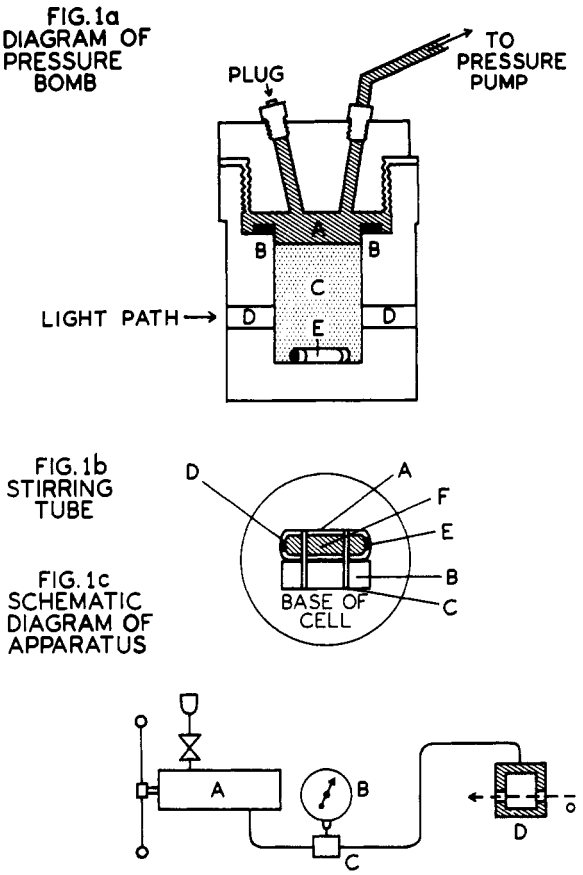

FIGURE 1: Diagrams of apparatus used. (a) Schematic diagram of the pressure bomb. A, high vacuum oil; B, nylon gasket; C, 0.01% myosin B; D, quartz windows; E, stirring arrangement containing 50 μl of ATP. (b) Diagram of the stirring-mixing device. A, glass tubing; B, magnetic bar; C, nylon string tie; D, silicone grease layer on end surfaces of tubing; E, oil drop seal; F, 50 μl of ATP. (c) General arrangement of the apparatus. A, hand-operated pressure generator; B, 0–~15,000 psi calibrated pressure gauge; C, tee; D, pressure bomb; O, optical path of spectrophotometer.

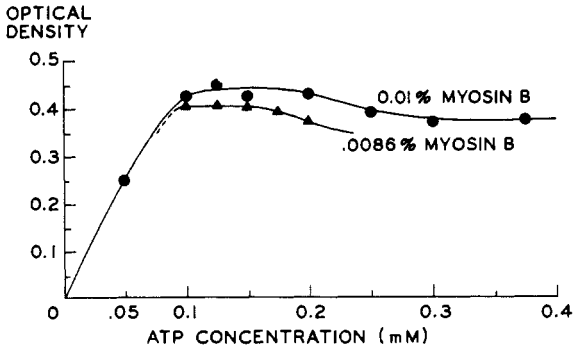

FIGURE 2: Final extent of superprecipitation of myosin B, $\Delta(\infty)$, as a function of ATP concentration. Myosin B, 0.01%; 0.06 M KCl; 0.04 M Tris; *p*H 7.0.

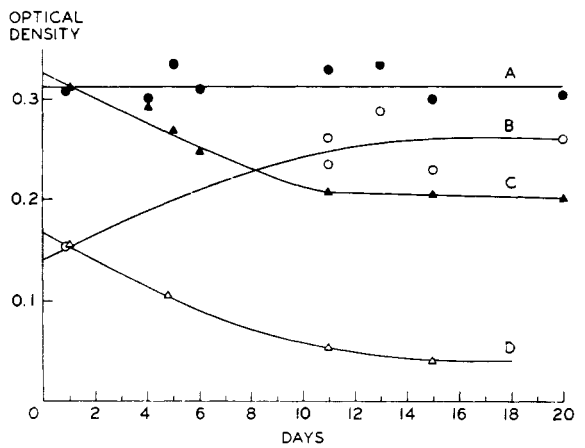

FIGURE 3: Final extent of superprecipitation, $\Delta(\infty)$, of myosin B as a function of age of preparation. A and B, immediate response of a fresh dilution of protein to saturation and half-saturation [ATP]. C and D, progressive response of an initial dilution of protein to saturation and half-saturation [ATP]. Myosin B, 0.01%; 0.06 M KCl; 0.04 M Tris, pH 7.0, $[ATP]_{sat}$ = 0.25 mM, $[ATP]_{1/2sat}$ = 0.05 mM.

(Figure 1b). Some 7500 psi could be applied in about 15 sec, and atmospheric pressure could be restored in 1 sec. A simple method of mixing protein and ATP solution in the bomb, with or without pressure, was devised. Since the walls of the bomb were diamagnetic it was possible to rotate a small magnetic stirring bar at constant speed (constant Variac setting calibrated with a stroboscope), at the bottom of the bomb, safely out of the light path. To such a bar was attached a piece of open-ended glass tubing about as long as the bar (Figure 1c). Both ends of the tubing were thinly coated with silicone grease. Fifty μl of ATP solution was delivered into the tube with a micropipet and held there by capillarity. Each end was then sealed by a small drop of oil, and the entire assembly was placed in the bottom of the cell. After slowly layering the aqueous phase and the oil phase and sealing, the bomb was placed in the light path, pressure lines were attached, and, when required, the desired pressure was imposed. Immediately below the bomb was a powerful magnetic stirrer. When the stirrer was activated, the bar in the bomb rotated and the contents of the tube was flung out by centrifugal force and was immediately mixed with the 7 ml of myosin B (aqueous phase).

Mixing was maintained for 15 sec, 3 at high speed and 12 at a slower speed; this span of time corresponds to about 2% of the time required for the superprecipitation phenomenon. Exact control was important since pilot experiments showed stirring to affect the nature of the precipitate.

Experiments to measure the volume change accompanying the reaction ATP + Mg → MgATP were carried out at 20° in a conventional dilatometer with

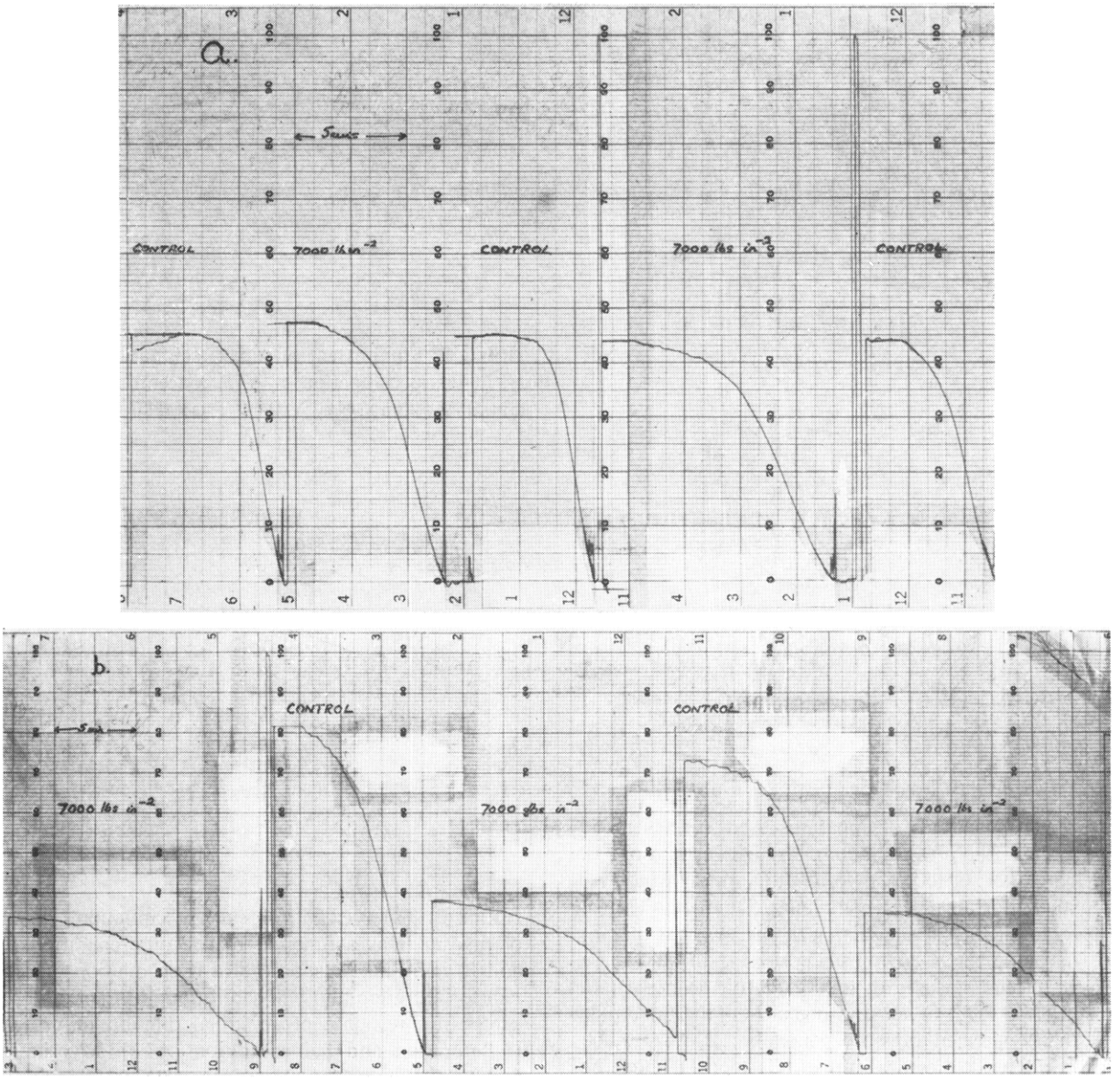

FIGURE 4: The effect of pressure on extent of superprecipitation. (a) Effect of $\Delta(t)$ of myosin B with saturation ATP. Pressure, 7000 psi; myosin B, 0.01%, ATP, 0.25 mM, 0.06 M KCl, 0.04 M Tris, pH 7.0. (b) Effect on $\Delta(t)$ of myosin B with undersaturation ATP. Conditions as for (a) but ATP 0.05 mM. Full scale reading for both traces, 1.0 OD units.

kerosene in the capillary. The Tris–KCl solvent used was as described, and the measured deviations from pH 7.00 after mixing were less than 0.1 unit. Displacements of the kerosene meniscus were *ca.* +23 mm (maximum reading error was 0.5 mm or less). The volume changes reported under Results were all corrected by the changes which accompany the individual dilutions of the reactants.

Results

$\Delta(\infty)$, the final extent of the change in OD_{550} which results when ATP is added to a suspension of myosin B, is a function of [ATP] (Figure 2). In this illustration $\Delta_{max}(\infty)$ is obtained above 10^{-4} M ATP, and the function defines $[ATP]_{1/2} = 5 \times 10^{-5}$ M, an [ATP] which elicits $\Delta(\infty) = \Delta_{max}(\infty)/2$. In this study we often refer to [ATP] values which are "saturation" [elicit $\Delta_{max}(\infty)$] and "undersaturation" [elicit $\Delta(\infty) < \Delta_{max}(\infty)$, *e.g.*, $[ATP]_{1/2}$]. It was observed incidentally that the quantities depend upon age of preparation and schedule of dilution, and knowledge of this dependence is essential for applying Ebashi's (1961) method semiquantitatively. In Figure 3 curve A shows $\Delta_{max}(\infty)$ measured each day immediately after the preparation of a fresh dilution from the stock, as described above. Over 20 days, the response, though somewhat erratic, was fairly reproducible. On the first day of this preparation $[ATP]_{1/2}$

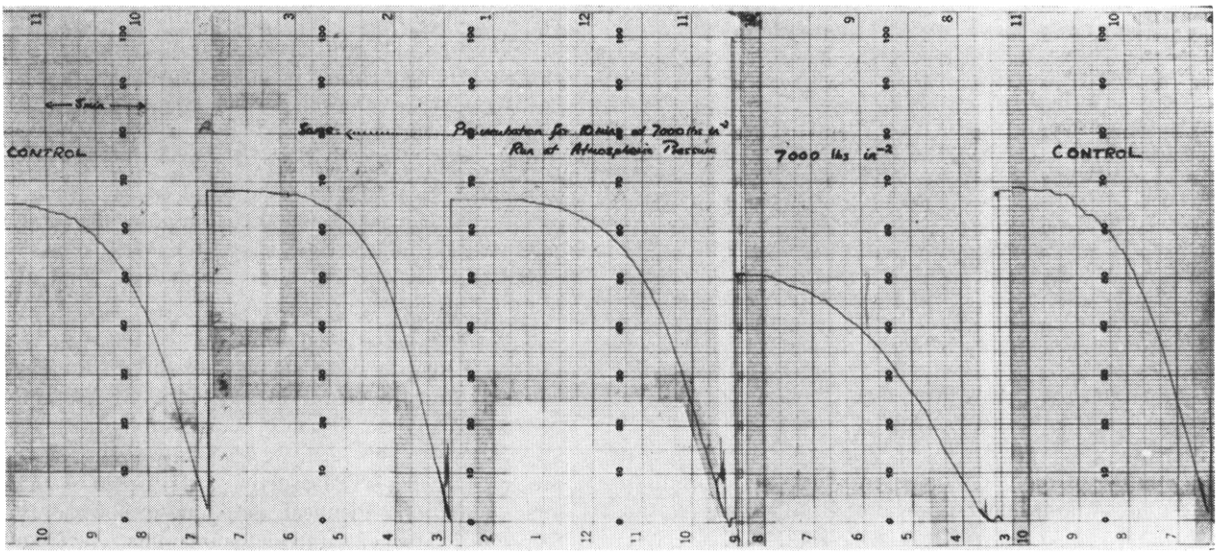

FIGURE 5: Effect of preincubation at high pressure on the subsequent ATP response of myosin B. Protein incubated for 10 min at 7000 psi prior to mixing with ATP at atmospheric pressure. ATP, 0.05 mM; 0.06 M KCl; 0.04 M Tris; pH 7.0; full scale 0.5 OD units.

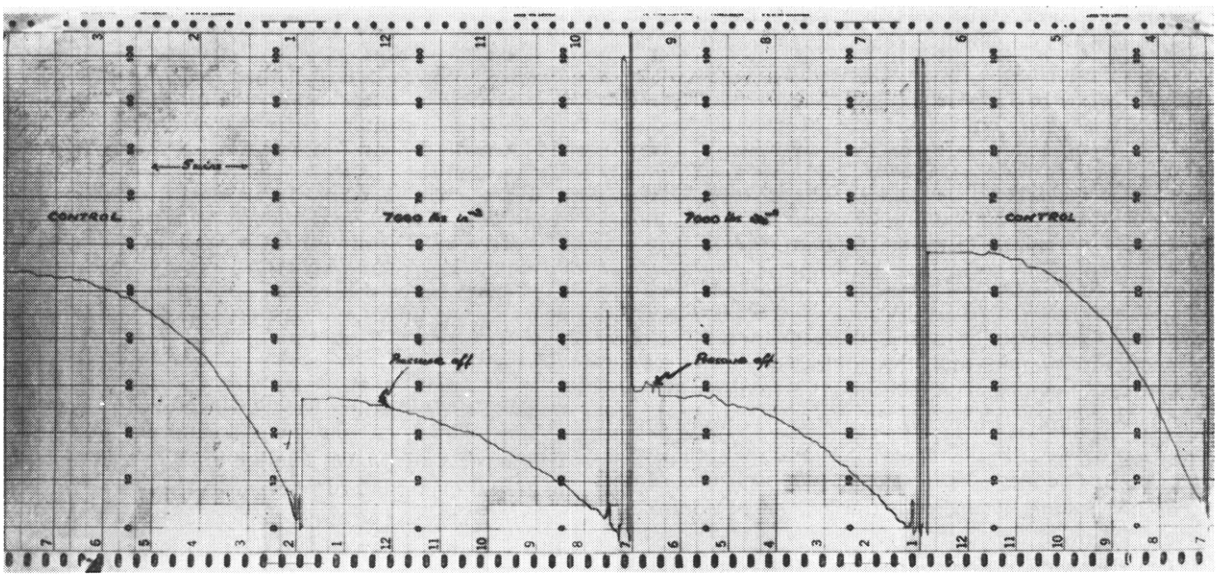

FIGURE 6: Effect on $\Delta(t)$ of pressure changes made during late stages in the superprecipitation of myosin B. Pressure 7000 psi returned to atmospheric at point indicated. Myosin B, 0.01%; ATP, 0.05 mM; 0.06 M KCl; 0.04 M Tris, pH 7.0; full scale 0.5 OD units.

was determined, from a curve such as is shown in Figure 2, and it elicited (by arrangement) $\Delta_{max}(\infty)/2$, as shown by the earliest point in curve B. It was found that, as the preparation aged, $[ATP]_{1/2}$ determined on the first day now elicited $\Delta(\infty) > \Delta(\infty)_{1/2}$. When using $[ATP]_{1/2}$ the immediate response was very erratic, as shown by curve B. The dilutions used to obtain the earliest points on curves A and B were stored, and curves C and D show the $\Delta(\infty)$ values obtained on successive days using $[ATP]_{max}$ and $[ATP]_{1/2}$, respectively. Clearly both $\Delta(\infty)$ values decayed with age. Another observation, not indicated on this figure but to be expected, was that as the stock protein became very old the $\Delta_{max}(\infty)$ of curves A and B fell and eventually $[ATP]_{1/2}$ (as determined the first day) elicited $\Delta(\infty) < \Delta(\infty)_{1/2}$. When the response of the protein became poor it was discarded and replaced by a new preparation.

The solutions used in the studies correspond to points on curves A and B, after the dilutions had

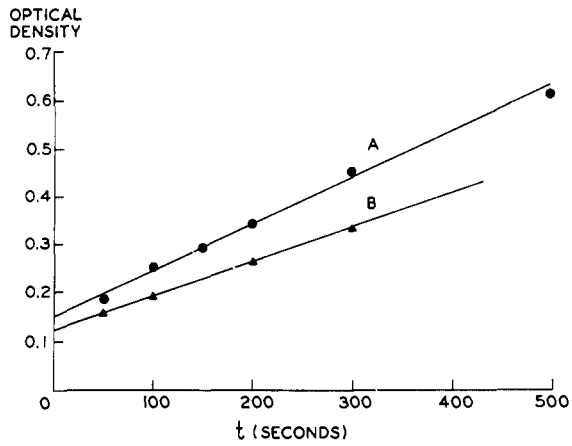

FIGURE 7: Graph of orthophosphate concentration *vs.* time. A, atmospheric pressure; B, 6500 psi; myosin B, 0.01%; 0.50 mM ATP; 0.06 M KCl; 0.04 M Tris; pH 7.0. A = 198 μM P_i g^{-1} sec^{-1}; B = 1.45 μM P_i g^{-1} sec^{-1}.

equilibrated for *ca.* 24 hr to ensure more stable responses. These foregoing phenomena explain, for example, why in different experiments the ratios of the $\Delta(\infty)$ values elicited by $[ATP]_{max}$ and $[ATP]_{1/2}$ may vary from one-half to three-fourths.

Figure 4a shows $\Delta(t)$ under atmospheric pressure and under 7000 psi. With "saturation" [ATP], high pressure reduces the initial rate $[\dot{\Delta}(0)]$ but not the final extent $[\Delta(\infty)]$ of superprecipitation, but with "undersaturation" [ATP], high pressure reduces both (Figure 4b).

The phenomena of Figure 4 are not due to an irreversible pressure destruction of the protein against which ATP may offer protection, because pressure applied for 10 min and then released in no way affects a subsequent superprecipitation under atmospheric conditions (Figure 5). If $\Delta(t)$ in "undersaturation" conditions at 7000 psi starts to level off, and the pressure is suddenly returned to atmospheric, $\Delta(t)$ does not resume its increase toward, *e.g.*, "control" levels (Figure 6).

The following observations show that this failure to resume an increase is due to the fact that by that time in the experiment the ATP is exhausted. First, Figure 7 shows the concentration of liberated orthophosphate as a function of time, *i.e.*, $[P_i](t)$ using "saturation" [ATP], myosin B, and two pressures (atmospheric and 6500 psi). Calculations made with the $[\dot{P}_i]$ values inferred from these graphs show that if $[\dot{P}_i](0)$ under pressure were maintained for a time long enough so that $\Delta(t)$ (high pressure) approximated $\Delta(\infty)$ (atmospheric pressure), the ATP originally present would have been hydrolyzed several times over (in practice, of course, $[\dot{P}_i(t)$ would fall toward the end of the run due to substrate depletion). Second, direct measurements after leveling off do show the ATP to be exhausted, under both atmospheric and high pressure conditions. These experiments show that under high pressure there is less superprecipitation accomplished per ATP molecule hydrolyzed than in the atmospheric case. In passing it should be noted that at high pressure $[\dot{P}_i]$ is 1/1.75 the atmospheric rate, and $\dot{\Delta}$ is one-half to one-third of the atmospheric rate.

Like $\dot{P}_i(t)$, $\dot{\Delta}(t)$ responds quickly to changes in pressure. Figure 8a shows the effect on $\dot{\Delta}(t)$ when the pressure is changed from 7000 psi to atmospheric pressure early in the experiment, and Figure 8b shows the result when the inverse occurs. Both show immediate responses to the changes in pressure.

It is often said that a superprecipitating (or "contracting") myosin B system is a mixture of an Mg^{2+}-activated "actomyosin ATPase" and a K^+-activated myosin A ATPase. Because below we consider some implications of this idea, we also studied the effect of pressure on the ATPase activity of myosin A under ionic conditions identical with those above, using recrystallized reagents and diethanolamine ATP (Figure 9). We confirmed the slowness of myosin A ATPase relative to myosin B ATPase (0.7 μmole g^{-1} sec^{-1}

TABLE I: Volume Change of Mg-ATP Binding.[a]

n_{ATP} (lobe 1)	n_{MgCl_2} (lobe 2)	n_{ATP_2} (calcd)	ΔV (obsd)	ΔV (Calcd)
6.0	1.5	1.31	32.3	24.6
6.0	3.0	2.50	57.5	23.0
6.0	6.0	3.83	90.9	23.7
6.0	12.0	5.43	108.0	19.9
6.0	24.0	5.80	119.0	20.5
1.5	6.0	1.31	19.1	14.6
3.0	6.0	2.50	39.3	15.7
6.0	6.0	3.83	90.9	23.7
12.0	6.0	5.43	137.0	25.2

[a] All experiments were conducted in 0.06 M KCl, 0.04 M Tris, pH 7.0, t = 20°. ATP solution in lobe 1 has a volume of 3 ml; $MgCl_2$ solution in lobe 2 has a volume of 15 ml. Quantities of reactants (n) are given in moles $\times 10^5$; observed volume changes are given in ml $\times 10^5$. Since it is assumed that the final ionization of ATP at this ionic strength has a pK of 6.5, $[ATPH^{3-}]$ is 24% of $[ATPH^{3-}] + [ATP^{4-}]$. Since it is assumed that the equilibrium constant of $ATP^{4-} + Mg^{2+} \rightarrow (ATPMg)^{2-}$ is 3×10^4 l. M^{-1}, and that Mg^{2+} has negligible affinity for $ATPH^{3-}$, the number of moles of ATP-Mg^{2-} formed is calculable from the mass law. In the calculated column ΔV is the observed volume change (ml) divided by the calculated number of moles of $ATPMg^{2-}$ formed. This quantity equals $\Delta V_M - 0.24(\Delta V_H - \Delta V_B) \cong \Delta V_M - 0.24\Delta V_H$, where ΔV_M is the true molar volume change of ATP^{4-}, Mg^{2+} complexing, and ΔV_B is the molar volume change of Tris + $H^+ \rightarrow TrisH^+$ (ΔV_B is probably negligible since no charge change is involved). In inhibiting complexation, what the hydrostatic pressure opposes is the *net* change ΔV, whose average value is 21.9 ml/mole.

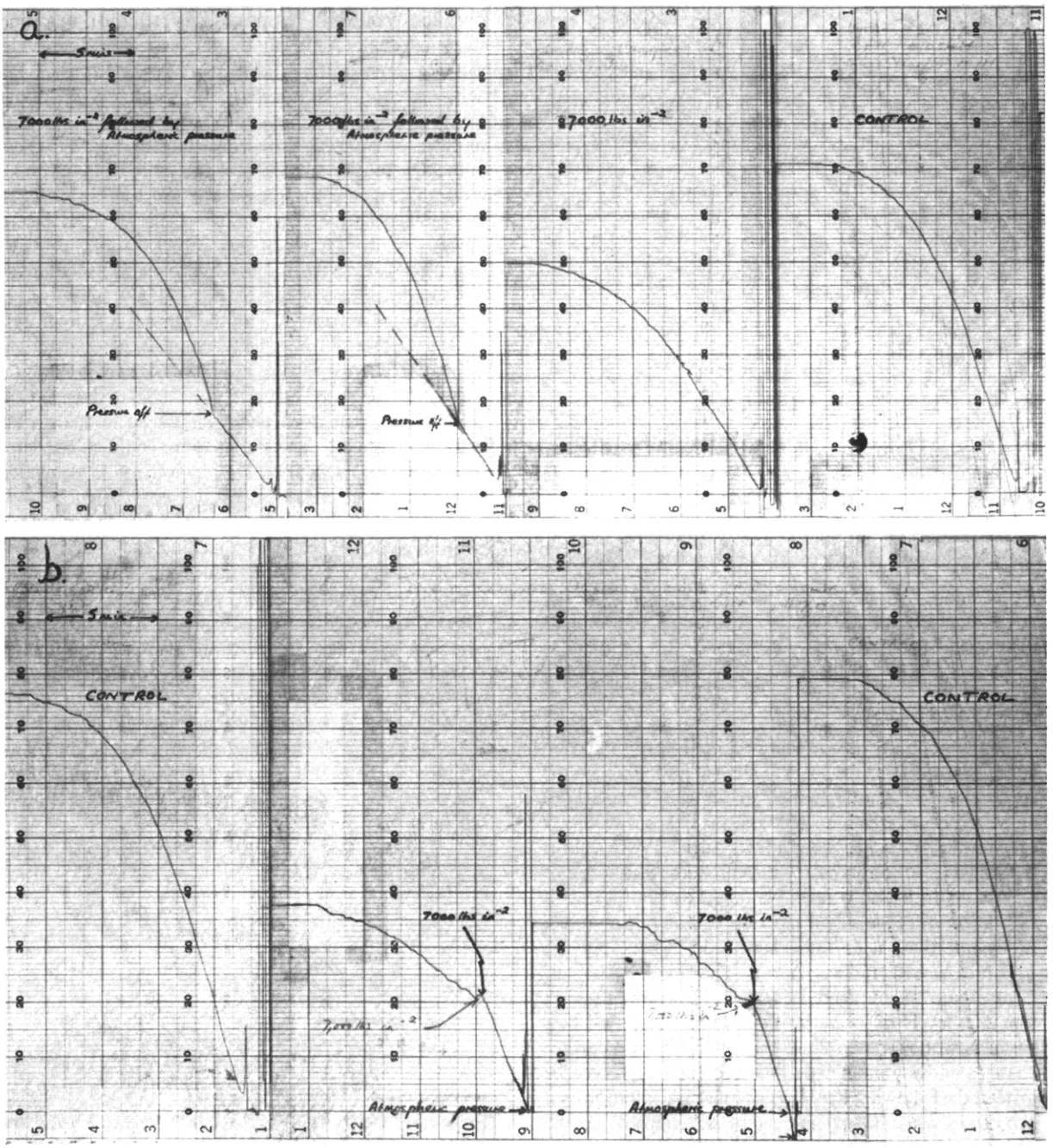

FIGURE 8: Effect of $\Delta(t)$ of pressure changes made during early stages in the superprecipitation of myosin B. (a) From 7000 psi to atmospheric pressure. (b) From atmospheric pressure to 7000 psi. Myosin B, 0.01%; ATP, 0.05 mM; 0.06 M KCl; 0.04 M Tris; pH 7.0.

compared to 1.98 μmoles g^{-1} sec^{-1}; considering the heterogeneity and age of the myosin B preparation this is probably the lower limit to the difference in rates). Contrary to what we expected, however, on the basis of Beardell's work (1954), we found no pressure effect on the "saturation" $[\dot{P}_i](t)$ of myosin A.

To investigate the possibility that trace Mg^{2+} might also be exerting some influence in the experiments with myosin B under pressure, we repeated several pressure runs using the recrystallized reagent and the diethanolamine ATP, and found the results to be the same as before under atmospheric and under high pressure conditions. "Mg^{2+} activation" could mean that for the enzyme in question (actomyosin) $(Mg\text{-}ATP)^-$, not ATP^{3-}, is the substrate. In this case complexation of Mg^{2+} by ATP^{3-} could be a pressure-sensitive process since the change in number of charges is thereby -4, and much water should be "unfrozen."

1963

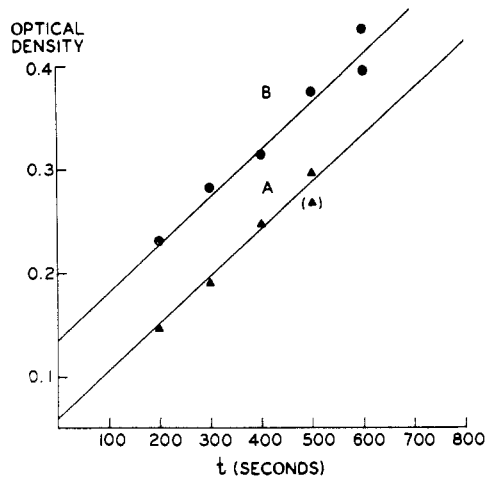

FIGURE 9: ATP hydrolysis of myosin A under pressure. A, atmospheric pressure; B, 7000 psi; myosin A, 0.013%; 0.06 M KCl; 0.05 M Tris; pH 7.0.

Accordingly we made a few measurements for the net ΔV of complexation and found it to be 21.9 ml mole^{-1} (Table I).

Discussion

Curves such as that of Figure 2 could arise from an equilibrium between myosin B elements to which some species of ATP is attached (high turbidity suspension) and elements to which no ATP is attached (low turbidity suspension). Were this so, and were the ATP–myosin B binding to result in a volume increase (*e.g.*, as a result of charge neutralization), then we might expect that, with saturation ATP, applying pressure would not affect $\Delta(\infty)$ but that, with undersaturation ATP, applying pressure would reduce $\Delta(\infty)$. The experiments of Figure 4a,b encouraged this view. However, the fact that $\Delta(t)$ levels off because ATP is exhausted and that $\Delta(\infty)$ is not predetermined but alterable by pressure changes midway in an experiment forced us to consider instead the following hypothesis: superprecipitation is a process in which a unit increment is "coupled" to the hydrolysis of an ATP molecule; because superprecipitation involves a transformation of elements of myosin B, it is a self-limiting process; thus superprecipitation can stop either because all the ATP has been hydrolyzed (undersaturation case) or because all the protein has been transformed (saturation case). Besides the reaction pathway "coupled" to structural change there is also a pathway along which ATP is hydrolyzed more slowly, and without causing a detectable structural change. At atmospheric pressure the flux of ATP over these two pathways is fixed by a set of physical constants. When high pressure is applied, the fast, coupled pathway is constricted and the slow, pressure-insensitive pathway is thereby favored. In these terms our observations are all understandable. With saturation ATP, pressure reduces the rate at which molecules are hydrolyzed over the "coupled" pathway ($\dot{\Delta}$ is reduced), but, since there is ample ATP, all the superprecipitation which can get done is done, and $\Delta(\infty)$ is the same as that achieved under atmospheric pressure. With undersaturation ATP, high pressure is able to reduce the total number of molecules hydrolyzed over the "coupled" pathway, so not only is Δ reduced, but also $\Delta(\infty)$ is reduced. Because the pressure is exerting an effect on the rates over the two pathways, its effect is instantaneous at any stage of superprecipitation, provided only that ATP is still being hydrolyzed. Speculatively we could assume that one pathway is slow hydrolysis by K^+-activated myosin A, and the other is fast hydrolysis by Mg^{2+}-activated myosin B, coupled, of course, with structural change. There seem to be two possibilities as to why pressure constricts the fast pathway. Conceivably the structural change induced in the protein could be reversible, and the pressure could be accelerating the reverse reaction. This hypothesis is equivalent to assuming a pressure-sensitive *equilibrium* between normal and contracted systems. The hypothesis is contradicted by the fact that just as much superprecipitation is achieved under high as under atmospheric pressure (it is only that the former is slower). Therefore, we can limit ourselves to discussing *irreversible* processes. Conceivably pressure could inhibit ATP dephosphorylation *per se*. Because different cations are involved, this hypothesis cannot be rigorously excluded, but it is certainly discouraged by the fact that the myosin A catalyzed dephosphorylation is not inhibited by pressure. The pressure effect would therefore seem to reside in the processes of the following scheme:

$$\begin{array}{l} \mathrm{HATP^{3-}} \\ {\scriptstyle K_h}\uparrow\downarrow \qquad\qquad\qquad\qquad\qquad\qquad \bar{K} \equiv k_1/(k_{-1}+k_2) \\ \mathrm{H^+} \qquad\qquad\qquad\qquad\quad \text{myosin B} \\ + \qquad\qquad\qquad\qquad\quad + \\ \mathrm{ATP^{4-} + Mg^{2+}} \overset{K_m}{\rightleftarrows} \mathrm{MgATP^{2-}} \\ \qquad\qquad\qquad\qquad\quad {\scriptstyle k_1}\uparrow\downarrow{\scriptstyle k_{-1}} \\ \qquad\qquad\qquad\qquad\quad \text{myosin B, } \mathrm{MgATP^{2-}} \overset{k_2}{\longrightarrow} \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad \text{products} \end{array}$$

Defining $\kappa \equiv (1 + K_h[H^+] + K_m[\text{total ATP}])/K_m[\text{total ATP}]$, one may show that under the conditions of our experiments $\dot{\Delta}(0)$ is proportional to $k_2\bar{K}/\kappa$. With "saturation" [total ATP], κ tends to very nearly unity and pressure insensitivity; with "undersaturation" [total ATP], κ remains primarily a function of pressure-sensitive K_m, and in the limit $\dot{\Delta}(0)$ becomes approximately proportional to k_2KK_m. It has been established in this work that pressure does have an effect on $\dot{\Delta}(0)$ using "saturation" [total ATP] (Figure 4a), and that using "undersaturation" [total ATP] the observed pressure effect is too large (apparent $\Delta V \sim$ 34–50 ml mole^{-1}) to be accounted for by an effect on K_m alone (ΔV = 21.9 ml mole^{-1}). Therefore, we must conclude that either or both (a) the combination of myosin B with substrate, (b) the activation of the enzyme–substrate compound, involve a sizable volume increase

Such a volume increase perhaps results from release of water molecules in the course of charge neutralization.

Acknowledgment

Dr. Richard Podolsky generously loaned us the pressure bomb for an extended period, thereby making possible these studies. As usual we are indebted to several colleagues. Miss Linda Stowring helped with several experiments and the interpretation of this work was greatly aided and stimulated by the suggestions of Dr. Jean Lecocq, Dr. Giuseppe Inesi, and Visiting Professor Yuji Tonomura.

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Kinetics of Iodination. III. Iodination of *N*-Acetyl-L-tyrosine and *N*-Acetyl-3-iodo-L-tyrosine Studied in a pH-Stat System*

W. E. Mayberry,† J. E. Rall, M. Berman, and D. Bertoli

ABSTRACT: The kinetics of the iodination of *N*-acetyl-L-tyrosine and *N*-acetyl-3-iodo-L-tyrosine has been studied in a pH-Stat system. A reaction model was proposed to represent the steady-state kinetics of the reactions. The model was based upon the concept of molecular iodine and the phenolate anion of each tyrosine derivative proceeding to a quinoid intermediate plus iodide. The rate-limiting step is then proton removal from the quinoid intermediate to form the iodinated phenolate anion. Using this model and a computer program, the rate k_6 representing the sum of the two steps (see equation 6) has been solved by a high-speed digital computer. The k_6 for each reaction is approximately inversely proportional to iodide concentration.

The values $k_6[I^-]$ are 1400–1600 sec^{-1} and 43–68 sec^{-1}, respectively, in the iodination of *N*-acetyl-L-tyrosine and *N*-acetyl-3-iodo-L-tyrosine. The reactions have previously been shown to be general base catalyzed, and the present values confirm the rate constants for the water-catalyzed reactions. The fit of the data to the model solution and of theoretical considerations to the results adds credence to the proposed mechanism of iodination.

The kinetics of iodination of *N*-acetyl-L-tyrosine and *N*-acetyl-3-iodo-L-tyrosine has been studied previously by following triiodide concentration spectrophotometrically (Mayberry *et al.*, 1964). Hydrogen ion concentration was maintained constant by the presence of buffers. In a subsequent report, evidence of general base catalysis for the two reactions was presented (Mayberry and Bertoli, 1965). In the present study, we wished to study the iodination reactions in the absence of buffer systems and to corroborate our previously determined catalytic constants for water as a base. Hydrogen ion concentration has been maintained by means of a pH-Stat system which has allowed monitoring of the iodination reaction by measuring the release of protons from the aromatic ring. The system is complicated by proton terms other than that of the release of the proton from the ring with iodination. A reaction model was designed, and a solution that fit the experimental observations was derived with the aid of a digital computer program.

Experimental

Materials. *N*-Acetyl-L-tyrosine, *N*-acetyl-3-iodo-L-tyrosine, and *N*-acetyl-3,5-diiodo-L-tyrosine[1] were the same preparations as those previously reported (Mayberry *et al.*, 1964). Water redistilled in glass was used in all experiments. Resublimed iodine and reagent grade potassium iodide were used. Sodium hydroxide solu-

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[1] The abbreviations *N*-acTY, *N*-acMIT, and *N*-acDIT will be used, respectively, for *N*-acetyl-L-tyrosine, *N*-acetyl-3-iodo-L-tyrosine and *N*-acetyl-3,5-diiodo-L-tyrosine.