nor RNA virus is capable of replication except in an environment that can supply the missing components. Such a requirement could well explain the apparent formation of infective RNA within the nucleus of the ascites-tumour cell and the transfer of this to the cytoplasm before synthesis of complete virus can begin.

SUMMARY

1. The effect of infection with encephalomyocarditis virus on the rate of incorporation of ^{14}C-labelled precursors into the protein and ribonucleic acid of the subcellular components of Krebs II ascites-tumour cells has been investigated.

2. The nucleus, which was the major site of ribonucleic acid synthesis in the cell, contained negligible amounts of virus. Infection caused a marked progressive inhibition of orotic acid incorporation into nuclear ribonucleic acid, and also some net loss of ribonucleic acid from the nucleus. It is suggested that this disruption of nuclear ribonucleic acid metabolism is related to the cell-killing properties of the virus.

3. Most of the virus sedimented with the mitochondrial fraction. The amount of mitochondrial ribonucleic acid increased progressively during infection by an amount approximately equivalent to that lost from the nucleus. Incorporation of orotic acid into mitochondrial ribonucleic acid was slightly inhibited for the first 3 hr. after infection; thereafter it was stimulated, reaching 320 % of the control at 6 hr.

4. Less virus appeared in the microsomal fraction. The pattern of incorporation into microsomal ribonucleic acid was similar to that of the mitochondria, but less pronounced. In both fractions, the increase in incorporation rate was apparently related to the amount of virus present, but estimates showed that only 5–8 % of this newly synthesized ribonucleic acid could be ascribed to viral ribonucleic acid formation.

5. In all cytoplasmic fractions, infection caused an initial slight stimulation of valine incorporation into protein, which was most marked in the cell-sap fraction. This was followed by a period of moderate inhibition, until appreciable amounts of virus had accumulated intracellularly, when incorporation into mitochondrial protein was again elevated.

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A Study of the Kinetics of the Fibrillar Adenosine Triphosphatase of Rabbit Skeletal Muscle

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One of the most puzzling features of the kinetics of the adenosine-triphosphatase activity of actomyosin and of the myofibrils in which it is contained is the so-called explosive phase of hydrolysis which occurs immediately after addition of substrate and which is followed under certain special conditions by a 'linear' phase of lower, but constant, velocity. These features were originally studied by Weber & Hasselbach (1954) in myofibrillar preparations at low ionic strengths ($< 0{\cdot}15$), but later Tonomura & Kitagawa (1957) showed that they were also characteristic of the hydrolysis of adenosine triphosphate by myosin B in the presence of Ca^{2+} ions, at high ionic strength ($> 0{\cdot}5$). Tonomura & Kitagawa (1960) have extended their observations on myosin B to include

the effect of other bivalent ions, such as Mg^{2+}, Mn^{2+} and Sn^{2+}, and of chelating agents such as ethylenediaminetetra-acetate, and have shown that both prolonged dialysis and treatment of myosin B with very low concentrations of ethylenediaminetetra-acetate ($< 10\ \mu$M) abolish the explosive phase almost completely, and that it can be restored by addition of small amounts of Mg^{2+} but not of Ca^{2+} ions.

In some respects the present study of the myofibrillar adenosine triphosphatase at lower ionic strengths (0·04–0·25) confirms the findings of Tonomura & Kitagawa, particularly the observation that the explosive phase is more prominent in the presence of Mg^{2+} ions than in their absence, even though Ca^{2+} ions may also be present in large amounts. Nevertheless, the extent and duration of this phase, with fibrils at low ionic strength, is further complicated under most conditions by pronounced inhibition by the products, so that a true stationary phase very rarely follows the so-called explosive phase, but rather a phase of steadily falling velocity. It so happens that the special conditions under which the reaction has usually been studied, that is at an ionic strength of $\sim 0{\cdot}15$ and at temperatures between 0° and 20° with magnesium as activator (cf. Weber & Hasselbach, 1954), are precisely those in which the phase succeeding the explosion is truly stationary and unaffected by addition of the products.

The question of product inhibition is one that seems to have been overlooked in most studies, although Kalckar (1944) and Green & Mommaerts (1954), using Ca^{2+} ions as activator at pH 6·4, have clearly demonstrated the inhibition of myosin adenosine triphosphatase by adenosine diphosphate. The myofibrillar enzyme shows very similar inhibition by the products under a wide range of conditions, e.g. with Ca^{2+} ions as activator, at all ionic strengths between 0·04 and 0·25 and at all temperatures from 0° to 35°, and with Mg^{2+} ions as activator, at all ionic strengths up to 0·25 at 35°, but only at the lowest ionic strength at 0° and 18°. From this it is clear that many false deductions can be made from progress curves of hydrolysis unless the effect of the products is taken into consideration, particularly in relation to the explosive phase. For example, the 'exponential' time curve, characteristic of product inhibition, can lead to the belief that a marked and extensive explosive phase is present, when in fact most of the effect is due to the gradual accumulation of products. Even in the presence of Mg^{2+} ions, which favour the explosive initial phase, it is much less extensive than Weber & Hasselbach (1954) originally thought, and with Ca^{2+} ions as the sole activator it either does not exist or is of such short duration that it cannot be distinguished from small, but inevitable, errors of estimation in the early stages.

METHODS

Abbreviation. P_i, inorganic orthophosphate.

Reagents. All reagents were of AnalaR grade (British Drug Houses Ltd. or L. Light and Co. Ltd.), except ATP and ADP, which were of the highest grade from Sigma Chemical Co. All reagents were treated by passage through a bed of the ion-exchange resin Dowex 50×8 (H^+ form), to remove traces of heavy metals, and, with the exception of $CaCl_2$, the reagents contained less than 1 p.p.m. of Ca. The water used was passed through a mixed-resin bed (Elgastat), and had an electrical resistance $> 2 \times 10^6\ \Omega$/cm³.

Preparation of glycerol-treated fibrils. Longissimus dorsi and psoas muscles (100 g.) from a freshly killed rabbit were homogenized in a small, high-speed homogenizer in lots of 2 g. in 7 ml. of ice-cold buffer (20 mM-imidazole, 80 mM-KCl and 5 mM-potassium oxalate, pH 7·2). Finally the volume of pooled homogenate was made up to 1 l. and stored at 0° overnight. Next day it was filtered through washed butter-muslin to remove fibre fragments and debris, and centrifuged at −1° at 2300*g* for 30 min. The supernatant was discarded and the fibrils were washed three times, by centrifuging and decantation at 0°, in a buffer consisting of 20 mM-imidazole, 80 mM-KCl, 5 mM-potassium oxalate and 4·1 M-glycerol. Finally, the fibrils were suspended in a volume of 1 l. of the glycerol buffer and stored at −9°. Prepared in this way, the fibrils are of uniform size and free of fibres and fragments of connective tissue. The method was originally due to Webster (1953). For use in enzymic studies portions of the fibril suspension were removed, and washed four times at 0° with the buffer solution appropriate to the experiment.

Determination of nitrogen content of fibrils. A portion (20 ml.) of fibril suspension (≡ 2 g. of muscle) was washed free of glycerol with 100 mM-KCl (three washes) and then made up to a final volume of 20 ml. in the latter solution. Portions (1 ml.) were digested, for 1 hr. after clearing, in 2 ml. of conc. H_2SO_4 + 80 mg. of a mixture of Se (1 g.) and K_2SO_4 (32·5 g.). The digest was made up to 100 ml. with water, and 1 ml. portions were removed for estimation of N content by direct nesslerization (1 ml. portion + 15 ml. of water + 2 ml. of Nessler's reagent + water to 25 ml.). The colour was measured in 1 cm. cells in a Unicam spectrophotometer (SP. 600) at 420 mμ, a digested reagent blank being used. The scatter of N values on five portions of the same fibril suspension was within 0·5% of the average value. The average N contents of the five glycerol-treated preparations used in this study were respectively 19·7, 19·3, 18·4, 18·4 and 18·9 mg. of N/g. of original muscle. The value given by Helander (1957) for myofibrillar protein N, free of stroma and connective tissue, is approx. 18·0 mg. of N/g. of fresh muscle.

Composition of buffer and substrate solutions, and their effect on ionic strength. The buffer used throughout was 40 mM-imidazole (p*K* 7·2) at pH 7·20 (final), which contributes 0·02 to the ionic strength (*I*). In the magnesium system the initial substrate (ATP) concentration in the control experiments was 4 mM and the $MgCl_2$ concentration 4 mM; 0·2 mM-$CaCl_2$ was also added to inhibit any traces of relaxing factor which might be present (cf. Bendall, 1953). From the formation of the complex ion, $(ATP–Mg)^{2-}$, calculated according to the stability constant of $\sim 3000\ M^{-1}$ given by Smith & Alberty (1956), this mixture contributes

$\sim$ 0·022 to I. The remainder of I, when $>$ 0·042, was made up by addition of KCl. In the calcium system, the initial ATP concentration was 4 mM and the $CaCl_2$ concentration 8 mM in the control experiments. These together contribute approx. 0·024 to I (cf. Smith & Alberty, 1956). The concentration of activator and substrate in both systems is in the optimum range given by the results of Perry & Grey (1956) and Weber (1959). Such substrate concentrations must be far above the Michaelis constant, K_s, of the reaction, since it was found in the present work that the rates of hydrolysis were still maximal, even at 1 mM-substrate, in either system.

Preparation of myokinase. Myokinase was prepared by the method of Kalckar (1943) but was not purified beyond the stage of the crude preparation employed by Bendall (1954). In the experiments cited it was added to the reaction mixture to give a final concentration of 0·04% of the crude protein. This concentration is twice that necessary to give the maximal rate of appearance of P_i, when added together with myofibrils to a mixture of 4 mM-ADP + 4 mM-$MgCl_2$ at I 0·15, and 35°.

Measurement of rate of hydrolysis. Portions of washed fibrils were pipetted into a 100 ml. beaker, held in a constant-temperature water bath, and provided with a mechanical stirrer. The buffer and activating ion were then added and finally the reactant solution, with vigorous stirring (final volume 72 ml.). The reactant was added in a volume of 4 or 6 ml. from a rapid-delivery pipette in $<$ 0·5 sec. Immediately after this addition portions of about 4 ml. were withdrawn in turn as quickly as possible, and each was dropped into 12 ml. of ice-cold 6% trichloroacetic acid contained in weighed flasks and stirred vigorously with a magnetic stirrer. The size of the samples was determined by reweighing the flasks. The method, with two operators, one pipetting and the other timing, allows withdrawal of a sample within 3·5–5 sec. of adding the reactant, and then every 4 or 5 sec. thereafter. The error in timing is less than 0·25 sec.

The P_i formed was estimated by the method of Allen (1940), appropriate enzyme and reagent blanks being included. The blank samples were put through exactly the same processes as the other samples, except that ATP was added to them after adding trichloroacetic acid to the fibril suspensions. The greatest care is necessary in the preparation of such blanks, particularly in view of the results of Marsh (1959), who found the blank value to vary independently and irregularly with the amount of ATP present. No such anomalies appeared in the present work, but, on the contrary, the blank value due to the small impurity of P_i in the ATP was always directly dependent on the amount of ATP added, even at high ATP concentrations. However, if the NaOH used to neutralize the trichloroacetic acid extracts had been stored in glass, rather than in polythene bottles, the blank values became anomalous, probably owing to interference with the colour development by complex silicates, leached from the glass.

RESULTS

The conditions for the control experiments have already been described under Methods. In experiments on addition of products, the concentration of activating ions (Ca^{2+} or Mg^{2+}) was kept in the same proportion to the combined concentrations of ATP + ADP as in the control experiments, i.e. 4 mM above this concentration in the calcium system and equal to it in the magnesium system. In these respects the experiments differ entirely from those of Perry & Grey (1956) on the magnesium system, where ADP was added in excess to a system containing equal concentrations of $MgCl_2$ and ATP. The units employed throughout to express the amounts of substrate or products initially present (a, Y) or of products formed (x) are μmoles of substrate or products/mg. of fibrillar N. The rates of hydrolysis are expressed as units of ATP hydrolysed to ADP/sec. ($\equiv$ units of P_i formed/sec.).

Nature of the progress curves for hydrolysis

The progress curves can be broadly divided into two types: (a) those which show a short explosive phase followed by a 'linear' phase of steady-state velocity (V_m), and (b) those which are curvilinear throughout. The two types are best illustrated from results with the magnesium system at 18·5°, where we see from Figs. 1 (a) and 1 (b) that a mere decrease of I from 0·15 to 0·042 changes the curve from type (a) to type (b). Besides the features described above, the type (a) curve is further characterized by the absence of inhibition by either of the products ADP or P_i, and, by the corollary of this, that addition of myokinase to remove ADP only slightly affects the shape of the curve, as the Figure shows. This type of curve is found only in the magnesium system where it is restricted to values of I from 0·15 to 0·25 and to temperatures between 0° and 20° (see Table 2 below). It does not occur at all at 35°, where all the curves are of type (b), from I = 0·04 to I = 0·25. Curves of type (b), besides being curvilinear throughout their course, differ from type (a) in showing no well-defined explosive phase in plots of x against t; in being more or less severely inhibited by addition of the products [see Fig. 1 (b)] and in being markedly affected by the addition of myokinase, which, by removing the main inhibitor, ADP, increases the rate of hydrolysis in the later stages. This latter effect is illustrated in Fig. 2 for the magnesium system at 35° and I 0·15. Curves of this type, as we have said, are found in the magnesium system at 35° and at low I at 0° and 18·5°, but are characteristic of the calcium system at all the values of I and temperatures studied (cf. Tables 1 and 2 below).

It follows from these results that plots of the rates of hydrolysis per unit volume against the enzyme concentration should give straight lines for curves of type (a), and curves indicating deceleration for those of type (b). This is illustrated in Fig. 3 for the magnesium system. The type (a) curve at

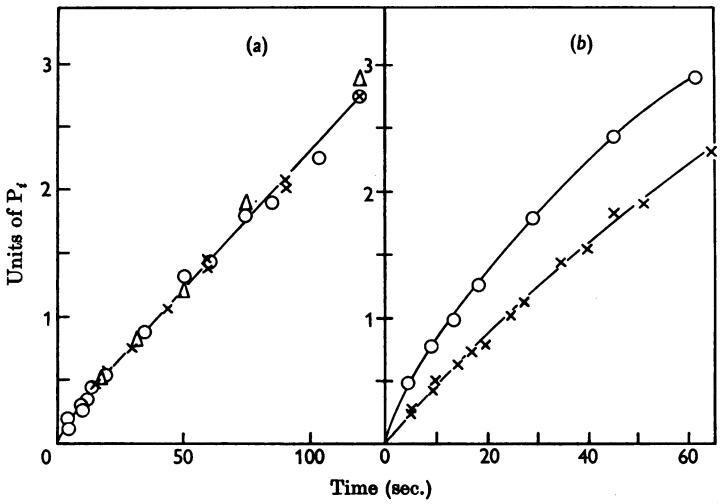

Fig. 1. Two types of progress curve found in the magnesium system, and the effect on them of adding either the product (ADP) or myokinase to remove it. Included in this and all other experiments with the magnesium system is 0·2 mM-$CaCl_2$. Temp., 18·5°; 4 mM-ATP + 4 mM-$MgCl_2$ for control, and 4 mM-ATP + 2 mM-ADP + 6 mM-$MgCl_2$ for product curve; initial substrate in both cases was 6·30 μmoles of ATP/mg. of fibrillar N. Crude myokinase was added where noted to give a final concentration of 0·04% of the crude protein. Curve (*a*) at *I* 0·15; curve (*b*) at *I* 0·042. ○, Control; △, control + myokinase; ×, control + ADP.

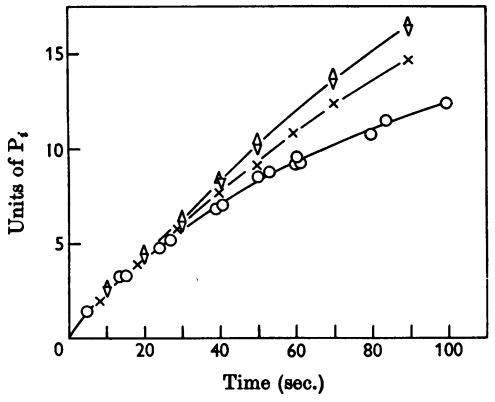

Fig. 2. Effect of myokinase on curves of type (*b*). Temp., 35°; *I* 0·15; 4 mM-ATP + 4 mM-$MgCl_2$; initial substrate, 25 moles of ATP/mg. of fibrillar N. Myokinase was added as in Fig. 1. For the observed values in the two lower curves: ○, control; ×, control + myokinase. For the calculated $V_m t$ values [see equations 9(*a*) and 9(*b*)]: △, control; ▽, control + myokinase.

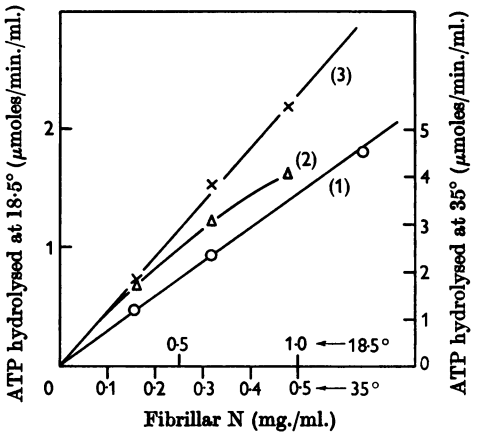

Fig. 3. Dependence of the steady velocity of hydrolysis (V_m) on the enzyme concentration for curves of types (*a*) and (*b*) in the magnesium system. Curve 1: *I* 0·15; temp., 18·5°; curves 2 and 3: *I* 0·042; temp., 35°. Velocities in curve 2 are average values of $\Delta x/\Delta t$ for first 30 sec. of reaction; velocities in curve 3 are calculated from equation (2) for the first 50 sec. of the reaction. Concentration of ATP 4 mM, and $MgCl_2$ 4 mM, throughout. Six points on the time curves were used to calculate each velocity.

18·5° is seen to be very nearly linear, whereas the type (*b*) curve at 35°, when expressed in terms of average rates for the first 30 sec. of the reaction, bends towards the abscissa, as expected. When, however, the rates are calculated from V_m values derived from the appropriate equation for product inhibition (see next section), they become directly proportional to enzyme concentration.

Theoretical analysis of the curves

Type (a). Curves of type (*a*) can be analysed simply by extrapolating the linear portion back to the time base, which gives one intercept, *X*, on the

product axis, which is generally positive, and one, t_s, on the time axis, which is generally negative. The stationary velocity of the linear phase is called V_m, by analogy with the calculated velocity of type (*b*) curves. The true initial velocity, V_o, cannot be estimated even approximately in most cases, because the initial rapid portion of the curve often lasts less than 5 sec., which is the earliest time at which a sample can be withdrawn for estimation by the method described here.

Type (b). Curves of this type are more difficult to analyse than those of type (*a*), because they are not only modified by addition of the products, but may contain a hidden explosive phase as well. In general, however, we can make use of an equation analogous to the integrated rate equation of Foster & Niemann (1955) derived from the reaction steps:

$$E+S \underset{k_2}{\overset{k_1}{\rightleftarrows}} ES \underset{k_4}{\overset{k_3}{\rightleftarrows}} E\begin{cases}P_1\\P_2\end{cases}$$

$$EP_1 \underset{k_6}{\overset{k_5}{\rightleftarrows}} E+P_1, \quad EP_2 \underset{k_8}{\overset{k_7}{\rightleftarrows}} E+P_2,$$

where E is concentration of enzyme, S of substrate, P_1 of ADP and P_2 of P_i. Making the assumption that $k_3 \gg k_4$, and putting

$$K_s = \frac{k_2}{k_1}, \quad K_1 = \frac{k_5}{k_6} \quad \text{and} \quad K_2 = \frac{k_7}{k_8},$$

the rate equation to be integrated then becomes

$$-V_m \frac{dt}{dS} = 1+\frac{K_s}{S}+\frac{K_sP_1}{K_1S}+\frac{K_sP_2}{K_2S}, \qquad (1)$$

where V_m is the maximum velocity and S, P_1 and P_2 are measured at time t. Now from our own results and those of Weber (1959), K_s is less than 0·1 mM for both the calcium- and magnesium-activated reactions, whereas S in the present experiments was initially $\geqslant 4$ mM and was never allowed to fall below 1·5 mM. Hence, even in the late stages of the reaction the term K_s/S was always less than 0·07, whereas the term $-V_m(dt/dS)$ had by that stage generally reached values in excess of 2·0. It follows that K_s/S can be ignored as a first approximation, and we may then integrate as follows:

$$V_mt' = (1-K')\,x+K'a\ln\frac{a}{a-x}, \qquad (2)$$

where a represents the initial amount of substrate/mg. of fibrillar N, x the amount of either product formed at time t (sec.), V_m the μmoles of ATP hydrolysed/mg. of fibrillar N/sec., K' equals $K_s/K_1+K_s/K_2$ and t' equals $t-t_s$.

Similar equations can be derived for the cases where both products are added initially in equal amount Y, or one product is added in amount y. For simplification, let a, $x = a_1$, x_1, and $a_1/(a_1-x_1) = \alpha$ for the experiment with substrate alone. Similarly, when products are added initially, let a, $x = a_2$, x_2 and $a_2/(a_2-x_2) = \beta$. Then for addition of both products in amount Y, we have

$$V_mt' = (1-K')\,x_2+K'(a_2+Y)\ln\beta. \qquad (3)$$

But, for addition of either product separately in amount y:

$$V_mt' = (1-K')\,x_2+\left(K'a_2+\frac{K_s}{K_1}y_1\right)\ln\beta \qquad (4a)$$

$$= (1-K')\,x_2+\left(K'a_2+\frac{K_s}{K_2}y_2\right)\ln\beta. \qquad (4b)$$

In any of these cases, however, there may be a hidden explosive phase in the initial stages, not taken into account by the equations. In such a case, t' is equivalent to $t-t_s$, where t_s is the negative intercept on the time axis of the linear curve derived from the appropriate equation. In practice, t_s can be eliminated by comparing the progress curves for substrate alone and substrate + products at fixed times from the start. It then follows from equations (2) and (3) that at time t

$$V_mt' = x_1+K'(a_1\ln\alpha-x_1)$$
$$= x_2+K'[(a_2+Y)\ln\beta-x_2]. \qquad (5)$$

Therefore

$$x_1-x_2 = K'[\{(a_2+Y)\ln\beta-x_2\}-(a_1\ln\alpha-x_1)]. \qquad (6)$$

We know all the terms except K', which is given by plotting a series of values of x_1-x_2 against the corresponding differences of the factors of K', and V_m which is then evaluated by plotting the two equivalent right-hand terms of equation (5) against t. Both sets of points should then lie on one line with slope V_m and a negative intercept on the time axis of t_s. An alternative method, useful if V_m does not change during the course of the reaction, is to take the times required in each case for a given amount of products, x, to be formed.

Then:

$$t_1'-t_2' = t_1-t_2$$
$$= \frac{K'}{V_m}[(a_1\ln\alpha-x)-\{(a_2+Y)\ln\beta-x\}]. \qquad (7)$$

From this we can evaluate K'/V_m. V_m can then be evaluated from

$$V_m = \frac{dx}{d\left[t_1-\frac{K'}{V_m}(a_1\ln\alpha-x)\right]}$$
$$= \frac{dx}{d\left[t_2-\frac{K'}{V_m}\{(a_2+Y)\ln\beta-x\}\right]}. \qquad (7')$$

Once we have evaluated V_m and K' in this way it is a simple matter to calculate K_s/K_1 and K_s/K_2 by means of equations (4*a*) and (4*b*) from the appropriate experiments with each product added separately.

Analysis of curves showing product inhibition

Calcium system. The above analysis applies most satisfactorily to the calcium system, where the explosive phase either does not exist or is of very short duration. The results at 18·5° provide an excellent illustration because we have examples, at *I* 0·042, 0·15 and 0·25, of three types of product equation, where $K' > 1{\cdot}0$, $K' = 1{\cdot}0$ and $K' < 1{\cdot}0$ respectively. Moreover, we have experimental verification at *I* 0·15 that K_m is very small in comparison with *S*, because the velocity, V_m, was found to be constant throughout a whole range of *S* values from 1 to 8 mM, when the initial amount of substrate per unit of enzyme was held constant. Fig. 4 shows the plots of $\ln [a/(a-x)]$ against *t* for the three cases where the concentrations of products and substrate, when these were initially added together, were so arranged that $a_1 \equiv a_2 + Y$. It will be noted that in the first case, where *I* is 0·042, the curves for the control and for the products both bend towards the time axis, and the latter is below the former, characteristic when $K' > 1{\cdot}0$, whereas in the second case, at *I* 0·15, both curves are rectilinear and coincident, characteristic when $K' = 1{\cdot}0$, and in the third case, at *I* 0·25, both curves bend away from the time axis, and the product curve is above the control, characteristic when $K' < 1{\cdot}0$. Analysis, by means of equations (6) and (7), showed that K' had a value of ~1·9 at *I* 0·042, of 1·0 at *I* 0·15, and of ~0·7 at *I* 0·25. The reliability of this analysis can be checked by plotting the values of $V_m t'$, calculated from the experimental points by equation (3), against *t* for the control and for the products in each case. This is done in Fig. 5, where all the curves are now rectilinear, and the experimental points lie very close to the curve in each case. Also all the curves pass very nearly through the origin, so that little or no explosive phase can have been present. This point is discussed in more detail below.

Once the relative inhibition constant for both products, K', was known, those for each product were evaluated separately from equations (4*a*) and (4*b*). In the present example of the calcium system at 18·5° such analysis showed that ADP was the more effective inhibitor at *I* 0·042 and *I* 0·15, where P_i contributed only about a third of the total inhibition, whereas at *I* 0·25 the inhibition was distributed equally between the two products. Similar results were obtained at 35°, as seen from Table 1, which summarizes all the results for this system at the three temperatures 0°, 18·5° and 35° and the three values of *I* 0·042, 0·15

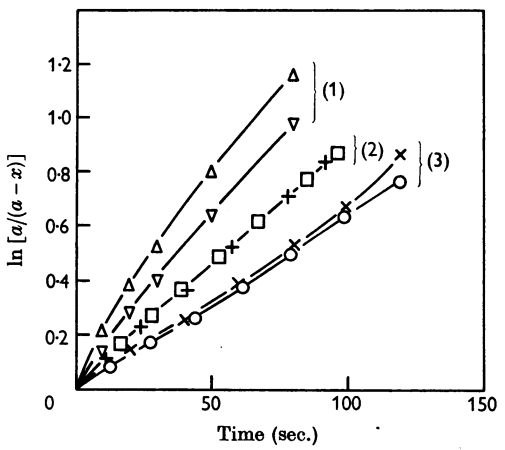

Fig. 4. Calcium system at 18·5°. The effect of plotting $\ln [a/(a-x)]$ against *t* at three values of *I*. For control curves: ATP, 4 mM; $CaCl_2$, 8 mM. For product curves: ATP, 3 mM; ADP, 1 mM; P_i, 1 mM; $CaCl_2$, 8 mM. The following pairs of curves are shown: Curve (1): *I*, 0·042; △, control; ▽, with products. Curve (2): *I*, 0·15; □, control; +, with products. Curve (3): *I*, 0·25; ○, control; ×, with products. *Note*: values for curve (1) have been multiplied by 2 to distinguish them from those for curve (2).

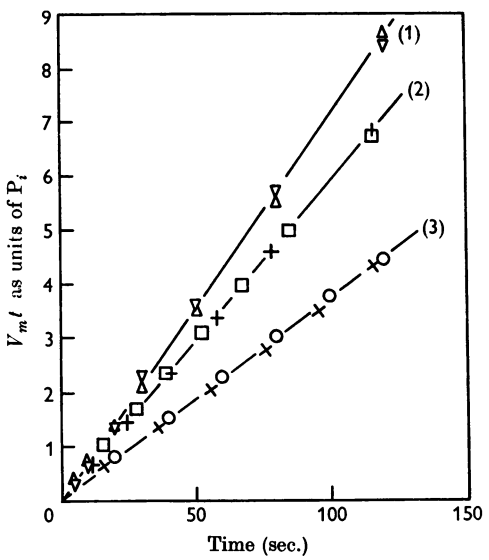

Fig. 5. Calcium system at 18·5°. The curves of Fig. 4 are expressed as $V_m t'$ against *t* calculated from equation (4). Conditions and symbols are as in Fig. 4. Initial substrate, *a*, in control curves 6·30, and in product curves 4·90 μmoles of ATP/mg. of fibrillar N. *Note*: $V_m t'$ values in curve (1) have been multiplied by 1·25 to distinguish them from those for curve (2).

Table 1. *Relative product inhibition constants for the calcium system at three temperatures and ionic strengths, with their standard errors*

Degrees of freedom are given in parentheses.

Temp.	I	K'	K_s/K_{ADP}	K_s/K_{P_i}
1·0°	0·042	1·00 (6)	1·00±0·001 (8)	0*
	0·150	0·83±0·004 (5)	0·52±0·025 (8)	0·31*
	0·250	0·86±0·003 (4)	0·46±0·04 (6)	0·40*
18·5	0·042	1·91±0·011 (5)	1·22±0·02 (6)	0·60±0·01 (5)
	0·150	1·00 (16)	0·63±0·04 (7)	0·34±0·04 (5)
	0·250	0·70±0·01 (8)	0·34±0·015 (6)	0·34±0·06 (6)
35	0·042	2·20±0·03 (6)	1·50±0·06 (7)	0·54±0·04 (8)
	0·150	1·38±0·046 (6)	1·34±0·026 (7)	0·16±0·016 (8)
	0·250	0·67±0·006 (15)	0·57±0·04 (12)	0·13±0·06 (13)

* By difference.

and 0·25. At 1·0°, unlike the higher temperatures, K' does not change much as I is increased, but remains close to 1·0 throughout, just as in the magnesium system at 0° it is always close to zero (cf. Table 2).

Magnesium system. The results of the studies of product inhibition in the magnesium system are more difficult to analyse than those for the calcium system, first because there is some evidence of a very early explosive phase, and secondly because a sudden decrease in slope appears after about 40 sec. in plots of $V_m t'$ against t. Analysis of this phenomenon at 35° is fortunately simplified at the lowest strength (I 0·042) because K' turns out to be unity, so that, following equation (5):

$$V_m t' = a_1 \ln \frac{a_1}{a_1 - x_1} \text{ for control}$$

$$= (a_2 + Y) \ln \frac{a_2}{a_2 - x_2} \text{ for initial addition of products.} \quad (8)$$

Furthermore, under the above conditions of I and temperature only ADP inhibits and not P_i, as shown in Fig. 6, where $V_m t'$, calculated from the experimental points by equation (8), is plotted against t for substrate only, substrate + ADP and substrate + ADP + P_i. The points from all these experiments lie closely on the curve, which has a sharp inflexion at ~40 sec. In this case, as in most others of this type, $V_{m,1}$ (up to 40 sec.) is ~1·4 times $V_{m,2}$ (from 40 to 150 sec.). Also the first straight line, $V_{m,1} t'$ against t, does not pass exactly through the origin, but cuts the axis at ~ -2 sec. ($= t_{s,1}$). This early non-linear phase, which lasts about 5 sec., is evidently the anomalous explosive phase of Weber & Hasselbach (1954). It will be discussed in detail below. Very similar diphasic curves are obtained at I 0·15 and I 0·25 at 35°, and at I 0·042 at 18·5° (Tables 2 and 3), although K' varies widely, being ~1·3, ~0·2 and ~0·5 respectively. In spite of these differences, however, $V_{m,1}/V_{m,2}$ lies between 1·24 and 1·50 in all cases (see Table 3). Also Table 2 shows that P_i acts as an inhibitor only at I 0·042 and 18·5° and at I 0·15 and 35°, another marked point of difference from the calcium system where such inhibition evidently occurs in all cases.

Effect of myokinase in relieving the inhibition by the adenosine diphosphate formed. It is of some interest to consider the kinetics of the reaction when myokinase is present, as in the experiment illustrated in Fig. 2 for the magnesium system at I 0·15 and 35°. The reactions now taking place can be written, according to Bendall & Davey (1957), as

$$2\text{ATP} \xrightarrow[\text{triphosphatase}]{\text{adenosine}} 2\text{ADP} + 2\text{P}_i, \quad \text{(i)}$$

$$2\text{ADP} \xrightleftharpoons{\text{myokinase}} \text{ATP} + \text{AMP}, \quad \text{(ii)}$$

$$\text{AMP} \xrightarrow{\text{deaminase}} \text{IMP} + \text{NH}_3. \quad \text{(iii)}$$

The overall reaction is therefore

$$\text{ATP} \longrightarrow \text{IMP} + 2\text{P}_i + \text{NH}_3. \quad \text{(iv)}$$

The only proviso is that myokinase must remove ADP as soon as it is formed in reaction (i), which was ensured in this experiment by adding a large amount of the active enzyme. Since neither IMP nor NH_3 appears to inhibit the fibrillar adenosine triphosphatase, it follows that P_i is now the only inhibitor present, two molecules of it being produced for each ATP molecule destroyed. Thus the integrated rate equation analogous to equation (2) becomes

$$V_m t' = \left(1 - 2\frac{K_s}{K_2}\right) x + 4a \frac{K_s}{K_2} \ln \left(\frac{2a}{2a - x}\right), \quad (9)$$

where K_s/K_2 is the relative inhibition constant for P_i, x the amount of P_i formed at time, t, in μmoles/mg. of fibrillar N, and V_m the velocity of reaction (i).

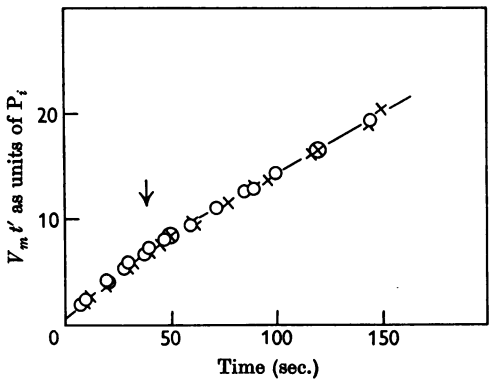

Fig. 6. Magnesium system at 35°. $V_m t'$ is calculated from equations (4) and (8) and is plotted against t for product inhibition at I 0·042, to illustrate the diphasic nature of the curves. Initial substrate, a, for control curves is 25·0, and for product curves, 18·8 moles/mg. of fibrillar N. O, Two control experiments, nine points from each, at ATP 4 mM and $MgCl_2$ 4 mM; ×, effect of both products, or ADP alone, nine points from each experiment. Conditions for both products: ATP, 3 mM; ADP, 1 mM; P_i, 1 mM; $MgCl_2$, 4 mM. Conditions for ADP only: ATP, 3 mM; ADP, 1 mM; $MgCl_2$, 4 mM.

Inserting the known values of a (25 μmoles/mg. of N) and of K_s/K_2 (0·31), given in Fig. 2 and Table 2 respectively, this equation becomes:

$$V_m t' = 0{\cdot}38\left(x + 81{\cdot}5 \ln \frac{50}{50-x}\right). \qquad (9a)$$

Similarly the equation for the control curve shown in Fig. 2 becomes:

$$V_m t' = 0{\cdot}28\left(-x + 114 \ln \frac{25}{25-x}\right). \qquad (9b)$$

Application of these two very different rate equations to the actual results gives the two sets of points on either side of the top curve in Fig. 2. They agree fairly closely, and thus give added proof of the accuracy of the inhibition constants given in Table 1 for these particular conditions. Here again the curve has a characteristic inflexion at about 50 sec.

Summary of the effects of temperature and ionic strength on the two systems. To summarize the complicated results described in this section: (i) the $V_m t'$ curves in the magnesium system always tend to show two phases, in addition to a very short explosive phase, particularly in cases where the

Table 2. *Relative product inhibition constants for the magnesium system at three temperatures and ionic strengths, with their standard errors*

Degrees of freedom are given in parentheses.

Temp.	I	K'	K_s/K_{ADP}	K_s/K_{P_i}
0·6°	0·042	0·26±0·02 (6)	—	—
	0·150	0 (4)	0	0
	0·250	0 (4)	0	0
18·5	0·042	0·52±0·008 (8)	0·42±0·006 (7)	0·11±0·02 (6)
	0·150	0 (6)	0	0
	0·250	0 (4)	0	0
35	0·042	1·00 (8)	1·00 (8)	0 (8)
	0·150	1·28±0·013 (7)	0·93±0·024 (10)	0·31±0·06 (4)
	0·250	0·17±0·005 (7)	—	—

Table 3. *Steady velocities of splitting ($V_{m,1}$, $V_{m,2}$) and time intercepts ($t_{s,1}$, $t_{s,2}$) for the magnesium system, at three temperatures and ionic strengths*

Standard errors are given in parentheses.

Temp.	I	$10^4 V_{m,1}$ (units/sec.)	$t_{s,1}$ (sec.)	Duration of phase with $V_{m,1}$	$10^4 V_{m,2}$ (units/sec.)	$t_{s,2}$ (sec.)	No. of expts.
0·6°	0·042	41·0 (1)	− 4 (7)	>240	*	*	4
	0·150	8·2 (1)	−17 (3)	~120	5·0 (0·5)	−100 (10)	2
	0·250	2·4 (0·2)	0 (1)	~240	1·6 (0·1)	−200 (50)	2
18·5	0·042	540 (20)	−2·3 (0·1)	45 (2)	430 (15)	−16 (1)	7
	0·150	230 (9)	−1·9 (0·5)	>240	*	*	9
	0·250	80 (20)	− 3 (0·5)	20 (5)	43 (5)	−22 (1)	2
35	0·042	1940 (20)	− 2 (0·2)	40 (2)	1190 (20)	−21 (1)	7
	0·150	2110 (30)	−1·4 (0·09)	38 (2)	1420 (10)	− 19 (1)	6
	0·250	890 (20)	0 (0·5)	25 (2)	660 (10)	− 14 (2)	2

* No detectable second phase.

products inhibit the reaction (cf. Tables 2 and 3), whereas in the calcium system there is no second phase and the early explosive phase is also absent or very restricted in extent (Table 4); (ii) the combined relative inhibition constants for both products (K') or for each product separately rise with increasing temperature and fall with increasing I in both systems, except in the calcium system at 1·0°, where K' is scarcely affected by I, and at I 0·25, where K' falls instead of rising with increasing temperature; (iii) the inhibition by the products, either separately or together, is always much greater in the calcium system than in the magnesium system, where indeed there is no inhibition at all at the lower temperatures and higher values of I (cf. Tables 1 and 2); (iv) increasing I has generally a far stronger inhibitory effect on hydrolysis in the magnesium system than in the calcium system, just as increasing temperature has a more marked accelerating effect (Tables 3 and 4). An exception to this rule occurs at 35°, where the velocity increases slightly in the magnesium system as I is raised from 0·04 to 0·15 and then decreases again at I 0·25, but the relative decrease is much less than at the lower temperatures. A similar increase in velocity as I is raised from 0·04 to 0·15 is a general feature of the calcium system.

Velocities of hydrolysis in mixed systems

As mentioned under Methods and as also shown by Bendall (1953) and Weber (1959), Ca^{2+} ions added in traces to the magnesium system have no effect on the shape of the progress curves, so long as all traces of relaxing factor have been removed in the process of washing. On the other hand, small amounts of Mg^{2+} ions added to the calcium system drastically modify the curves, and, in cases where the two systems are clearly distinguished, change them toward the pattern characteristic of the magnesium system, and away from that of the calcium system. This is particularly well illustrated at I 0·15 and 18·5°, by curves (1) and (4) in Fig. 7, where the curve for calcium alone (1) has almost double the velocity of that for a system to which 1 mM-$MgCl_2$ has also been added (4), and the latter has been modified to the 'linear' form characteristic of the magnesium system, instead of the 'exponential' form of the calcium system at this I. The effect is not so dramatic at I 0·042 and 18·5° [curves (2) and (3)], because both systems in any case give curves of exponential type under these conditions (cf. Tables 1 and 2), but K', calculated from an experiment in which the products were added, falls on addition of magnesium to the calcium system from the characteristically high value of 1·9 to 0·35, which is of the same order as K' for the magnesium system at this temperature (cf. Tables 1 and 2). At about 0°, the effect is even more striking, the stationary velocity of the calcium system being reduced by addition of $MgCl_2$ from $1{\cdot}5 \times 10^{-2}$ to $\sim 2{\cdot}3 \times 10^{-3}$ unit/sec. at I 0·042,

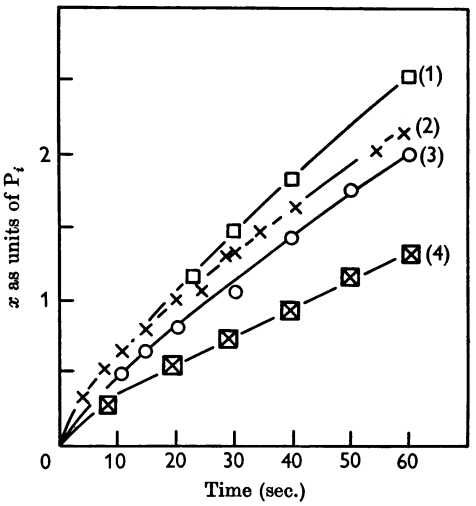

Fig. 7. Effect of adding $MgCl_2$ to the calcium system, at 18·5°. Plot of x against t for ATP, 4 mM, and a, 6·30 μmoles of ATP/mg. of fibrillar N. Curve (1): I, 0·15; $CaCl_2$, 8 mM. Curve (2): I, 0·042; $CaCl_2$, 8 mM. Curve (3): I, 0·042; $CaCl_2$, 7 mM; $MgCl_2$, 1 mM. Curve (4): I, 0·15; $CaCl_2$, 7 mM; $MgCl_2$, 1 mM.

Table 4. *Steady velocities of splitting (V_m) and time intercepts (t_s) for the calcium system*

Standard errors are given in parentheses.

Temp.	I	$10^4\ V_m$ (units/sec.)	t_s	No. of expts.
1·0°	0·042	151 (6)	−8·6 (1·3)	3
	0·150	171 (7)	−0·7 (0·7)	3
	0·250	128 (4)	−13·0 (2)	3
18·5	0·042	530 (13)	−0·7 (0·33)	10
	0·150	550 (5)	+0·12 (0·20)	8
	0·250	360 (12)	+0·50 (0·30)	4
35	0·042	1410 (25)	−0·50 (0·10)	7
	0·150	1680 (30)	−0·40 (0·30)	5
	0·250	1210 (40)	−1·00 (0·30)	6

Table 5. *Effect of pretreating fibrils at* 18·5° *with* 4 mM-*adenosine triphosphate*–4 mM-*magnesium chloride on the subsequent rate of splitting in the magnesium system*

Average values are for two experiments in each case.

		Control				Pretreated			
Temp.	I	$10^4\ V_{m,1}$ (units/sec.)	$t_{s,1}$ (sec.)	$10^4\ V_{m,2}$ (units/sec.)	$t_{s,2}$ (sec.)	$10^4\ V_{m,1}$ (units/sec.)	$t_{s,1}$ (sec.)	$10^4\ V_{m,2}$ (units/sec.)	$t_{s,2}$ (sec.)
18·5°	0·042	580	1	420	28	490	0·2	380	11
	0·150	230	3	*	*	230	0	*	*
35	0·042	1790	3	1290	20	1690	2	1340	11·0
	0·150	2100	1·4	1420	19	2000	0	1920	3·0

* No detectable second phase.

and from $1{\cdot}8 \times 10^{-2}$ to $\sim 6{\cdot}5 \times 10^{-4}$ unit/sec. at I 0·15, with a change in both cases from 'exponential' to 'linear' type (not shown).

Analysis of the so-called explosive phase and the effect upon it of pretreatment of the fibrils with adenosine triphosphate

In describing the kinetics of the hydrolysis reaction we have so far confined the discussion to the post-explosive phase, which we have shown to be inhibited more or less strongly by the products, ADP and P_i. It is now necessary to inquire whether indeed an explosive phase exists at all in the sense used by Weber & Hasselbach (1954), when these inhibitory effects are taken into account. This phase can best be described in terms of t_s, which for curves of type (*a*) is the intercept on the time axis of straight lines of best fit in plots of x against t, and for curves of type (*b*) is the intercept of plots of $V_m t'$ against t, where $V_m t'$ is calculated from the experimental points by means of the appropriate equation for product inhibition. Clearly t_s will be negative when an explosive phase exists, whereas in its absence it will be zero with more or less marked positive and negative deviations due to experimental errors. A complication arises in the magnesium system where there are sometimes two well-defined linear phases after the explosion proper (see Fig. 6). The time intercept of the first of these is $t_{s,1}$ and of the second $t_{s,2}$ with velocities $V_{m,1}$ and $V_{m,2}$ respectively. Since the former lasts for at least 40 sec. and often much longer, it is clearly quite distinct from the much earlier explosive phase described by Weber & Hasselbach (1954) and by Tonomura & Kitagawa (1960) and for this reason will be treated separately below.

The mean values for $t_{s,1}$, $t_{s,2}$, $V_{m,1}$ and $V_{m,2}$ for the magnesium system are given in Table 3, and the values of t_s and V_m for the calcium system in Table 4. It is seen that t_s is generally very small indeed for the latter system, except in two anomalous cases at 0°, I 0·04 and I 0·25, where the high values are possibly due to swelling of the fibrils under these conditions. In all other cases the standard error is so large in comparison with the means that t_s frequently becomes positive instead of negative, and in two cases at 18° even the mean is positive. This is exactly what would be expected if t_s were really zero and the deviations were due to experimental error. We may conclude that no explosive phase exists in the calcium system when due allowance is made for inhibition by the products.

The magnesium system is rather more complex; Table 3 shows that t_s is generally negative, although often very small, and with a high standard error. At 0° swelling effects may again become a complicating factor, but even here there is generally a well-defined stationary phase lasting 2–4 min., of which the time intercept, $t_{s,1}$, is small in comparison with the very low velocities of hydrolysis. The essential difference between the two systems lies, however, in the much more pronounced shortening of the fibrils which occurs at the higher temperatures in the magnesium system as soon as ATP is added (cf. Marsh, 1952; Bendall, 1958). This shortening, which probably occurs during the first 5 sec. of the reaction and which often amounts to 70 % or more of the original length of the fibrils, would be expected to lower the velocity of hydrolysis, because in the supercontracted state many of the active enzyme sites would be masked by other inactive groups (see Hanson & Huxley, 1955). In spite of this, Weber & Hasselbach (1954) claimed in their original work that fibrils pretreated with ATP, i.e. pre-shortened, still showed an explosive phase on readdition of substrate. The present experiments do not confirm this view, but indicate exactly the contrary, as we see from Table 5, where the fibrils were pretreated with ATP + $MgCl_2$ under the appropriate ionic conditions at 18·5° and then washed free of substrate and products before the actual measurement of the rate of hydrolysis. The centrifuged volume of such fibrils was approximately half that of untreated fibrils, showing that considerable synaeresis had occurred. In every case, in Table 5, $t_{s,1}$ is reduced or abolished by the

pretreatment and $V_{m,1}$ is also generally lower than the control values. $V_{m,2}$, however, is not so much affected, and, at 35° and I 0·15, is actually higher than the control value and nearly equal to $V_{m,1}$. The effect of these changes is, of course, to reduce $t_{s,2}$ markedly in every case. We may conclude from these observations that the so-called explosive phase mainly represents the decreasing velocity of hydrolysis as the fibrils shorten and supercontract. Because this happens so quickly it becomes impossible to estimate accurately the true initial velocity of hydrolysis, V_o, although it is doubtful whether this could ever exceed $2V_{m,1}$. Fibrils pretreated with ATP and $CaCl_2$ and subsequently activated with $CaCl_2$ show no change in V_m, whereas pretreatment with ATP and $MgCl_2$ reduces V_m from ~14 to 13 units/sec. when hydrolysis is subsequently measured in the presence of Ca^{2+} ions at I 0·15 and 35°.

Energy of activation of hydrolysis

It is of some interest to calculate the apparent energy of activation (E_A) of the hydrolysis of ATP by the fibrils in the magnesium and calcium systems, because so far there are very few reliable data in the literature upon it. This can be done by means of the differential form of the Hood–Arrhenius equation

$$E_A = -R\frac{d(\ln V_m)}{d(1/T)}, \qquad (10)$$

where V_m is the steady velocity of hydrolysis and E_A is given in kcal./mole. The results based on the average $V_{m,1}$ or V_m values given in Tables 3 and 4 respectively are plotted as log V_m against $1/T$ in Fig. 8, and the E_A values calculated for the two temperature ranges 0–18·5° and 18·5–35° are summarized in Table 6. The average E_A values are always much higher in the magnesium than in the calcium system and moreover behave in an anomalous manner, falling in every case by ~6 kcal. as the temperature is raised from one range to the other. The values for the calcium system on the other hand are of the order to be expected of a straightforward chemical or enzyme-catalysed reaction (Moelwyn-Hughes, 1947; Dixon & Webb, 1958), that is ~11 kcal./mole, although they are also anomalous at low I, whereas at the higher they actually increase with increasing temperature (cf. Fig. 8 and Table 6). This second type of anomaly, an increase of E_A with temperature, occurs rarely, even in enzyme reactions (Dixon & Webb, 1958). The case of the fumarase reaction at high pH, reported by Massey (1953), was the only example of it known when Dixon & Webb were writing in 1958, and in that case it was probably due to a change of state of the enzyme.

It would be of great theoretical interest to be able to calculate the heat of combination of substrate or products with the enzyme, but this cannot be done from the data because only relative constants are available, i.e. K_s/K_{ADP}, K_s/K_{P_i} and the combined constant K'. This stresses the need for an accurate method for the measurement of K_s or K_m in this system without which neither the free energy nor the entropy of the substrate–enzyme or product–enzyme equilibria can be calculated. Some preliminary experiments have shown that K_s in the magnesium system at 35°

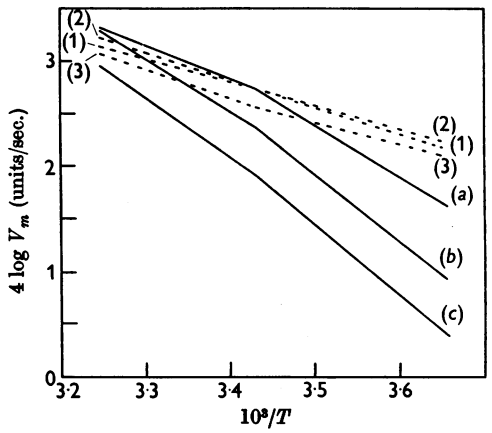

Fig. 8. Plot of log V_m against $1/T$ for the magnesium and calcium systems, based on the average $V_{m,1}$ and V_m values given in Tables 3 and 4 respectively. - - -, Calcium system: curves (1), (2) and (3) at I 0·042, 0·15 and 0·25 respectively. ——, Magnesium system: curves (*a*), (*b*) and (*c*) at I 0·042, 0·15 and 0·25 respectively.

Table 6. *Apparent energy of activation (E_A) of the splitting process, calculated from $V_{m,1}$ and V_m in Tables 3 and 4 respectively*

E_A is calculated as an average value over the stated range of temperature.

		E_A (cal./mole)		
Temp. range	Ion	I 0·042	I 0·150	I 0·250
0·6–18·5°	Mg	21400	28600	30100
18·5–35·0		14800	24200	26400
1·0–18·5	Ca	11400	10600	9400
18·5–35·0		10700	12100	13300

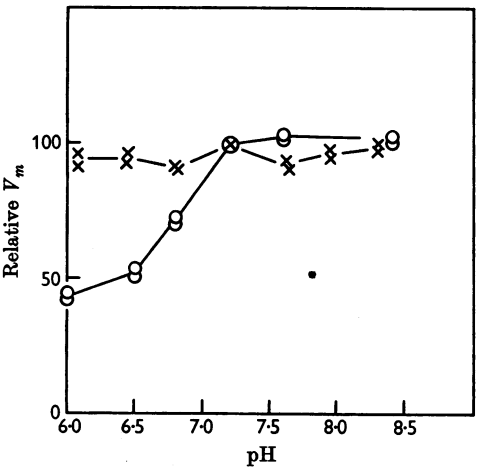

Fig. 9. Effect of pH on the steady velocity of hydrolysis, V_m, in the calcium and magnesium systems at 18·5°, I 0·15 and ATP 4 mM. Relative V_m values are given as % of V_m at pH 7·20. There were two experiments at each pH. ×, ATP + 8 mM-$CaCl_2$; ○, ATP + 4 mM-$MgCl_2$. Absolute V_m at pH 7·2 for calcium system was $5{\cdot}30 \times 10^{-2}$ unit/sec., and for magnesium system $2{\cdot}30 \times 10^{-2}$ unit/sec.

and I 0·04 is 0·089 mM (S.E. ± 0·007, n 15), but values at other temperatures have not yet been obtained.

Effect of pH

The effect of pH has been studied only under the rather special conditions of I 0·15 and 18·5°. Fig. 9 shows that the optimum range of pH for the magnesium system extends from pH 8·0 down to about 7·00, below which the relative velocity falls to a value of ~ 50 % of the maximal at pH 6·5 and is not much further reduced even at pH 6·0. In the calcium system, on the other hand, changes of pH within this range seem to have little or no effect on the rate of hydrolysis. Although it is unwise to generalize from only one value of I and temperature, it is probable, at least, that a pH of 7·2 is optimum for both systems. A wider range of values is clearly required, however, to establish whether the pH effect is due to dissociation of groups on the enzyme or on the substrate, which is an $(ATP–Mg)^{2-}$ complex in the magnesium system, but probably the free ion, ATP^{4-}, in the calcium system (Weber, 1959).

DISCUSSION

Most of the studies of the adenosine-triphosphatase activity of rabbit-muscle fibrils or of their constituent proteins, myosin and actomyosin, have been carried out at temperatures between 0° and 25° and often at very high or very low I (cf. Perry & Grey, 1956; Hasselbach, 1956; Morales & Botts, 1956; Blum, 1955). Such conditions diverge more or less widely from the physiological and this divergence has been further exacerbated in some cases by studying the reaction at pH 6·5 (e.g. Weber, 1959), which is certainly below the optimum pH for the magnesium-activated reaction (Fig. 8) and is also below the pH which can actually be measured in surviving muscle (Bate-Smith & Bendall, 1949; Bendall & Davey, 1957; Caldwell, 1958). From evidence that has accumulated over many years, it seems likely that the actual conditions in resting rabbit muscle, i.e. without an appreciable content of lactic acid, are a pH of 7·10–7·30, an I of 0·15–0·20 (Dubuisson, 1950), a concentration of Mg^{2+} ions of ~ 10 mM and of Ca^{2+} ions of ~ 2 mM, a concentration of ATP of ~ 10 mM and of ADP of ~ 1 mM, and a temperature of ~ 38° (cf. Hasselbach, 1957; Bendall & Davey, 1957). Of the soluble constituents, the ADP is bound to actin and most of the Ca^{2+} ions to actomyosin or the sarcoplasmic proteins, whereas most of the Mg^{2+} ions and ATP are not so bound, but are present as an $(ATP–Mg)^{2-}$ complex (Smith & Alberty, 1956; Hasselbach, 1957). It has been attempted here to approach these conditions as closely as possible or at least to cover a sufficient range to include them, because otherwise it is impossible to relate the results to the real state of affairs in living, contracting mammalian muscle.

Other points of great technical importance which have often been overlooked in previous studies are first the necessity of preparing adequate blanks and secondly of measuring the rate of hydrolysis from the earliest seconds of the reaction onwards. Lack of attention to the first point can lead to quite false deductions about the extent of the explosive phase, and to the second to an erroneous and incomplete interpretation of the effects of changing the environment, particularly in cases where the products inhibit the forward reaction. In the present study it became very noticeable, as the work proceeded and insight was gained in overcoming these difficulties, that the magnitude and duration of the explosive phase progressively decreased, until its apparent time intercept (t_s) was reduced from values of the order of tens of seconds to the very low values shown in Table 3 for the magnesium system and to the vanishingly small values in Table 4 for the calcium system. Coupled with this marked decrease was the observation that the explosion in the magnesium system could be virtually abolished by pretreatment of the fibrils with ATP; in other words, the falling velocity, characteristic of this phase of the reaction, appears to be entirely an effect of the concomitant supercontraction of the fibrils. It is significant that this effect is unique to the magnesium system because it is precisely here that the hydrolysis process

probably involves sites on both the actin and myosin filaments of the fibril, whereas in the calcium system it involves only sites on the myosin filaments (Bárány & Bárány, 1960; Nagai, Makinose & Hasselbach, 1960). If such is the case, it would be expected that supercontraction in the former system would have a considerable disorientating effect on the two enzyme sites involved, leading to a fall in the hydrolysis rate, but would have little or no effect in the latter, where only one type of site was involved. The view of Bárány & Bárány that only myosin sites are involved in hydrolysis in the calcium system is, incidentally, confirmed by the present results, because the relative inhibition constant, K_s/K_{ADP}, for this system at I 0·15 and 18·5° is ~0·70, which is close to the value of 0·90 obtained by Green & Mommaerts (1954) for highly purified myosin under nearly identical conditions. By contrast, the magnesium system shows no inhibition by the products under these conditions.

The extent of the disorientation during supercontraction in the magnesium system can best be envisaged from the sliding-filament model of Hanson & Huxley (1955), from which it is clear that even at ~30% shortening the I-bands of the sarcomeres have already vanished, the actin filaments of each half sarcomere have met in the middle of the A-band and are piling up into a contraction band, and the Z-disks are just in contact with the edges of the A-band. Shortening beyond ~70% of the initial length leads to further folding and buckling of the actin filaments and to buckling of the myosin filaments of the A-band. It is quite clear that these changes will rapidly and drastically reduce the number of opposing active sites on the two sets of filaments with a consequent fall in the velocity of hydrolysis of ATP from V_o to $V_{m,1}$ in the early 'explosive' phase. It is quite possible that further buckling of the filaments will occur in the supercontracted fibrils with time, which would account for the subsequent characteristic fall of velocity from $V_{m,1}$ to $V_{m,2}$ at ~40 sec. from the start (cf. Fig. 6). These changes in velocity are more clearly defined with frog-sartorious fibrils than rabbit fibrils, because V_o takes longer to decay to a plateau, $V_{m,1}$, and this plateau lasts only a short time before decaying at a slower rate to $V_{m,2}$. The two decay curves, $V_o \rightarrow V_{m,1}$ and $V_{m,1} \rightarrow V_{m,2}$, are of typical exponential form, $dV/dt = k(V_1 - V_2)e^{-kt}$. This, in itself, indicates that the change in velocity is unlikely to be due to a faulty product-inhibition equation, because there are no terms, even in the equation for non-competitive inhibition, which could account for the time course of the changes.

The general question of inhibition by the products at low I seems to have been studied only by Green & Mommaerts (1954), using myosin adenosine triphosphatase, and no data have hitherto been available on actomyosin or on the myofibrillar adenosine triphosphatase. The results presented here are unfortunately incomplete because they include no measure of K_s, so that only relative constants can be given, and these are subject to error because of the differential method used to calculate them. They give, however, a fairly clear idea of the general course of the reaction under the different conditions. Tables 1 and 2 show that K_s/K_{ADP} and the combined constant, K', generally tend to rise and K_s/K_{P_i} to fall with increasing temperature, whereas all three constants fall with I. The latter phenomenon is very striking in the magnesium system, where K' falls to zero at the two higher I values at temperatures of 18·5° or below, but is measurable in all cases at the lowest I. An exception to these general rules occurs in the calcium system at the highest I, where K' falls, instead of rising, as the temperature is increased, and this reversal is accompanied by an anomalous rise in the energy of activation of hydrolysis with temperature (cf. Table 6). By analogy with other systems this strongly suggests a change of state of the enzyme as the temperature is increased (Dixon & Webb, 1958), which in this case may be a change in the myosin filaments from the gel to the sol state or vice versa.

The energy of activation of the hydrolysis process has not been studied systematically in previous work, although Hasselbach (1952) and Perry & Grey (1956) have noted that the magnesium system in the temperature range 0–25° is much more strongly activated by increasing temperature than is the calcium system. The question seems worthy of more detailed investigation than this, however, because it bears not only on the nature of the process, but also on its role in the concentration cycle *in vivo*. As we have shown, the effect of Mg^{2+} ions always dominates that of Ca^{2+} ions in determining the initial velocity and the precise course of hydrolysis when both ions are present together *in vitro*, and this dominance is likely to be emphasized *in vivo* by the higher total concentration and the much higher 'free' concentration of Mg^{2+} ions than of Ca^{2+} ions, the latter being mainly bound to protein (Hasselbach, 1957). It would seem obvious, therefore, that it is the magnesium-activated process which is relevant in interpreting the contractile process *in vivo* and not the calcium-activated one, which has nevertheless been so persistently studied from this point of view (Green & Mommaerts, 1954; Mommaerts & Green, 1954; Morales, Botts, Blum & Hill, 1955). Table 6 shows that it is precisely the magnesium system that shows a high apparent energy of activation (E_A), particularly at physiological I values of ~0·15,

where it lies in the range 22–28 kcal./mole, and the calcium system which shows a normal E_A of ~11 kcal./mole, characteristic of many straightforward enzymic reactions (Dixon & Webb, 1958). Further, the magnesium system is anomalous in showing a very high average value for $\Delta E_A/\Delta T$ of more than −200 cal./mole/degree, which is at least three times the value for most of the so-called anomalous chemical and enzymic reactions (Moelwyn-Hughes, 1947; Dixon & Webb, 1958). Anomalies of this type are generally due to side reactions concurrent with the main one, or in enzyme-catalysed reactions to many reaction steps. In the present case, it has been suggested that the reaction takes place between active sites on the actin and myosin filaments of the fibril (Weber, 1958; Bárány & Bárány, 1960), and, if this is so, we can easily imagine that a number of steps would be necessary. Starting, as we must, in the system *in vitro*, with the fibrils in rigor and the overlapping actin (A) and myosin (M) filaments bonded together in a complex, A–M, we can formulate the initial steps as follows:

$$\text{A–M} + \text{ATP} \underset{K_2}{\overset{K_1}{\rightleftarrows}} \text{A–ATP–M}, \qquad (a)$$

$$\text{A–ATP–M} \underset{K_{b,2}}{\overset{K_{b,1}}{\rightleftarrows}} \text{A} + \text{ATP–M}, \qquad (b)$$

$$\text{A–ATP–M} \underset{K_{c,2}}{\overset{K_{c,1}}{\rightleftarrows}} \text{A–ATP*–M}, \qquad (c)$$

$$\text{A–ATP*–M} \underset{K_4}{\overset{K_3}{\rightleftarrows}} \text{A} \begin{matrix} \diagup \text{ADP*} \\ \diagdown \text{P}_i \end{matrix} \text{M}, \qquad (d)$$

$$\begin{matrix} \text{ADP*} \\ \text{A} \\ \text{P}_i \end{matrix} \text{M} \underset{K_6, K_8}{\overset{K_5, K_7}{\rightleftarrows}} \text{A–M} + \text{products}, \qquad (e)$$

where we have kept to the numbering of the constants in the simple formulation given in the section on theoretical analysis of the curves, and where the asterisks indicate activated complexes. We must also envisage, of course, a contraction step somewhere in the series, possibly at step (*d*) or between steps (*c*) and (*d*), because we know that supercontraction takes place at 18·5° and above. Similarly, step (*b*), an alternative type of dissociation of the enzyme–substrate complex, is obligatory because swelling of the fibrils, that is separation of the actin and myosin filaments by water of hydration, occurs under some conditions, particularly at the higher I values at 0° (unpublished work) and it also occurs whenever the forward reaction of step (*d*) or of earlier steps is artificially inhibited (Marsh, 1952; Bendall, 1958). It seems to be represented in the behaviour of intact fibre models by the process of relaxation, which also occurs whenever the early steps are inhibited (Weber, 1958). Indeed, quite apart from the contribution of the many other steps which could be formulated, this dissociation of actomyosin is probably the one which mainly determines the high E_A of the overall process. As will be shown, it occurs to a greater or lesser degree at all three values of I even in the absence of ATP, when it has a negative heat of dissociation in every case, that is the dissociation of A–M decreases with temperature. Taking all these considerations into account, it is not surprising that the E_A of the magnesium-activated process is extremely high and, moreover, rises as I increases, that is as the actomyosin dissociation increases. The calcium-activated process is, by contrast, very much simpler, first because little or no contraction occurs concomitantly with it and secondly because it behaves as if it involved only myosin filaments. In line with this view, we have shown that it is disturbed only slightly by previous supercontraction of the fibrils and further that its E_A is in the normal range, despite the considerable swelling accompanied by actual extraction of actomyosin from the fibrils, which can occur, particularly at 0° and at 18·5° and I 0·250. We can formulate its first steps as

$$\text{AM} + \text{ATP} \underset{K_2}{\overset{K_1}{\rightleftarrows}} \text{AM–ATP} \longrightarrow \text{AM–ATP*}, \qquad (a')$$

$$\text{AM–ATP*} \underset{K_4}{\overset{K_3}{\rightleftarrows}} \text{AM} \begin{matrix} \diagup \text{ADP*} \\ \diagdown \text{P}_i \end{matrix}, \qquad (b')$$

and a dissociation step

$$\text{AM–ATP} \rightleftarrows \text{A–ATP–M} \rightleftarrows \text{A} + \text{ATP–M}. \qquad (c)$$

The dissociation reaction, however, has only a minor disturbing effect on V_m here in contrast with its very large effect in the magnesium system. Nevertheless, it may be this reaction which leads to the second and unique type of anomaly found in this system at I 0·150 and I 0·250, where E_A rises with temperature (cf. Table 6). We can easily imagine that the partial change of state of the myosin from gel to sol, which occurs respectively at 0° and 18·5° at these values of I, would increase the rate of hydrolysis out of proportion to the rate at 35°, where the myosin filaments probably remain intact during the course of the reaction.

We may conclude from the present results how important it is to study the reaction under conditions as nearly physiological as possible. To give an example of the confusion that may otherwise result, we have only to consider the steady velocities of hydrolysis (V_m) at physiological I (0·150) in the two systems. At 18·5°, for instance, the

ratio of V_m for the calcium system to V_m for the magnesium system is > 2, but at 35° it is only 0·8, because of the great difference in the energies of activation (cf. Tables 3, 4 and 6). Indeed, the true initial velocity of uncontracted fibrils in the magnesium system at 35° is probably at least 1½ times the V_m for the supercontracted fibrils in Table 3, so that under living conditions this system hydrolyses ATP nearly twice as fast as the calcium system, which is of course unaffected by the process of supercontraction. Allowing for a temperature of 38°, we may calculate the maximum velocity of hydrolysis in the living animal to be ~ 0·44 μmole of ATP/sec./mg. of fibrillar N, or ~ 5×10^{-4} mole/g. of fresh muscle/min. This is of the same order as the value of 10×10^{-4} calculated by Mommaerts (1950) from data for oxygen consumption, which necessarily involves a great number of assumptions. It is unfortunate, indeed, that we possess no reliable values for the heat production in a mammalian twitch with which to compare these rates of hydrolysis, whereas in the frog we have Hill's very accurate data for heat production (Hill, 1949) but little or no data for adenosine-triphosphatase activity. It is only when such data become available that it will be possible to test critically the various hypotheses concerning the role of ATP in contraction.

SUMMARY

1. The time course of the hydrolysis of adenosine triphosphate by rabbit myofibrils has been studied at temperatures of 0°, 18·5° and 35° and at ionic strengths (I) between 0·04 and 0·25, with Ca^{2+} ions, Mg^{2+} ions or a mixture of both as activators.

2. It has been found that the products inhibit the reaction, the inhibition being more pronounced in the calcium than in the magnesium system. The combined relative inhibition constant,

$$K_s/K_{P_i} + K_s/K_{ADP},$$

varies in the former system from ~ 1·0 to 0° to about 2·2 at 35° for I 0·042, and from ~ 0·8 to ~ 0·7 for the same temperature range at I 0·250. In the magnesium system, no inhibition by products occurs at $I \geqslant 0{\cdot}150$ at the two lower temperatures, but the inhibition constant at $I \leqslant 0{\cdot}150$ reaches values of ~ 1·00 at 35°.

3. When allowance is made for inhibition by the products in the calcium system, the so-called early explosive phase of the reaction is either absent or restricted to < 2 sec.; in the magnesium system a short explosive phase is always detectable, lasting for < 5 sec. at 18° or above, but is either absent or much reduced in fibrils supercontracted by pretreatment with adenosine triphosphate. It is suggested that the declining velocity during the explosive phase in this system is entirely due to reduction in the number of active enzyme sites during the process of supercontraction.

4. The progress curves of the calcium system and the product inhibition constants characteristic of it can be entirely converted into those characteristic of the magnesium system by addition of Mg^{2+} ions to one-seventh of the concentration of the Ca^{2+} ions. The explosive phase of the magnesium system and the characteristic supercontraction appear concomitantly. It is deduced that the Mg^{2+} ion is always the dominant ion when both ions are added together. It follows that it is the characteristics of the magnesium system which must be taken into account *in vivo*, where most of the Mg^{2+} ions are free and most of the Ca^{2+} ions are bound to protein.

5. The energy of activation of the hydrolysis process is ~ 11 kcal./mole in the calcium system, whereas it always exceeds 15 kcal./mole in the magnesium system, and *in vivo* is > 20 kcal./mole. It is suggested that these large differences are due to participation of sites on both the actin and the myosin filaments in the magnesium-activated process, but of only myosin sites in the calcium-activated.

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The Isolation of a New Lipid, Triphosphoinositide, and Monophosphoinositide from Ox Brain

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The classic work of Folch (1942) showed that inositol was a constituent of brain phospholipids and an inositol phosphatide fraction was isolated which was rich in these inositol lipids. This inositol phosphatide was subsequently subfractionated with solvents, and an inositol-containing phospholipid was isolated which was designated diphosphoinositide because it produced inositol diphosphate as a major product on acid hydrolysis (Folch, 1949). Also, a trypsin-resistant protein fraction was isolated from brain tissue. This fraction contained a phosphoinositide bound to it by a salt-like linkage so that it was not extracted with lipid solvents unless these were acidified (Folch, 1952; Lebaron & Folch, 1956).

The starting point of the present investigation was an attempt to prepare pure diphosphoinositide as a substrate for metabolic experiments. It was soon realized that the brain phosphoinositide fraction was heterogeneous and from it were isolated two other inositol-containing phospholipids, monophosphoinositide and a new lipid designated triphosphoinositide because it contains inositol and phosphate in the molar ratio 1 : 3. The fractionation of these inositol phospholipids was followed by examining ionophoretically the products they formed after deacylation by mild treatment with alkali (Dawson, 1960). This showed that only the monophosphoinositide was extracted from brain tissue with neutral lipid solvents such as chloroform–methanol unless the tissue was pretreated with acetone. This has led to a convenient method for the isolation of triphosphoinositide by removing it with acidified lipid solvents from brain tissue previously extracted with neutral solvents. The protein–triphosphoinositide complex obtained is then disrupted to liberate the free lipid.

This paper is concerned with the isolation of triphosphoinositide from brain tissue and describes some properties of the new lipid. It also reports the isolation and analysis of monophosphoinositide from the same tissue. A preliminary account of this work has been given (Dittmer & Dawson, 1960). Confirmation of the existence of triphosphoinositide in brain has also been recently obtained independently by Grado & Ballou (1960, 1961), who isolated inositol triphosphate from acid hydrolysates of purified diphosphoinositide preparations.

EXPERIMENTAL

Materials

Ox brain was obtained from the animal as soon as possible after death and transported to the laboratory packed in ice. No attempt was made to separate white and grey matter for the fractionation. Guinea-pig brains were removed from the animals a few minutes after killing with chloroform and accumulated in solid CO_2.

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