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A Study of the Adenosinetriphosphatase Activity of Developing Chick Muscle

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In 1939, Engelhardt & Lyubimova (1939) first made the important observation that a close association exists between the enzyme adenosinetriphosphatase (ATP-ase) and the myofibrillar protein, myosin, and they tentatively suggested that the enzyme might in fact form part of the myosin molecule itself. Since that time their observations have been amply confirmed by other workers. Moreover, the significance of the connecting link which is thus provided between the chemical events during muscle contraction and the protein systems presumed responsible for the mechanical changes has been widely appreciated. The muscle ATP-ase studied by Kielley & Meyerhof (1948, 1950) appears to be a distinct enzyme present in water extracts, and associated with the cell particulate bodies rather than with the myofibrils (Perry, 1952).

A study of the muscle ATP-ase during the early stages of development of the chick was begun in conjunction with other work on the developing tissue (Robinson, 1952*a, b*). The homogenized muscle was extracted with 0.6M-potassium chloride in 0.067M-borate buffer at pH 7. A sample of the extract was diluted to 0.05M-potassium chloride, when the myofibrillar proteins precipitated, and could be centrifuged down. The enzyme activity was determined in the whole extract, in the precipitate on dilution and in the residue left after extraction with the 0.6M-potassium chloride solution. The presence of a high proportion of the total ATP-ase activity in water extracts from muscle early in embryonic development led to a further more detailed study of such extracts.

EXPERIMENTAL

Most tissues possess a variety of enzymes whose action results in the liberation of inorganic phosphate. Thus, alkaline and acid phosphatases, pyro-

phosphatases and myokinase might interfere with any attempt to estimate a specific ATP-ase activity, and it is necessary to try to define conditions for an ATP-ase estimation which will minimize any interference by such systems.

In view of the importance of the myosin ATP-ase system, many workers have investigated its properties. Not all the results are in agreement, particularly with respect to the pH optimum and the effect of ions upon the activity. However, Mommaerts & Seraidarian (1947) have been able to account for most of the differences in terms of the extreme sensitivity of the myosin ATP-ase towards electrolytes, and to show that, provided the ionic conditions are strictly controlled, it is possible to develop procedures for the specific assay of myosin ATP-ase. It is activated by calcium ions at low potassium chloride concentrations and has its maximum activity around pH 9, though the behaviour is in general too complex to be described in terms of a constant pH optimum. Myokinase will have little effect on the initial velocity if this is measured in the presence of excess substrate; moreover, the specificity of a preparation and its freedom from alkaline and acid phosphatases and from pyrophosphatases can readily be tested.

It is more difficult to define conditions under which the Kielley & Meyerhof (1948, 1950) ATP-ase of muscle will not be estimated. This enzyme is, however, relatively unstable in the absence of cyanide ions and has a maximum activity in the presence of magnesium ions at pH 6.8: the test conditions to be described are such as to minimize its contribution to the activity measurements.

Preparation of adenosinetriphosphate. The barium salt of adenosinetriphosphate (ATP) was prepared by a method similar to that described by Needham (1942) by Mr E. J. Morgan. It was converted to the sodium salt as recommended by Bailey (1942). The salt solution was freed from

iron by shaking with 8-hydroxyquinoline in CHCl_3 at pH 7 until no coloration was given by the 2,2'-dipyridyl test for iron. The solution was then shaken with pure CHCl_3 to remove 8-hydroxyquinoline and finally the CHCl_3 was removed *in vacuo*. The free inorganic phosphate, estimated by the Fiske & Subbarow (1925) method, was negligible in these preparations. ATP concentrations are expressed in terms of acid-labile phosphorus, which forms two-thirds of the total phosphorus, and hence twice the amount liberated by myosin ATP-ase. It is estimated by hydrolysing at 100° for 10 min. in N-HCl under which conditions both pyrophosphate linkages are broken (Bailey, 1949).

Preparation of adult fowl myosin ATP-ase. Muscle dissected from the breast was finely cut with scissors in an extractant having the following composition: 0.6 M-KCl, 0.067 M-borate, pH 7.4. After homogenization in the Waring Blendor for 30 sec. the suspension was stirred for 15 min. and finally centrifuged for 30 min. at 1200 g. The supernatant fluid was diluted to 0.05 M-KCl and the precipitate centrifuged down. Further purification was achieved by dissolving the precipitate in 0.8 M-KCl and, after centrifuging off any insoluble material, precipitating by dilution to a molarity of 0.05 M. The preparations were twice reprecipitated in this way so as to be practically free of any myokinase activity. Glass-distilled water was used throughout and the temperature was maintained at 0°.

Preparation of fractions from embryonic muscle. The extracting solution had the same composition as in the preparation of the adult ATP-ase. From 4 to 8 g. of breast muscle were harvested and a muscle extract prepared as before in 0.6 M-KCl-borate.

A sample of the extract was diluted to 0.05 M-KCl and the precipitate spun down on the centrifuge and retained. This myofibrillar fraction was expected to contain most of the ATP-ase activity and was not further purified. The whole extract of embryonic muscle, the precipitate on dilution of the extract and the residue left after extraction were tested for their ATP-ase activity. The total enzyme content of the residue was never more than 5% of that of the myofibrillar fraction.

Preparation of aqueous extracts of embryonic muscle. Muscle dissected from the breast was homogenized in the Waring Blendor with an equal volume of glass-distilled water for 30 sec. and the suspension stirred for 15 min. at 0°. After centrifugation for 30 min. at 1200 g, second and third extractions were carried out and the supernatants combined at neutral pH.

Conditions of enzyme tests. These were developed on the basis of an examination of the ATP-ase associated with the adult myofibril. It was assumed that the enzyme present in the developing tissue would behave similarly, and consequently the test conditions were such as to be optimal for the adult ATP-ase. However, in order to increase the specificity of the test, the activity was measured at two pH values, 8.8 and 6.6. If the ratio of activities at these two values remains constant it provides additional evidence that the same enzyme system is present throughout in all the fractions.

The incubation system recommended by Bailey (1942) was used. The final concentrations in all the tests were as follows: ATP, 500–600 μg . acid-labile P/ml.; CaCl_2 , 0.02 M; KCl, 0.1 M; buffer solution, 0.066 M. Glyoxaline (buffering range: pH 6.2–7.8), 4-methylglyoxaline hydrochloride (buffering range: pH 7.0–8.25) and 2,4-dimethylglyoxaline hydrochloride (buffering range: pH 8.0–9.3) were used as

buffers covering the whole range pH 6.2–9.3. The tubes were incubated at 25° for periods of 1, 2, 3, 4 and 5 min. at two pH values, 8.8 and 6.6, and the reaction arrested by adding trichloroacetic acid to a final concentration of 5% (w/v).

Under the above conditions excess ATP was present throughout, and there was no falling off in the rate of liberation of inorganic phosphate. This method of activity determination seems preferable to the single reading generally used, since any decrease in activity with time is immediately apparent.

RESULTS

Preliminary investigations of the activity of adult fowl myosin adenosinetriphosphatase

The behaviour of adult fowl myosin ATP-ase appears to be directly akin to that associated with rabbit myosin. The pH-activity curve shows two peaks, at pH values of 8.8 and 6.6, in glyoxaline buffers containing 0.1 M-potassium chloride and 0.02 M-calcium chloride at 25° (Fig. 1). The enzyme is

Fig. 1. pH-Activity curve of adult fowl myosin ATP-ase at 25° in glyoxaline buffers containing 0.1 M-KCl and 0.02 M- CaCl_2 .

relatively unstable at high pH values and with periods of 10 min. incubation considerable inactivation occurs even at pH 8.5. The varied values for a pH optimum reported in the literature (Banga, 1942; Laki, 1943; Biro & Szent-Györgyi, 1949) may be due to such partial inactivation and it seems essential that only initial velocities should be used in the determination of the myosin ATP-ase pH curve.

In the presence of low concentrations of potassium chloride the enzyme shows marked calcium activation and very slight magnesium activation, in general agreement with previously published observations on rabbit ATP-ase (Bailey, 1942). In a typical experiment in 0.1 M-potassium chloride at pH 8.8, 15 μg . P were liberated in the absence of calcium and magnesium ions, 18 μg . P in the presence of 0.02 M-magnesium chloride and 47 μg . P in the presence of 0.02 M-calcium chloride, in 2 min. at 25°. Glyoxaline buffers have consistently given the highest activity.

The adenosinetriphosphatase activity of developing muscle

The results will be presented (a) in terms of the activity of the proteins associated with the myofibril; that is, the sum of the activities of the precipitate obtained on dilution of the 0.6M-potassium chloride extract and of the residue left after the extraction; and (b) in terms of the activity of the sarcoplasmic proteins not precipitated on dilution, the latter being calculated from the difference between the activities of the whole extract and the myofibrillar precipitate.

studied almost all of the ATP-ase remains in the supernatant solution on dilution of strong salt extracts of the muscle. As growth continues there is a progressive decrease in the proportion of ATP-ase in this fraction and a corresponding increase in the proportion associated with the precipitated myofibrillar proteins. Even at hatching, however, only 65% of the total ATP-ase, as measured under the specified test conditions, is associated with the myofibrillar protein precipitate.

When the activity of the myofibrillar fraction is expressed in terms of its protein content (using the data of Robinson, 1952*a*, *b*) it rises to a peak prior to

Fig. 2. The distribution of ATP-ase activity in the muscle of the developing chicken.
x, myofibrillar ATP-ase; ⊕, sarcoplasmic ATP-ase.

Table 1. *The adenosinetriphosphatase activity of the myofibrillar protein fraction of developing muscle*

Average weight of chick (g.)	Activity (mg. P/min./g. myofibrillar protein at 25°)*
10.3	36.7
14.7	38.6
23.3	55.8
25.3	53.3
Hatching	
134	34.0
750	35.0

* Corrected for the nucleoprotein contribution.

The percentage of the total ATP-ase possessed by each of these fractions at different periods is shown in Fig. 2. In the earliest developmental stages

hatching (Table 1). A similar peak value was reported by Herrmann, Nicholas & Vosgian (1949) in studies on developing rat muscle.

The adenosinetriphosphatase activity of water extracts of embryonic chick muscle

The forms of the two curves in Fig. 2 would suggest at first sight that a process exists whereby the ATP-ase activity passes into the myofibrillar fraction and becomes closely associated with the fibril as development proceeds; in other words, that a water-soluble ATP-ase becomes associated with the myofibril and gives rise in the adult to an ATP-ase firmly combined with the myosin proper. However, no direct estimation of the ATP-ase in

water extracts had thus far been made, and a detailed examination of these was therefore undertaken.

pH-Activity curve. This was determined in the presence of 0.2M-potassium chloride and 0.001M-calcium chloride; it again shows two peaks, a large one in the alkaline region about pH 8.8–9.0 and a smaller peak around pH 6.6–7.0. Although the preparation is rich in myokinase, as almost 100% of the total acid-labile phosphorus is released on long incubation at neutral pH, this activity should have no effect on the pH curve which is based on measurements of the initial velocities.

Specificity of the preparation. The activity of the preparation towards inorganic pyrophosphate and β -glycerophosphate was tested. Pyrophosphate was not attacked either at pH 8.8 or at pH 7.0, at which latter pH pyrophosphatase activity is usually maximal. At pH 8.8 the activity towards β -glycerophosphate was very slight amounting to only 5% of that towards ATP.

Influence of ions. The activity in the presence of different ions suggested a closer relationship to the adult myofibrillar ATP-ase than to the Kielley & Meyerhof (1948, 1950) enzyme (Table 2), since the activity is raised markedly in the presence of calcium ions but little in the presence of magnesium ions.

Table 2. *The influence of ions upon the activity of the adenosinetriphosphatase present in water extracts of chick embryo muscle*

(Activity measured at pH 8.8 in 2:4-dimethylglyoxaline buffer.)

Ions present in addition to K ⁺ and Cl ⁻	Activity (μ g. P released/3 min.)
No addition	21.5
Mg ⁺⁺ (0.02M)	29.0
Ca ⁺⁺ (0.02M)	76.0

Miscellaneous observations. When stored at pH 7 at 0° the enzyme loses activity only at a slow rate, but is much less stable at lower pH values. Thus at pH 5, 80% of the activity is lost in 5 min. at 0°. Fractionation with ammonium sulphate shows that the enzyme is precipitated over a wide range between 20 and 50% saturation. Some of the enzyme activity is lost after precipitation with ammonium sulphate and a further diminution occurs on dialysis. Using the unit of activity, *Qp*, defined by Lyubimova & Engelhardt (1939) as the volume of a hypothetical gas (μ l.) equivalent to the amount of P (in μ g.) liberated by 1 mg. of myosin in 1 hr., freshly prepared water extracts give values as high as 1500 units at 25°. These can be compared with average values for freshly prepared fowl myosin ATP-ase which range from 1300 to 1500 at 25° and are similar to those obtained by Bailey (1942) for chick myosin at 37°. Extraction of the

tissue with 0.1M-potassium chloride instead of water gave an extract of comparable activity.

In all these respects the activity of water extracts shows a closer resemblance to the adult myosin ATP-ase than to the Kielley & Meyerhof (1948, 1950) system, though the latter is associated with the cell granules which would also aggregate and come out of solution at similar ammonium sulphate concentrations.

High-speed centrifugation of water extracts

Centrifugation of a water extract of embryonic muscle at 13 000 *g* for 60 min. left some 60% of the ATP-ase in the supernatant while about 40% appeared to be associated with the precipitate.

Some preliminary electron microscope studies of this precipitate, made in collaboration with Mr R. W. Horne of the Cavendish Laboratory, Cambridge, showed that in addition to the cell granules, it contained fragments of elementary myofibrillar material. Such material has been studied in some detail in preparations from the whole embryonic muscle. Although fibrillar in nature it appears to be very fragile and tenuous, and has a much simpler banding system than can be observed in similar preparations from the adult muscle.

Examination with the electron microscope of the residue remaining after water extraction of embryonic muscle shows that this fraction contains similar rudimentary myofibrils and, in addition, considerable amounts of reticular material, the presence of which was anticipated from histological knowledge of the composition of the embryonic tissue (Robinson, 1952*a*, Fig. 8).

DISCUSSION

The present studies have shown that in the earlier developmental stages of the chick only a small proportion of the total muscle ATP-ase is present in the precipitate formed on dilution of strong salt extracts of muscle, most of the activity remaining in the supernatant solution. With further development a progressive change in the distribution occurs until in the adult practically the whole of the ATP-ase activity is associated with the myofibrillar precipitate.

In previous work on the protein distribution in embryonic muscle (Robinson, 1952*a*) low concentrations of ethanol have been used to complete the precipitation of the myofibrillar proteins on dilution of a strong salt extract of the muscle. Because of this difference in procedure no direct comparisons can be made between the protein distribution found then and the ATP-ase distribution studied here, since in the embryonic extracts ethanol may have caused the precipitation of the

sarcoplasmic ATP-ase and its estimation with the myofibrillar proteins.

In view of the activity of the Kielley & Meyerhof (1948, 1950) ATP-ase in adult muscle, it is necessary to examine more closely the estimation procedures which have been used. It seems clear from the well defined calcium activation and optimum activity around pH 8.8 shown by all the enzyme preparations from embryonic muscle, that the main enzyme studied is very similar to that first described by Engelhardt & Lyubimova (1939). The Kielley & Meyerhof ATP-ase is magnesium-activated and has an optimal activity around pH 6.8. On these grounds our observations support the view that in our studies on the embryonic tissue we have been mainly concerned with the Engelhardt-Lyubimova enzyme. It seemed likely that the Kielley & Meyerhof ATP-ase might have been active in that fraction precipitated on high-speed centrifugation of water extracts. However, detailed studies of the activity of this fraction show that in the embryonic preparations the calcium-activated enzyme is responsible for most of the activity even at pH 6.6. As anticipated, in similar preparations from the adult, the magnesium-activated system prevails. The author is grateful to Dr S. V. Perry (1952) for information concerning the tests he has elaborated to distinguish between these two enzymes.

Herrmann & Nicholas (1948) studied the Ca^{++} -activated apyrase activity of developing rat muscle and found that after the 16th day of incubation the total activity, expressed in terms of the dry weight of the muscle, begins to rise from a low level and about 3 weeks after birth reaches an approximately constant value. Later studies (Herrmann *et al.* 1949) of particular protein fractions, showed that at the earlier developmental stages only a small amount of the total apyrase activity was contributed by the myofibrillar fraction, in general agreement with our results.

The finding that water extracts of embryonic muscle contain an adenosinetriphosphatase which is apparently closely related to that associated with myosin in the adult, is particularly striking in view of the evidence for the possible identity of myosin and that enzyme (Bailey, 1942; Bailey & Perry, 1947; Szent-Györgyi, 1947). At present, however, it does not seem possible to decide whether we are concerned with a true separation of myosin and its ATP-ase activity, or whether the results are explicable in terms of the elaboration of the myo-

fibrillar elements of muscle without assuming such a separation of protein and enzyme.

An interpretation in the latter sense is certainly possible. For instance, a water-soluble prototype of the myosin molecule possessing ATP-ase activity can be envisaged, or possibly the elaboration of the adult myofibril may proceed by some form of aggregation of elementary protofibrillar structures which in the earlier embryonic stages might be present in water extracts. The electron microscope work described may be quoted in support of such a view. Certainly such concepts would account for the ATP-ase distribution with which we have been concerned without the necessity of questioning the identity of myosin and ATP-ase.

SUMMARY

1. The distribution of adenosinetriphosphatase activity has been studied in the developing chick embryo.

2. Muscle extracts containing the sarcoplasmic and myofibrillar proteins have been prepared in 0.6M-potassium chloride-borate solution and the myofibrillar proteins precipitated on dilution to 0.05M-potassium chloride. In the adult most of the muscle adenosinetriphosphatase is present in the precipitate in association with the protein, myosin.

3. The adenosinetriphosphatase activity of the whole extract and of the precipitate has been determined at different developmental stages. In the earliest embryos studied the percentage of the total activity in the sarcoplasmic fraction is high in comparison with that in the myofibrillar protein fraction alone. As development proceeds the distribution changes and the enzyme becomes more closely associated with the myofibril.

4. The adenosinetriphosphatase activity of the sarcoplasmic fraction has been studied in water extracts of embryonic muscle. Its relation to the adult myosin adenosinetriphosphatase and to that associated with the cell particulate systems of muscle has been considered. The work cannot be related to previous studies on the protein distribution in chick muscle since the methods of preparation of the fractions are different. Some possible interpretations of the results are discussed.

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The Amino-Acids and other Ampholytes of Urine

1. A GENERAL METHOD OF ISOLATION

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The nitrogenous constituents of urine have been studied by many workers since the earliest days of biochemistry. Various methods have been devised for the estimation of the amino-acids and other nitrogenous components, but the actual isolation of the large variety of substances present in relatively small amounts has proved to be tedious and time-consuming. The invention of paper chromatography and the production of the newer synthetic ion-exchange resins has now provided a means whereby further studies of the nitrogenous substances which occur in urine have become more practicable. New interest in such methods has been stimulated by the discovery that in the routine examination of normal and pathological urines by paper chromatography carried out by Dent (1947, 1948) and his co-workers during the past 5 years, ninhydrin-reacting spots have been observed which could not be ascribed to any of the known naturally occurring amino-acids. Moreover, in almost every case, the positions of the unknown spots on the paper chromatograms were unchanged after hydrolysis by acid and this indicated that the unknown constituents were probably amino-acids and not peptides. Several of these substances have now been isolated from urine and preliminary communications have been published (Crumpler, Dent, Harris & Westall, 1951; Dent, Fowler & Walshe, 1951;

Searle & Westall, 1951). The early stages of the method used for the isolation of these amino-acids were essentially the same in each case, and in order to avoid unnecessary repetition the general method of separation will be described in this paper, whilst the details of the isolation of the individual substances will be given in later papers of this series.

The procedure employed has evolved from studies on displacement chromatography on the synthetic ion-exchange resins Zeo-Karb 215 and De-Acidite E (Partridge & Westall, 1949; Partridge & Brimley, 1949). The fractionation of the amino-acids of acid-hydrolysed proteins of egg white has been described (Partridge, 1949) and further application has led to the isolation of γ -aminobutyric acid from beetroots (Westall, 1950*a*) and for the preparation in quantity of glutamine from the same source (Westall, 1950*b*). The application of the method, now modified by using the anion-exchange resin Dowex 2 instead of De-Acidite E, to the isolation of amino-acids from urine has not, so far, been described in detail.

General principles

The direct application of diluted urine to the Zeo-Karb columns resulted in the production of a gel within the column which disturbed the even flow of the liquid. This was due partly to traces of protein but also to some other ultrafilterable substance.