THE CYTOLOGICAL DISTRIBUTION IN PIGEON SKELETAL MUSCLE OF ENZYMES ACTING ON PHOSPHORYLATED NUCLEOTIDES*

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The elaboration of high energy phosphate bonds has assumed a crucial role (1) in the energy conversions associated with the maintenance of biological systems. The transfer of this bound energy is mediated principally through the turnover of adenosinetriphosphate¹ which is derived either from aerobic respiration or by glycolysis in animal tissues when integrated enzyme systems catalyze the esterification of adenine nucleotide acceptors with inorganic phosphate. The bulk of the evidence for this concept is derived from studies on tissue dispersions or "cell-free homogenates" which are essentially suspensions of cellular particles obtained by mashing or grinding tissues until few intact cells remain. Several workers have shown that the metabolic activity of mashed tissue is brief but can be slightly prolonged by procedures which generate and conserve the high energy phosphorylated intermediates. It is not oxidation alone but the extent of accompanying oxidative phosphorylation and the utilization of phosphorylation energy which determine the structural and metabolic perpetuation of the constituents of tissue dispersions (2). Under such severe treatment as maceration it is not very remarkable, as Barron, Ardao, and Hearon (3) so aptly demonstrate in their studies, that the metabolic patterns and pathways of disrupted and whole cells are very different.

It is pertinent, therefore, to inquire into the circumstances which contribute to alterations in metabolism when cells are ruptured. The injurious effect of breaking cells has been attributed to the dissolution of organization and the consequent release of autolytic enzymes normally segregated in the cell among the different discrete cellular particles. A rapid depletion of ~P reserves en-

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¹ The following abbreviations are used in this article:—

ATP—adenosinetriphosphate

ADP—adenosinediphosphate

A-5MP—muscle adenylic acid

A-3MP—yeast adenylic acid

~P—high energy phosphate

P—phosphate group

sues because the most active enzymes in this group are those which operate on the ATP and ADP compounds (4). Because of this vigorous dephosphorylation which accompanies the dispersion of cytoplasm, the efficiency of oxidative phosphorylation is difficult to assess accurately, and the $\sim$ P trapped by the use of artificial hexokinase systems is an approximate estimation of the synthesis of bonds which escape cleavage. The suggestion has been offered (1), therefore, that the complete separation of subcellular constituents from one another is highly desirable prior to their use in metabolic studies. Careful isolation of the different particles is most likely to prevent the intervention of destructive phosphorolytic enzymes and offers a closer approximation toward normal intracellular separation of enzymes. The present study is oriented to determine the pattern of localization in the pigeon breast muscle of several enzymes which specifically act upon the adenine nucleotides.

The details of the myofibrillar and sarcomeric structure of skeletal and cardiac muscle have been amply documented and the fine interrelations established by electron microscopy (5). On the other hand, the interfibrillar structures have been less emphasized, although the presence of a discrete sarcoplasm and sarcoplasmic granules was observed (6) contemporaneously with the earliest comprehensive description of the fibrillary structure of skeletal muscle (7). Koelliker (8) accurately described the occurrence of the sarcoplasmic particles and called them interstitial granules, signifying thereby certain large, irregular or oval particles which lie between the fibrils, swell in dilute salt solutions, and constitute an essential part of muscle tissue. Later Floegel (9) found a second small, isodiametric granule which he distinguished from the Koelliker granule because of differences in size and shape. Holmgren, a pupil of Retzius, grouped these 2 granules together as "sarcosomes" and classified them into Q and J granules depending on the position they occupied adjacent to the Q and J segments of the sarcomere (10). This nomenclature permitted the development of an hypothesis which specified a direct intervention of the granules in the cycle of contraction and restitution. Because of the comparative cytologic investigations of several workers (11, 12), the synthetic schemata of Holmgren were partly stultified when the large "true interstitial granules" of Koelliker were identified with Altmann's "bioblasts" (13) or the mitochondria. Current studies with more comprehensive morphologic and biochemical criteria have fortified this identification (14). Less has been said of the smaller granules, except that Jordan (15) preferred to regard them as precursors of the larger granules on histologic evidence alone.

We should, perhaps, use a comprehensive term to include all the types of granules found in muscle, so we have selected the term cytochondria used by Opie (16) to describe "all of the cytoplasmic bodies that are recognizable. They are essential constituents of the cytoplasm and include those that stain or fail to stain as mitochondria." In this we adhere to the original opinion of Koelliker that the cytoplasm in the muscle fibers is chiefly between and not in the myofibrils which are primarily contractile. The cytochondria of skeletal muscle are comprised, therefore, of the mitochondria and the small particles of Floegel, which later will be designated specifically as sarcosomes. Fat granules or liposomes may also be included under cytochondria and, perhaps, contain the structures described as Golgi bodies in other types of cell.

Preparation of the Tissue Fractions

1. Preliminary Procedure.—Adult pigeons were decapitated and exsanguinated. The pectoralis major muscle was removed, sliced coarsely, and immersed in ice-cold water. During all subsequent manipulations the preparations were kept at temperatures between 0–4°C. To obtain a consistent number of granules in each preparation, care was taken to use only the pectoralis major muscle because the pectoralis minor, located just beneath it, contained too few granules. 20 gm. of the muscle was finely chopped with scissors and homogenized for 45 seconds in a precooled stainless steel Waring blender (250 ml.) in 150 ml. of cooled (0–4°C.) 0.25 M sucrose containing 1.5 ml. of 0.04 M sodium bicarbonate. A further 150 ml. of 0.25 M sucrose was added; the homogenate was stirred, strained through 1 layer of gauze (type II, 32 by 28 mesh) to remove fibrous tissue and collected into cooled pear-shaped centrifuge tubes (Fig. 1). After an initial period of 2 minutes of centrifugation at 125 g in the free swinging horizontal head, the speed was rapidly increased to apply a force of 500 g for a further 10 minutes. This alternation of force was applied to effect a differential layering of the heavier particles into separate layers and to facilitate clean harvesting of the uppermost layer.

The supernatant (S_1) (Text-fig. 1 and Fig. 1), consisting of mitochondria, sarcosomes, microsomes, and a few myofibrils, was transferred to another set of pear-shaped centrifuge tubes. The residue consisted of 2 separate layers. The upper layer (A, Fig. 1) yellow in color with a reddish tint contained numerous mitochondria with some myofibrils and nuclei. The lower layer, (B, Fig. 1) which was usually in the stem of the tube, contained a larger proportion of nuclei, a considerable number of myofibrils, and both red blood cells and some unbroken fibers. The upper layer was stirred up in 0.25 M sucrose and redispersed for 10 seconds in the blender to break coarse clumps. This fraction and S_1 were recentrifuged in separate sets of pear-shaped centrifuge tubes at 500 g for 15 minutes to remove contaminating myofibrils. The supernatant (S_2), consisting of mitochondria, microsomes, and sarcosomes, was transferred to 50 ml. lusteroid tubes for further centrifugation, and the residue (R_2) was resuspended in 0.5 M sucrose and saved for the preparation of the nuclear myofibril fraction.

2. Preparation of the Cytochondrial (C) Fraction.—The supernatant (S_2) was centrifuged at 2250 g for 15 minutes. The tightly packed residue was resuspended in 0.25 M sucrose and recentrifuged at 2250 g for 15 minutes. The final residue was suspended in 0.25 M sucrose to make a final volume of 4 ml. cytochondrial suspension.

3. Preparation of the Nuclear Myofibrillar (NM) Fraction.—The suspension of R_2 was homogenized in the blender for 3 minutes to separate the myofibrils from one another and also from other structures. Crushed ice was added during this process to maintain proper cold conditions during the longer period of comminution. The dispersate was passed through one layer of surgical gauze to remove the ice and centrifuged at 500 g for 6 minutes in the pear-shaped centrifuge tubes. The supernatant was carefully removed with a small pipette, and the loosely packed reddish residue was resuspended in 0.5 M sucrose and recentrifuged at 500 g for 6 minutes. This process was repeated to purify the nuclear myofibrillar fraction. The final residue, consisting of a reddish, loosely packed layer, was suspended in 0.5 M sucrose to make a final volume of 4 ml. of the nuclear myofibrillar suspension.

Analytical Procedures

Unless otherwise stated, dephosphorylation of the adenine nucleotides was measured in a medium consisting (in final concentrations) of sodium diethyl barbiturate buffer at pH 7.6, 1.8×10^{-2} M, sucrose, 0.25 M, and either calcium or magnesium chloride at optimal levels as determined experimentally. Substrates were used in variable amounts as determined by the levels of activity.

The temperature of the medium was raised to 37°C. before 0.1 ml. of the cold enzyme was

TEXT-FIG. 1.

² In the various centrifugations an International refrigerated SB-1 centrifuge was used. The centrifugal forces designated indicate those at the middle of the tubes.

added to make the total volume of 3.5 ml. Incubation was done in a Dubnoff shaking incubator at 37°C. for varied periods. The reaction was stopped by adding trichloroacetic acid to a final concentration of 5 per cent. After the protein precipitate was removed by centrifugation, 1.0 ml. aliquots of the clear supernatant was taken for the determination of orthophosphate by the method of Lowry and Lopez (17) using a wave length of 660 $m\mu$ in a Coleman universal spectrophotometer No. 14.

Adenylic acid deaminase activity was measured in a medium of 0.25 M sucrose containing 1.0×10^{-2} M sodium diethyl barbiturate buffer pH 7.6 and a final concentration of 10^{-2} M adenylic acid. After the temperature of the medium had been raised to 37°C. in a Dubnoff shaking incubator, 0.1 ml. of the cold enzyme suspension was added to make a total volume of 1.0 ml. At the end of the incubation period 0.1 ml. samples were transferred directly to the outer chambers of Conway's micro-diffusion units containing 0.1 ml. of saturated KOH which stopped the enzymatic reaction and released ammonia (18). The diffused ammonia was absorbed in the central chambers containing 5 per cent boric acid and Conway's indicator. A standard N/10 HCl was titrated against the content of the central chambers to determine the amount of ammonia present in the samples. Conway's special horizontal micro-burette was used throughout for the titration. Suitable blanks were run, and the differences between the ammonia content of the blanks and the samples were taken as the amount of ammonia formed during the period of incubation.

For purposes of comparison the activities of different suspensions were calculated on the basis of the contained nitrogen. The nitrogen contents of the samples of the various fractions were determined according to the method of Johnson (19). Readings were taken in a Coleman universal spectrophotometer No. 14 at 480 $m\mu$ as recommended. The ATPase activity is then expressed as Q_P (N), which represents P in microliters of gas at NTP liberated per hour by an enzyme preparation containing 1 mg. of nitrogen. Similarly, the adenylic acid deaminase activity is expressed as Q_{NH_3} (N), representing microliters of ammonia gas at NTP liberated per hour by a suspension containing 1 mg. of nitrogen. The activity values presented in this paper are the highest values obtained when the systems are saturated with substrates.

The Q_P value proposed by various authors (20), based on the conversion of nitrogen content of the preparation into a protein value, is applicable to a pure myosin preparation. However, in preparations consisting of cell particulates the protein values obtained by such procedure do not represent the actual protein content of the suspensions because of the presence of various types of protein which require different conversion factors.

Materials Used.—Disodium ATP (Pabst), containing 0.04 μM of inorganic phosphate per mg. of ATP as an impurity, was used in this study. The easily hydrolyzable phosphorus content was determined by hydrolysis in 1 N HCl at 100°C. for 7 minutes.

Barium ADP (Sigma) was converted into a potassium salt by removal of barium as a sulfate salt in 0.1 N H_2SO_4 and neutralization with 0.1 N K_2CO_3 . The easily hydrolyzable phosphorus was determined as in ATP.

Muscle and yeast adenylic acid were obtained from the Schwarz Laboratories, Inc. Glucose-1-phosphate (Nutritional Biochemical Corp.), as the potassium salt, was neutralized with 1.0 per cent KOH. Phenolphthalein phosphate (Paul Lewis Laboratories), as the sodium salt, was neutralized with 0.1 N HCl.

Cytological Techniques

The various stages of differential centrifugation and particle separation were checked as a routine with phase microscopy.³ This examination is essential at each definitive step to ensure

³ A Spencer (American Optical Co.) phase microscope combination, including a turret condenser attached to a Spencer binocular microscope model 15, was used in these studies. The eye pieces were compensating 15.0 Huyghenian type. The objectives used were the 1.0

the purity of the suspensions. Employment of standard gravitational forces and times of operation occasionally failed to achieve clear partition of the different components so that additional brief centrifugations and washes were necessary. The most reliable criteria for identifying the cytological constituents were the specific morphologic features which characterize them, such as the sarcomeric structure of the myofibrils, the composite internal structure of the mitochondria (21), and the appearance peculiar to the nuclei (22). Because of the rapidity required for examination during the fractionation procedures, the slowness with which supravital dyes adhered to particles precluded their use. It has also been our experience that the staining by Janus green B, pinacyanole, and cresylecht violet in cell-free dispersions is not limited to the mitochondria. We find the affinity of these dyes both low and not selective for isolated mitochondria. Pinacyanole is especially liable to color nuclei more intensely than the mitochondria. Nile blue sulfate also very slowly stains the mitochondria.

For the protracted study of cytological detail, thin slices of intact muscle tissue and samples of suspensions were fixed in Regaud's fluid and in buffered 2 per cent osmium tetroxide, embedded in paraffin, and sectioned at a thickness of $4\ \mu$. Several procedures of staining were selected for their ability to distinguish the mitochondria (23). For the sharpest distinction between myofibrils and mitochondria, the aniline-fast green FCF procedure was modified by including 2.5 per cent phloxine B or rose bengal as a counterstain and by passing directly from this dye solution through a wash of absolute acetone into xylol before mounting in clarite. The mitochondria acquired the specific green hue; the myofibrils, a contrasting red purple color. The suspensions which were mixed with equal volumes of buffered 2 per cent osmium tetroxide for 30 minutes were centrifuged and obtained free of the osmium by several washes in distilled water. They were suspended in a small volume of distilled water and either examined directly by phase microscopy unstained or were first smeared on a slide and selectively stained by the aniline-fast green procedure.

RESULTS

Cytology

The pigeon muscle tissue used for these studies contains two varieties of myofibers, one filled with granules and a second in which granules are rare (Figs. 2 to 4). Counts on fixed preparations give an estimate of 20 per cent as the incidence of non-granulated fibers in this muscle. Figs. 5 to 7 reveal that the granules or cytochondria *in situ* are intimately related to one another and to the myofibrils. The relation of the sarcomeres to the cytochondria is inconstant, but an alternate disposition of mitochondria and sarcosomes in linear regularity is conspicuous, though many mitochondria are not separated from their adjacent neighbors by sarcosomes (Figs. 8 to 10). This cytological distribution shows the close relationship of the cytochondria to one another as well as the myofibrils.

As is illustrated in Figs. 11 to 13 the nuclei and myofibrils in the NM suspension are well preserved. The former have sharp membranes, distinct nucleoli, and appear intact. In view of the tremendous shearing force of the Waring blender and the ice crystals used for cooling purposes, the ability of the nuclei

mm. B-minus M, dark contrast-M, dark contrast-L, and light contrast-M. As a light source the Spencer illuminator 735 was suitable. For photography a Bausch and Lomb hyperplane $10\times$ ocular was preferred and a Spencer R-4L research microscope employed.

to survive in such large numbers, up to 10 per cent of the particles, is a criterion of their extraordinary toughness. The striations in the separated myofibrils are conspicuous and indicate that the conventional intrafibrillary structure is not drastically altered. Most fibrils are in a relaxed or slightly contracted state. On the addition of 0.01 M ATP in final concentration to a coverslip preparation in the presence of 0.01 M KCl and 0.05 M $MgCl_2$, they are observed to enter into rapid syneresis which verifies the integrity of the actomyosin component of the myofibrils in these suspensions. The NM suspensions contain very few mitochondria, either in the free state or locked between the small clusters of myofibrils. Some vascular strands and nerve fibers are seen, but they comprise a small percentage of the total.

On the other hand, the suspension of cytochondria consists of mitochondria and sarcosomes which separate out together and is referred to as the C suspension. The C suspension is composed of a mixture of these two granules in about equal proportion and is almost completely free from the grosser nuclei and myofibrillar fragments. It has not been feasible with the present centrifugal procedures to prevent some contamination of C suspensions with the finest fragments of myofibrils which approximate in size the dimensions of the cytochondria and sediment with them. Such fragments usually constitute about 1 to 2 per cent of the particles in the C suspensions in either fresh or stained preparations.

In a dark field illustration of a typical C suspension (Fig. 14), the tiny highly refractile particles represent the sarcosomes and the larger, vaguer granules the mitochondria. It is pertinent that no intermediate granules occur (Figs. 15 to 17) as transitional forms between those two and that they differ radically in size, shape, position, osmophilia, staining capacity, and swelling reaction to dilute salt environment. The salient morphologic differences between the two types of particles are compared in Table I to emphasize their disparity. The capacity of the mitochondria to swell and their composite internal structure are the most characteristic features which distinguish them from the osmotically inert and isodiametric sarcosomes.

Dephosphorylating Activity of the C and NM Suspensions

The purified C suspensions cause a rapid liberation of orthophosphate from ATP. If aliquots of substrate at various concentrations are reacted with the standard 0.1 ml. aliquots of C suspensions containing 1.7 to 1.9 mg. N/ml., each during an incubation period of 5 minutes, the amount of phosphate released is maximal when 7 μM . of ATP are present in the system (Text-fig. 2). A further increase of available ATP permits no additional liberation in the amount of orthophosphate. Consequently, a system containing ATP in excess of 7 μM . is considered to be saturated with this substrate during the 5 minute incubation period selected for routine analysis.

It is necessary to be certain that the reaction is caused by a specific enzyme

TEXT-FIG. 2. Rates of ATP dephosphorylation by C suspension with respect to various concentrations of the substrate. Standard conditions were used without additional activating cations. The total amount of ATP in the reacting system is represented on the abscissa. The ordinate represents orthophosphate liberated in 5 minutes by 0.1 ml. of the C suspension.

TABLE I
Comparison of Morphologic Characteristics of Mitochondria and Sarcosomes of Pigeon Breast Muscle

Property	Mitochondria	Sarcosomes
Size.....	2 to 10 μ	0.1 to 0.3 μ
Shape.....	Irregular rods	Round or elliptical
Refractility.....	Slightly refringent	Strongly refringent
Osmophilia*.....	Slight	Strong
Chromophilia (aniline dyes)....	Specific	Slight
Osmotic swelling.....	Characteristic	Absent
Solubility in organic solvents....	Slight	Complete
Orientation.....	Parallel to myofibrils	Transverse to myofibrils

* Osmophilia is the property of certain cellular structures of staining black with tetroxide of osmium.

which acts only upon ATP and not by any of several isodynamic phosphatases. In answer to this possibility the C suspension when allowed to act in the presence of a series of selected substrates is able to release orthophosphate from only ATP and ADP in significant quantity (Table II). This eliminates the presence of pyrophosphatase, non-specific phosphatases, and adenylase at the

pH of 7.6 used in the system, but it leaves open the question of whether there is a mixture of ATPase and ADPase activity since both ADP and ATP are attacked, though at different rates, or whether there is a single enzyme or apyrase (24) which splits both these linkages without discrimination between the di- and triphosphorylated nucleotides.

The enzymatic dephosphorylation by the C suspension is further elucidated by study of reaction rate by the suspension in the presence of an ATP concentration below the saturation level. With such restriction and in the presence of optimal concentration of calcium ions, orthophosphate is rapidly split off from ATP in the first 10 minutes. In its subsequent behavior it is apparent that the C suspension is bimodal and exhibits two distinct periods of enzymatic ac-

TABLE II
Dephosphorylating Activities of the C and NM Suspensions on Various Substrates

Substrates 5 μ M (Total)	Amount of P liberated*		
	C suspension	NM suspension	
	1 $\times 10^{-3}$ M Mg.†	7 $\times 10^{-3}$ M Ca†	5 $\times 10^{-3}$ M Mg†
	μ M	μ M	μ M
ATP	5.38	3.79	2.56
ADP	1.96	0.00	0.00
A-5MP	0.00	0.00	0.00
A-3MP	0.00	0.03	0.00
Glucose-1-P	0.00	0.00	0.00
Na-P-P	0.13	0.00	0.00
Phenolphthalein-P	0.20	0.00	0.00

* The activity was measured under standard conditions as described in the text with an incubation time of 15 minutes.

† Final concentrations of various ions are as listed.

tivity. Nearly half of the total labile phosphate is liberated within 10 minutes, after which with depletion of ATP the reaction becomes almost asymptotic. After 30 minutes the rate of dephosphorylation rises again and attains a second peak by 40 minutes (Text-fig. 3). This second rise corresponds with the release of the second half of the available labile phosphate and is manifested in the curve by the orthophosphate released in excess of one equivalent of ATP. When magnesium is present in place of calcium, the dephosphorylation follows the same pattern, although the liberation of the labile phosphate occurs more rapidly. The latent period (arrow in Text-fig. 3) is brief and is followed in 20 minutes by the second period of dephosphorylation which reaches maximum by 30 minutes. Whereas in the medium containing calcium only 15 per cent of the second labile phosphate is split during 60 minutes of incubation, nearly 65 per cent is freed during the same period of action in the presence of

magnesium. When the C suspension is then allowed to act upon ADP, it is found that calcium and magnesium have the same relatively proportionate effect on the rate of dephosphorylation as they exert on the release of the second moiety from ATP (Text-fig. 4). After 60 minutes of incubation 19 per cent of labile phosphate is released under the influence of calcium and 66 per cent is split away in the presence of magnesium; these figures correspond closely with

TEXT-FIG. 3. Dephosphorylation of ATP by C suspension. Standard conditions, and $5 \mu\text{M}$ of ATP were used. A and B represent systems containing $1 \times 10^{-3} \text{ M}$ MgCl_2 and $8 \times 10^{-3} \text{ M}$ CaCl_2 (final concentrations), respectively. C is a system in which no cations were added. The horizontal broken line at the level of $5 \mu\text{M}$ P represents orthophosphate liberated in amount equivalent to ATP initially present in the system. Arrows indicate a second rise in the rate of dephosphorylation.

the 15 per cent and 65 per cent respectively for the separation of the second labile phosphate of ATP in association with the 2 ions. Because of the bimodality of behavior, the answer seems, therefore, to be more in favor of 2 separate enzymes acting upon ATP rather than the cleavage of the pyrophosphate bond by a non-specific apyrase.

It is clear, therefore, that the dephosphorylation of ATP by the C suspension is not caused by a non-specific phosphatase but that 2 enzymes appear to be involved in the reaction. As alternative mechanisms both the action of a

specific ADPase and the dismutation of ADP by myokinase are possible as follows:—

The application of several experimental procedures in accordance with the known kinetic properties of the myokinase enzyme has established the occurrence of the latter mechanism in the present instance.

TEXT-FIG. 4. Dephosphorylation of ADP by C suspension. Standard conditions, and 5 μM ADP were used. A and B represent systems containing 1×10^{-3} M MgCl_2 and 8×10^{-3} M CaCl_2 (final concentrations) respectively.

From a consideration of the myokinase reaction it is feasible to expect a retardation of activity when A-5MP is accumulated or added in excess and Kalckar has demonstrated this (25). In Text-fig. 5 it is manifest that A-5MP in the system under study exerts an appreciable inhibitory effect in the period when the second labile phosphate is under attack. For further proof of the identity of the second enzyme, its acid and heat stability are assessed. When myokinase and ATPase are boiled in an acid medium, the latter is destroyed whereas the former is still active. The C suspensions subjected to this maneuver are without ATPase activity or ADPase activity. When this suspension is preincubated for 30 minutes with ADP, the subsequent addition of the NM suspension effects a rapid splitting of orthophosphate (Table III). From the fact that the NM suspension has a powerful ATPase activity and no ADPase

TEXT-FIG. 5. Effect of A-5MP on the dephosphorylation of ATP by C suspension. Standard conditions were used. A is a system containing $5 \mu\text{M}$ ATP; B, a system containing, in addition to $5 \mu\text{M}$ of ATP, an equivalent amount of A-5MP. The horizontal broken line represents orthophosphate liberated in amount equivalent to ATP initially present in the system.

TABLE III
Comparison of Dephosphorylating Activity of Untreated C Suspensions with Boiled and Acidified C Suspensions

System	Substrate $5 \mu\text{M}$ (Total)	Boiled and acidified C suspensions	NM suspension	Untreated C suspension	Amount of P liberated
					μM
1	ADP*	+	—	—	0.00
2	ATP*	+	—	—	0.00
3	ADP	—	+	—	0.00
4	ADP*	+	+	—	0.92
5	ATP	—	+	—	3.79
6	ADP	—	—	+	1.96
7	ATP	—	—	+	5.38

The activities were measured under the standard conditions during an incubation time of 15 minutes in the presence of Mg^{++} at its optimal levels for the enzyme preparations. The boiled and acidified C suspension was prepared by boiling 3 ml. of the standard C suspension for 1 minute in the presence of 0.1 ml. of 0.1 N HCl. In the system containing the acidified and boiled cytochromes (Nos. 1, 2, and 4), 0.5 ml. of the suspension was added to the standard medium containing $1 \times 10^{-3} \text{ M}$ Mg^{++} . The reaction was stopped after 30 minutes of incubation except for No. 4 in which, at the end of this period, the Mg^{++} level was raised to the optimal level of the myofibrillar ATPase ($5 \times 10^{-3} \text{ M}$) and 0.1 ml. of the fresh NM suspensions added. After further incubation of 15 minutes the reaction was stopped.

effect (Table II) it is evident that ATP is formed from ADP by the acidified and boiled mitochondria and that the addition of NM suspension reveals the accumulation of this ATP. The untreated C suspension completes the dephosphorylation of ATP down to A-5MP by the combined activity of a specific ATPase and myokinase.

On the other hand, the NM suspension contains only an ATPase (Table II and Text-fig. 6) which is specific and differs in many ways from the ATPase found in the C suspensions. It contains no other phosphotransferase or dephosphorylase active at pH 7.6. Both the cytochondrial and myofibrillar

TEXT-FIG. 6. Dephosphorylation of ATP by NM suspension. Standard conditions, and $5 \mu\text{M}$ of ATP were used. The final concentration of CaCl_2 present in the system was $5 \times 10^{-3} \text{ M}$.

ATPases occur in high concentration and may be distinguished especially by their selectively different behavior in the presence of various cations.

Effect of Cations on ATPases of C and NM Suspensions

ATPase of C Suspension.—The influence of ions was tested in a system saturated with ATP and incubated for only 5 minutes to minimize the myokinase activity which becomes appreciable after longer incubation periods (Text-fig. 3) as indicated in the preceding experiments. Under these conditions when the effects of various concentrations of different cations on the rate of dephosphorylation are assayed, it is clear (Text-figs. 7 and 8) that the degrees of activation incurred and the optimal levels required for the greatest activation are very different for the several ions tested. Magnesium is singular

TEXT-FIG. 7. Effect of cations on the ATPase activity of the suspension. Standard conditions, and $10\ \mu\text{M}$ of ATP were used. The abscissa represents the final concentration of cations; the ordinate indicates orthophosphate liberated in 5 minutes. A and B represent systems containing MgCl_2 and CaCl_2 respectively.

TEXT-FIG. 8. Effects of Na and K on the ATPase activity of C suspension. Standard conditions, and $10\ \mu\text{M}$ of ATP were used. A is a system activated with $1 \times 10^{-3}\ \text{M}$ MgCl_2 before the additions of NaCl and KCl ; B, a system in which no magnesium was added. Broken lines show the effect of sodium; solid lines that of potassium. The ordinate represents orthophosphate liberated during a period of 5 minutes.

in the considerable augmentation of rate it induces over a relatively wide range of concentrations, although its stimulatory effect is sharpest at a level of 1×10^{-3} M. Calcium ions, on the contrary, produce some inhibition in the lower ranges of concentration and evoke only slight activation at the optimal level. Other cations tested are similar to calcium in the minimal stimulation which they effect.

If the system already contains an optimal concentration of magnesium, however, the introduction of additional sodium or potassium ions greatly depresses the rate (Text-fig. 8). This depression is proportional to the quantity of sodium and potassium added, although the potassium ion has a more profound effect than an equal concentration of sodium. It is apparent that in the presence of magnesium, the potassium and sodium ions dampen the dephos-

TABLE IV
The Activities of the Cytochondrial and Nuclear Myofibrillar ATPases in the Presence of Optimal Levels of Cations

Cations	Cytochondrial ATPase		Nuclear-myofibrillar ATPase	
	Molarity (final)	$Q_F(N)$	Molarity (final)	$Q_F(N)$
None	0.0	5,000	0.0	3,000
K ⁺	0.2 M	6,500	0.08 M	4,000
Ca ⁺⁺	8×10^{-3} M	7,500	9×10^{-3} M	11,000
Mg ⁺⁺	1×10^{-3} M	21,000	5×10^{-3} M	7,000
Mg ⁺⁺ and Ca ⁺⁺	Optimal*	6,300	Optimal*	7,500
Mg ⁺⁺ and K ⁺	Optimal*	10,000	Optimal*	7,000

* In experiments with mixtures of ions the same optima for Mg⁺⁺, Ca⁺⁺, and K⁺ are used as were employed in the single ion experiments.

phorylation and alter the rate toward the level obtained in the "magnesium-free" system.

In a study of the activating effects of the different cations, it is not logical to compare their rates at the same ionic concentrations. There is no biological precedent for this, and in fact, the disparity in the concentrations of the ions in biological systems is not consistent with such a comparison. Therefore, their activities are matched at their optimal concentrations without implication that such levels are operative *in vivo*. In Table IV the correlated maximal or *in vitro* optimal activities are arranged for the different ions. The accentuated response of the enzyme to magnesium establishes the specific effect of this ion for the cytochondrial ATPase.

ATPase of the NM Suspension.—In contrast with the ATPase activity of the cytochondria, calcium and magnesium ions affect the dephosphorylation process of NM suspensions in an entirely dissimilar manner. With calcium the ATPase activity is especially enhanced and has a maximal activity extending

over an exceptionally wide range of ionic concentration (Text-fig. 9). Both magnesium and the potassium ions display sharper maximal concentrations, but with these ions the enzyme has a much lower optimal rate in comparison with the calcium-activated system.

One of the most significant phenomena which occurs under the agency of ions is the effect of combining the optimal levels of calcium and magnesium. Since each by itself enforces a stimulation, their combination might be expected to be additive, if not synergistic. In Text-fig. 10 it is shown that such a mixture, however, produces a profound depression of the activity. It is,

TEXT-FIG. 9. Effects of cations on the ATPase activity of the NM suspension. Standard conditions, and 5 μ M of ATP were used. The dephosphorylation of ATP during a period of 15 minutes in the presence of various concentrations of cations are shown as indicated by A, CaCl_2 and B, MgCl_2 .

moreover, a peculiarity of this enzyme that potassium restricts the rise in the calcium activation.

The ATPase found in the NM suspensions is manifestly different from that situated in the C suspension in relation to cation activation. The evidence adduced in the establishment of the discrete behavior of the enzymes, as well as their separate localizations, is brought together in Table IV. In view of the fact that differently located enzymes are under consideration, the summation analyses of the type exemplified by Schneider (26) merit consideration but are not used here for several reasons. The most salient difficulty which militates against such a study of summation is the unusual toughness of the tissue. Application of the comminution which can effectively separate out all

the mitochondria from the myofibrils leads to excessive destruction of the fragile mitochondria and, consequently, stultifies the purpose of the operation. Consequently, the partition of the mitochondria and the nuclei and myofibrils into pure suspensions with widely divergent activities has obviated the necessity of an elaborate summation which elsewhere has subserved a useful purpose. It is, moreover, obvious in Table IV that since the optimal ionic concentrations required for quantitation of the differently located enzymes are very disparate, the value of summation experiments is equivocal.

TEXT-FIG. 10. The effect of Mg on the ATPase activity of the NM suspension in a system containing Ca. Standard conditions, and $5 \mu\text{M}$ of ATP were used. The ATPase activity during a period of 15 minutes is indicated by A, in various final concentrations of CaCl_2 , and B, in various concentrations of CaCl_2 and of $5 \times 10^{-3} \text{ M}$ MgCl_2 .

Distribution of Adenylic Acid Deaminase

Adenylic acid deaminase activity is detected in both the C and NM suspensions. The distribution between them, however, is unequal, with the enzyme located preponderantly in the NM suspensions. The action in the NM suspension is rapid and effects almost complete deamination of the substrate after an hour. In contrast, the C suspension has feeble activity (Text-fig. 11) which splits off ammonia from only 7 per cent of the available substrate in an hour. It is possible that the environment of the enzyme, *i.e.* its cytological position, affects its activity because the deaminase associated with the NM suspension is linear until the substrate is nearly exhausted when it tends to an asymptote. On the contrary, the deaminase in the C suspensions has completed its maximal action within a few minutes and rapidly approaches an

asymptote long before substrate depletion becomes a limiting factor. This suggests a quick inactivation or inhibition of the cytochondrial enzymes under the applied experimental conditions.

The difference in activity of the 2 suspensions is conspicuous when the deamination rates are compared on the basis of their Q_{NH_3} (N) values. The NM suspensions had a Q_{NH_3} (N) of 11,800 whereas that for the C suspensions was only 814 or less. It is evident that adenylic acid deaminase is predominantly located in the NM suspensions where its survival under *in vitro* conditions is

TEXT-FIG. 11. Adenylic acid deaminase activity of the C and NM suspensions. Standard conditions were used. The ordinate represents micrograms of $\text{NH}_3\text{-N}$ liberated by an enzyme preparation containing 0.05 mg. protein-N. A is the deaminase activity of the NM suspension; B, that of the C suspension.

prolonged. The preexistence of the enzyme among the cytochondria prior to comminution may be challenged because of the exceptionally low Q_{NH_3} (N) value compared with that in the NM suspension. In view of the occasional myofibrillar fragments of size approximating mitochondrial dimensions which can be encountered in the C suspensions as illustrated above in the section on cytology, it is not unlikely that the slight activity might be attributable to contamination with myofibril fragments or actomyosin.

DISCUSSION

The present studies demonstrate that several enzymes acting on phosphorylated nucleotides are selectively located in the cytochondria or the myofibrils

of pigeon skeletal muscle. Although the magnesium activated ATPase is found among the cytochondria, the evidence does not suggest exclusive presence in either the mitochondria or the sarcosomes. Perry (27) has shown that a magnesium-activated ATPase, similar to that of Kielley and Meyerhof (28), is present among the lipoprotein granules isolated from rabbit muscle, but his electron microscope studies have not exactly identified the granules. Examinations by phase microscopy of rabbit psoas and thigh muscles and rat leg muscles in this laboratory reveal that there are cytochondria which are composed of many sarcosomes and a few mitochondria. It is uncertain which muscles Perry used as a routine, and precise selection is important because there is considerable difference in the granular composition among various rabbit muscles. According to the centrifugal forces he used for the isolation of the granules it is probable that his preparations contained a large proportion of sarcosomes, some "microsomes," and a few mitochondria. Yet, although our cytochondria are predominantly mitochondrial and his lipoprotein granules are mainly microsomes and sarcosomes, as he implies, both preparations contain considerable magnesium-activated ATPase. It seems likely, therefore, that a similarly activated enzyme is present in both the sarcosome and the mitochondrion. On this account the development of the granules from a common precursor or the transformation of one into the other type, as originally suggested by Jordan (15), merits consideration.

Our findings have paralleled essentially those of others (27, 28), except for a few discrepancies. There are differences in the optimal levels of activating ions required for our cytochondrial ATPase and for the muscle ATPases of these workers. This can possibly be explained by the differences in media used for isolation and suspension of the granules. Other factors which may contribute to the diversity in ion activation are the buffers used in assay, the pH levels, and the several types of animal employed. Some of these agents may account also for the "lability" found by other workers, but which is not a feature of our cytochondrial enzyme.

SUMMARY

A procedure has been described for the centrifugal fractionation of the cytological components of pigeon breast muscle. An analysis of the distribution of enzyme activity among the different particles reveals a predominant location of magnesium-activated ATPase and myokinase in the cytochondria. The myofibrillar nuclear components are the site of calcium-activated ATPase and adenylic acid deaminase.

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EXPLANATION OF PLATES

PLATE 27

FIG. 1. The conical centrifuge tube contains a homogenate of pigeon breast muscle in 0.25 M sucrose, which has been subjected to 500 g for 10 minutes. Three phases are illustrated, a supernatant suspension of mitochondria, a buff colored sediment *A* composed of both mitochondria and myofibrils, and a red sediment *B* consisting of few mitochondria and mostly of myofibrils, unruptured cells, and nuclei. $\times \frac{1}{4}$.

FIG. 2. Two fibers of pectoralis major muscle. One fiber is rich in interstitial granules. Stained with aniline-fast green FCF and rose bengal. $\times 1000$.

FIG. 3. Fibers of *M. pectoralis major*. The one fiber is replete with granules; the other is free of granules, has conspicuous conventional cross-striations. Stained by Regaud's iron hematoxylin and safranin. $\times 1000$.

FIG. 4. A pair of fibers of pigeon *M. pectoralis major*, stained by Benda's aniline gentian violet technique. The non-granular fiber has evident transverse striations. $\times 1000$.

(Kitiyakara and Harman: Enzymes acting on phosphorylated nucleotides).

PLATE 28

FIG. 5. Portion of an unfixed myofiber contains transversely striated myofibrils between which are discrete, dense, rod-shaped mitochondria and a few round, dense sarcosomes. Phase contrast, dark L. $\times$ 2700.

FIG. 6. Several myofibrils with interstitial mitochondria in fresh homogenate. Phase contrast, dark L. $\times$ 2700.

FIG. 7. The myofibrils are in contracted state, with conspicuous Z line and vague A disc. Several mitochondria are seen, one spanning up to four sarcomeres. Phase contrast, dark L. $\times$ 8100.

FIG. 8. Portion of a fiber is so focused to obliterate details of myofibrils and define the cytochondria clearly. The linear arrangement of the units is evident. Intercalated between the rod-shaped mitochondria are small, isodiametric, spherical, and highly refractile sarcosomes. Phase contrast, dark L. $\times$ 2700.

FIG. 9. The alternately arranged mitochondria and sarcosomes are manifest. Some sarcosomes are paired. Phase contrast, dark L. $\times$ 8100.

FIG. 10. A mitochondrion is shown with a highly refractile sarcosome adherent to both extremities. Phase contrast, dark L. $\times$ 8100.

FIG. 11. A fresh NM suspension. Both nuclei and discrete, myofibrillar fragments comprise the preparation. No mitochondria are seen. Phase contrast, dark L. $\times$ 1080.

FIG. 12. The myofibrils of an NM suspension are in relaxed, in partly contracted and strongly contracted state. Transverse striations are conventional. Phase contrast, dark L. $\times$ 5100.

(Kitiyakara and Harman: Enzymes acting on phosphorylated nucleotides)

PLATE 29.

FIG. 13. An NM suspension is depicted by dark field illumination. Numerous, finely striated myofibrils and occasional nuclei are seen. $\times 1080$.

FIG. 14. A fresh C suspension in 0.5 M sucrose is seen with dark field illumination. The larger, vague, ring forms represent the mitochondria. The minute, brilliant light points are sarcosomes. Because of the relatively greater Brownian movement of the sarcosomes many of their shapes appear angular. $\times 2700$.

FIG. 15. Linearly arranged mitochondria and sarcosomes comprise the only visible particles. Intervening myofibrils are not seen. A group of three sarcosomes separates one pair of mitochondria. Phase contrast, light M. $\times 8100$.

FIG. 16. Alternately situated mitochondria and sarcosomes. Myofibrils are inconspicuous. Phase contrast, light M. $\times 8100$.

FIG. 17. The discrepancy in refractility between the rodlet mitochondria and round sarcosome is seen. A few mitochondria are not separated by sarcosomes. Phase contrast, light M. $\times 8100$.

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