# Liberation of Inorganic Phosphate from Adenosinetriphosphate by Fractions Derived from Developing Rat Muscle.* (17466)

HEINZ HERRMANN, J. S. NICHOLAS, AND M. E. VOSGIAN

*From the Osborn Zoological Laboratories, Yale University, New Haven, Conn.*

The systematic investigation of chemical changes occurring in differentiating muscle tissue was begun[1,2] by assaying quantitatively the main protein fractions at different stages of development. The direct continuation of this analysis was not immediately possible because the preparative characterization of the most significant muscle proteins, the myosin group, was impeded by the similarity in solubility of the nucleoproteins[3] and the discrepancy between the increase in quantity of the myosin proteins and the activity of adenosinetriphosphatase which usually are thought to be closely associated. As an explanation of this discrepancy (1) an appreciable increase in the enzymatic activity in fractions other than the myosin proteins, or (2) an increase in activity of the myosin fraction itself or (3) a simultaneous occurrence of both have been considered. These possibilities have been tested experimentally and the results are here described.

*Experimental*: ATPase activity determinations. About 500 mg of fresh, minced muscle tissue were homogenized in 10 cc of .5 M KCl in the cold. The pH was adjusted to 7.5 and the homogenate was divided into two 5 cc portions. One was stored in the cold for a determination on the whole (W). The other was subjected to protein fractionation as follows: the homogenate was stirred for one hour at 40°F, and then centrifuged for ½ hour at 3,000 r.p.m. The residue is R. The supernatant was carefully adjusted to pH 5.7 by adding split drops of .04 N HCl. The insoluble material was separated by centrifugation for ½ hour and denoted as $M_1$. The supernatant was diluted with 2 volumes of water, and then centrifuged for ½ hour. The residue is $M_2$. The supernatant is S. The fractions so separated were rehomogenized in 5 cc of .5 M KCl, and the pH was adjusted to 7.2-7.5. After dilution of each fraction and the whole, so that the activity would fall within a measurable range, .2 cc portions were taken for ATPase activity determinations, which were performed by the procedure of Dubois and Potter.[4] The ATP was prepared according to the method of Dounce, *et al.*[5]

Tubes set up for phosphate determination contained the following: .60 cc of 2.5% ammonium molybdate in 5 N $H_2SO_4$, .25 cc of sample, distilled water to dilute to 3.00 cc, and lastly, 15 cc of .25% 1-amino, 2-naphthol, 4-sulfonic acid. Phosphate standards and a blank were run concomitantly. The contents were thoroughly mixed and heated in a water bath at 37°C for 5 minutes. After cooling the samples to room temperature, colorimetric readings were taken on the Beckman spectrophotometer at 660 mμ.

Nitrogen determinations for each fraction in suspension were made by the Kjeldahl method modified by Boell[6] after precipitation with trichloracetic acid (100 g to 100 cc) to yield a solution concentration of 5%.

*Results and discussion*: The enzymatic liberation of inorganic phosphate from adenosine triphosphate by 4 protein fractions obtained from mesoderm or muscle tissue at different stages of development is shown in Table I. The activities are computed as phosphate liberated *either* per mg of N in the

* Supported in part by a grant from the American Cancer Society through the NRC Committee on Growth.

1 Herrmann, Heinz, and Nicholas, J. S., *J. Exp. Zool.*, 1948, **107**, 177.

2 Herrmann, Heinz, and Nicholas, J. S., *J. Exp. Zool.*, 1948, **107**, 165.

3 Herrmann, Heinz, and Nicholas, J. S., *J. Exp. Zool.*, 1949, **112**, 253.

4 Dubois, K. P., and Potter, V. R., *J. Biol. Chem.*, 1943, **150**, 185.

5 Dounce, A. L., Rothstein, A., Beyer, G. Thannhauser, Meier, R., and Freer, R. M., *J. Biol. Chem.*, 1948, **174**, 361.

6 Boell, E. J., *Trans. Conn. Acad. Arts and Sci.*, 1945, **36**, 429.

TABLE I. Activities in μg Phosphorus Liberated in 15 Min. at 37.5°C per mg N.

| Fraction | 14 days gestation | | | 19 days gestation | | | 1-2 days after birth | | | 9-10 days after birth | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | No. rats | Range | Avg | No. rats | Range | Avg | No. rats | Range | Avg | No. rats | Range | Avg |
| | | | | | | Activity of individual fraction | | | | | | |
| R | 2 | 66-80 | 73 | 2 | 480-500 | 490 | 3 | 720-1000 | 850 | 3 | 610-720 | 670 |
| $M_1$ | 3 | 55-140 | 91 | 2 | 550-680 | 615 | 4 | 720(1400)*-1700 | 1300(1500) | 3 | 860-1300 | 1050 |
| $M_2$ | 3 | 50-88 | 70 | 2 | 82-88 | 85 | 4 | 110(560)-870 | 530(670) | 4 | 420-850 | 570 |
| S | 4 | 170-300 | 220 | 2 | 410-420 | 415 | 4 | 460(630)-850 | 660(720) | 3 | 720-1100 | 920 |
| W | 2 | 120-130 | 120 | 2 | 400-890 | 645 | 4 | 830-980 | 910 | 4 | 800-1300 | 970 |
| | | | | | | Activity calculated per mg N of whole homogenate | | | | | | |
| R | | 47 | | | 200 | | | 360 | | | 300 | |
| $M_1$ | | 5 | | | 54 | | | 180(190) | | | 280 | |
| $M_2$ | | 15 | | | 40 | | | 48(62) | | | 50 | |
| S | | *66* | | | *250* | | | *170(190)* | | | *220* | |
| Σ | | 133 | | | 544 | | | 758(802) | | | 850 | |
| W | | 120 | | | 490 | | | 910 | | | 970 | |
| (W) | | 95 | | | 570 | | | 570 | | | 950 | |

Σ = The sum of activities of single fractions per mg N of the whole homogenate.
(W) = Values recalculated from previous data for activities of the whole homogenate corrected for increase in activity in the presence of KCl.
* The wide spread is due to one determination which is out of line with the others. If this experiment were discarded the spread would be as indicated by the figures in parentheses.

fraction (fractional activity) or per mg of N in the whole homogenate (relative activity). The first way of computing indicates the changes of activity which occur during the development within the respective fraction, independent of the changes of the quantity of this fraction. The second method of computation provides an index for the contribution of enzymatic activity of one particular fraction relative to the enzymatic activity of the whole homogenate of muscle tissue. If the enzymatic activities for the single fractions computed for the total nitrogen are added, the activity obtained theoretically should be equal to the activity of the whole unfractionated tissue homogenate. The latter data correspond to the figures represented in the graph in the previous paper.[1] The figures obtained by computation for the total nitrogen, (relative activities) are dependent not only upon the activity in the respective fraction but also upon the quantity of this fraction relative to the other fractions composing the total homogenate. In order to show the scattering of our results we have listed in Table I the numbers of samples used and the range for the fractional activities and also the averages for single fractions and for the whole homogenate as well as the relative activities for the single fraction and for the sum of the fractions to be compared with the activity of the whole unfractioned tissue. From these figures it is quite evident that all fractions show a marked increase in activity. It can be noticed that in the 3 fractions, R, $M_1$, $M_2$, almost the entire increment is found for the development from the 14th to the 19th day of gestation, the period in which the most pronounced steps of structural and functional differentiation are taking place.

Fraction S increases at a steadier rate over the whole developmental period investigated. The increase in the relative activities (calculated per mg nitrogen of the total homogenate) differs from the fractional activities by its dependence upon the quantity of the respective fraction. A closer correlation of the two sets of figures was not attempted. However it can be clearly recognized that in the case of fractions R, $M_2$, and S the increases in the relative activities are of the same order of

magnitude as the increases in fractional activities. In contrast to these fractions an exceptionally great increase in the relative activity is found in the $M_1$ fraction. In this instance an increase of 14 times for the fractional activity is to be compared with an increase of about 56 times for the relative activity. This difference is due to the rapid increase in the quantity of the $M_1$ fraction which has been described before.

The values for the relative activities in Table I, when added (R, $M_1$, $M_2$, S) should equal the activities found in the whole homogenate (W). Since the two sets of figures are in close agreement, it can be concluded that the values secured for the relative activities give a correct measure for the proportion in which these individual fractions contribute to the activity of the whole homogenate. The increase in activity for the whole homogenate found in this series corresponds closely to the values obtained previously.[3] The present values are consistently somewhat higher than the previous ones since the activity of the whole when homogenized in KCl is 18% higher than when homogenized in water, due to activation by the K ion. For the first day after birth the earlier value (W), which is more than 35% lower than the recent value W, forms a "dip" in the curve in previous experiments. Since the rate of increase in activity is greatest up to the time of birth, the present higher value, W, is the more reasonable one. The difference between the rapid increase in enzymatic activity and the slow increase in the quantity of the total fraction of myosin proteins (the sum of the present fractions $M_1 + M_2$) which appeared previously as a discrepancy can now be explained. The former assumption was that most of the apyrase activity in muscle at all stages of development should be linked to the myosin protein fraction and should remain fairly constant for the unit of this fraction. It now appears that only a minor part of the total activity is contributed by the myosin proteins; although a large increase in activity does occur within the myosin fraction itself.

The problem of the nature of the proteins of the myosin group as well as the relationship of the different enzymes with apyrase activity is at present in a state of flux. As to the former, discussion centers around Szent-Gyorgyi's[7] separation of the myosin complex into actin and myosin, and recently Bailey seems to have found evidence for a new member of the myosin family (his tropomyosin). Enzymes with apyrase activity and distinctly different properties recently have been described from various sources[8–10] and even in muscle a distinction of ATPase enzymes was recently claimed. The fact that there is an increase during embryonic differentiation of all fractions of muscle tissue is in agreement with the observations of Potter,[11] Flexner[12] Barth,[13,14] and Moog[15] who find an increase in apyrase enzymes during differentiation in a great variety of mammalian and even invertebrate tissues. In these instances the increase means a more or less indirect role of apyrases in the elaboration and maintenance of specific structures and functions. In the $M_1$ fraction this relation is a more direct one since the development of enzymatic and structural properties of the myosin proteins in muscle represents directly the products of differentiation in a highly specialized tissue.

*Summary.* In a quantitative chemical study of developing muscle there is a greater activity of adenosinetriphosphatase than is warranted by the increase in amount of myosin proteins. In order to ascertain the reason for this discrepancy determinations of phosphatase activity were made on fractions of devel-

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oping muscle obtained from rat embryos of 14 days, fetuses of 19 days, rats of 1-2 days and 9-10 days of age. The activity was calculated in micrograms of phosphorus liberated in 15 min. at 37.5° per mg N.

The activity was determined for each of 4 fractions and the sum of these, the activity of the whole homogenate, was in close agreement with previous determinations of the whole homogenates.

All 4 fractions increase in activity during development; the $M_1$ fraction, containing the purest preparation of the myosin proteins develops in activity 6 times faster than the other fractions.

While the apyrase activity was formerly thought to be limited to the myosin protein fraction this contributes but a minor part of the total activity.

Received August 8, 1949. P.S.E.B.M., 1949, **72.**

## Role of Pyridoxine in the Production of Leucocytes in Normal and Leukemic Mice.* (17467)

DAVID R. WEIR, ROBERT W. HEINLE, AND ARNOLD D. WELCH

*From the Departments of Medicine and Pharmacology, School of Medicine, Western Reserve University, and the University Hospitals of Cleveland.*

Morgan, Groody, and Axelrod[1] showed that pyridoxine-deficient dogs developed a leucocytosis with an absolute increase in the numbers of circulating neutrophiles and an absolute decrease in the numbers of lymphocytes. These abnormalities could be corrected by giving adequate doses of pyridoxine. In the same year McCall, Waisman, Elvehjem, and Jones[2] described similar leucocyte changes in pyridoxine-deficient and in pantothenic acid-deficient monkeys. They stated that the reversal of the polymorphonuclear to lymphocyte ratio also occurred in riboflavin deficiency. In the pyridoxine-deficient animals the reversal could not be corrected completely by pyridoxine but could be corrected by whole liver. Wintrobe and his associates[3] observed a tendency of the granulocytes to increase in pyridoxine-deficient swine, but there was no reversal of the ratio of polymorphonuclears to lymphocytes. Stoerk, Eisen, and John[4] described thymus atrophy and a reduction in the number of fixed and circulating lymphocytes, and Stoerk[5] described lymphoid atrophy and regression of rat lymphosarcoma in pyridoxine-deficient rats. In order to facilitate the production of pyridoxine deficiency, Ott[6] investigated the antipyridoxine activity of desoxypyridoxine hydrochloride (2,4-dimethyl-3-hydroxy-5-hydroxymethylpyridine). He found that two molecules of the analogue were sufficient to offset the vitamin activity of one molecule of pyridoxine, as measured by chick growth. Under other conditions[7] much larger quantities of the analogue are necessary. These observations prompted us to investigate the effect of both pyridoxine deficiency and pyridoxine excess on the circulating leucocytes and hematopoietic organs of normal mice and of mice having various abnormalities of the leucocyte pattern.

*Pyridoxine Deficiency in Normal Mice.*

* This research was made possible by grants from the National Vitamin Foundation and the Price McKinney Memorial Fund.

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