Levels of Phosphate Compounds in Experimental Cardiac Hypertrophy

By Paul R. Minton, M.D., Paul M. Zoll, M.D., and Leona R. Norman, M.D.

The close association of cardiac hypertrophy with congestive heart failure is well established: cardiac enlargement is common clinically in patients with various types of chronic heart disease and is the only pathologic change almost invariably present in patients dying with chronic congestive heart failure. No single theory satisfactorily explains the mechanism of cardiac hypertrophy and the exact relationship of cardiac hypertrophy to congestive heart failure, as cause, result, or common effect of some other factor, remains obscure.

Olson and Schwartz suggested that metabolic failure of the heart occurs because of disturbances in energy production or utilization. A critical evaluation of these 2 mechanisms involves determination of the high-energy phosphate stores in the failing heart: normal levels of high-energy phosphate compounds in failing hearts would suggest normal energy production and defective energy utilization; low levels would indicate failure of energy production or conservation. Much data have been accumulated on the biochemical changes in cardiac failure, but little is known about the changes in high-energy phosphate that may accompany cardiac hypertrophy. Knowledge of these changes would clarify the pathogenesis of cardiac hypertrophy, especially its relation to heart failure. In this study we have investigated the changes in creatine phosphate and adenosine triphosphate in experimental cardiac hypertrophy and have attempted to correlate our findings with the results of similar investigations in experimental heart failure.

Methods

Development of Hypertension

White male rats of the Wistar strain, weighing between 80 and 120 Gm., were used. Arterial hypertension was produced by 3 methods: bilateral application of constricting latex capsules about the kidneys (group A); subcutaneous injection of desoxycorticosterone acetate (DOCA) (group B); and intramuscular implantation of desoxycorticosterone acetate pellets (group C). The pellet-treated groups received from 70 to 141 mg. desoxycorticosterone acetate. Pellets containing 15 or 25 mg. of the drug were implanted bilaterally in the dorsal musculature of the lumbar region. The injection group received 2.5 mg. desoxycorticosterone acetate in oil, 5 times weekly until a total of from 145 to 265 mg. was administered. In addition to the usual laboratory diet, both DOCA groups received physiologic saline (0.85 per cent) as their sole source of drinking water.

Measurement of Blood Pressure

Blood pressures were periodically measured by a modification of the indirect microphonie method of Friedman and Freed. Briefly, this method involved warming the animals at a temperature of 37 C. for 10 minutes, then placing them in a heated restrictive device, while the tail was placed through a small pressure cuff connected to a bulb and manometer. A small microphone was taped to the ventral surface of the tail, distal to the cuff, and the characteristic, rapid pulsations of the caudal artery (200 to 300 beats/min.) were heard by means of an amplifying stethoscope when the cuff pressure fell below arterial pressure. According to Friedman et al., the values obtained by this method do not represent systolic, diastolic, or mean pressure, but they do offer reliable relative measures of blood pressure in comparable groups.

Criteria for Hypertension and Hypertrophy

The criterion for hypertension was based, in part, on the observations of Friedman et al., in which an average indirect blood pressure of 121 mm. Hg was obtained in 8 control rats. In our ex-
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experience with more than 15 normal rats, the blood pressure ranged between 80 and 120 mm. Hg and rarely exceeded 130 mm. Hg. Severe hypertension was considered present in our series if the blood pressure exceeded 200 mm. Hg.

Cardiac hypertrophy was considered to be present if the ventricular weight exceeded the normal ventricular weight predicted on the basis of body weight. The predicted ventricular weight was obtained by deducting 5.5 per cent of total heart weight from Donaldson's predicted values for weight. The predicted ventricular weight was obtained by deducting 5.5 per cent of total heart weight from Donaldson's predicted values for weight. This corrective factor was used because in 13 normal animals of varying age and ranging in weight from 86 to 410 Gm., we observed that the combined atrial weight averaged 5.59 per cent of total heart weight. In 3 instances, the observed body weight exceeded the largest value in Donaldson's tables. The predicted ventricular weight in these cases was calculated from the formula of Hatai for heart weight of both sexes combined.

For statistical purposes the ratio of ventricular weight/total body weight was used, and a statistically significant increase in this ratio was found between the control (2.93) and hypertrophied (4.04) groups (p < 0.01). On this basis we classified the entire hypertensive group as cardiac hypertrophy, even though in 2 instances the observed ventricular weight did not exceed the predicted ventricular weight.

Chemical Methods

Animals were anesthetized with intraperitoneal pentobarbital (50 mg./Kg.); the ventricles were removed while still beating and frozen in a mixture of dry ice and ether. Not more than 5 seconds elapsed between severance of tissue and immersion in the liquid. The frozen tissue was crushed in a chilled steel pounder, transferred to a Potter-Elvehjem homogenization tube containing cold 10 per cent trichloroacetic acid (TCA), and quickly weighed on a triple beam balance. Homogenization of the tissue was performed in a cold room at approximately 4 C. The extract containing the phosphate fractions soluble in TCA was separated from the homogenate by centrifugation and made just alkaline (pH 8.2 to 8.8) to phenolphthalein. Homogenization and separation of the material remaining in the homogenization tube was repeated in a similar manner with 5 per cent TCA. Not more than 2 minutes elapsed from the beginning of homogenization to alkalinization of the separated extract. All extracts were kept chilled until alkalinization was completed.

The alkalinized TCA extract was analyzed for free inorganic phosphate, creatine phosphate, and the labile phosphate of adenosine triphosphate. The procedure used for fractionation and separation of the phosphate fractions was adapted from Fiske and Subbarow, as suggested by Uihlebreit, Burris, and Stauffer. Inorganic phosphate and adenosine triphosphate were precipitated at pH 8.2 to 8.8 with 10 per cent CaCl₂ saturated with Ca(OH)₂; creatine phosphate is soluble in CaCl₂ in this pH range. The precipitate containing inorganic phosphate and adenosine triphosphate was removed by centrifugation and washed with the CaCl₂ reagent, and the wash was combined with the supernatant fluid. Following addition of 1.0 N HCl, to dissolve the precipitate, and dilution with water, aliquots were removed for determination of inorganic phosphate and adenosine triphosphate.

Measurement of inorganic phosphate was made directly on a spectrophotometer; the labile phosphate of adenosine triphosphate was measured after 15 minutes' acid hydrolysis at 100 C. The labile phosphate of creatine phosphate was measured after 30 minutes' acid hydrolysis at room temperature. Final phosphate determinations in the inorganic form were made simultaneously in duplicate with a phosphate standard.

In preliminary experiments with known quantities of synthetic inorganic phosphate, creatine phosphate, and adenosine triphosphate, Ba(C₂H₅₀)₂ was used as a precipitating agent according to the method of LePage. Barium precipitation, however, involved a loss of 20 per cent total phosphate. The good recoveries obtained with CaCl₂ made it our choice as a precipitating agent.

New reagents were prepared every 4 to 5 weeks. To insure reliability of reagents control analyses on normal rat hearts were performed with them, and simultaneously with each analysis of a hypertrophied heart; if the simultaneous control analysis did not fall within the established range of normal values, all the results were discarded.

Precision and Validity of Methods

Periodic recovery analyses of known quantities of synthetic inorganic phosphate, creatine phosphate, and adenosine triphosphate were performed to test the accuracy of the method. The mean per centages of analytical recovery ± S.D. in 8 analyses are creatine phosphate: 94.7; adenosine triphosphate: 85.0; inorganic phosphate: 100.2. The high mean per cent recovery of inorganic phosphate (100 per cent) is not a true value but includes approximately 10 per cent additional labile phosphate liberated from adenosine triphosphate before separation. The average loss of 10 per cent adenosine triphosphate in recovery analyses suggests that the method may not be sensitive enough to demonstrate small differences in adenosine triphosphate between control and hypertrophied hearts. Actually a significant decrease in adenosine triphosphate was found between normal and hypertrophied hearts, but it did not exceed that which could be produced by dilution by edema.

*Coleman Model No. 14 at 660 μ.
Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Creatine phosphate</th>
<th>Adenosine triphosphate</th>
<th>Inorganic phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12.3</td>
<td>2.4</td>
<td>2.4</td>
</tr>
<tr>
<td>No. duplicate analyses</td>
<td>(74)</td>
<td>(79)</td>
<td>(78)</td>
</tr>
<tr>
<td>Hypertrophied</td>
<td>11.7</td>
<td>3.5</td>
<td>9.5</td>
</tr>
<tr>
<td>No. duplicate analyses</td>
<td>(38)</td>
<td>(42)</td>
<td>(44)</td>
</tr>
</tbody>
</table>

*Calculated from the formula $V = 100 \times \frac{\text{error of S.D.}}{X}$,

\[
\text{in which error of S. D.} = \frac{(a^2 + b^2) - \frac{a + b}{n}}{n},
\]

where $a$ and $b$ equal respective duplicate values.

The discrepancy in the number of duplicate analyses for the 3 phosphate fractions is due to individual experiments in which one or the other duplicate value was lost through technical error.

Duplicate analyses were performed routinely with all phosphate fractions for both control and hypertrophied hearts. The variations of duplicate analyses expressed as coefficient of variation are summarized in table 1. It was not possible to obtain satisfactory duplicate results in all instances due to loss of a duplicate aliquot through technical failure.

Although the method is generally considered to be reliable when standardized by the individual laboratory, it is subject to criticism, especially since nonspecific acid hydrolysis fails to distinguish between the labile phosphates of adenosine triphosphate and adenosine diphosphate. Furthermore, in addition to the biologic variation of the experimental animal used, there is a considerable degree of variation inherent in the chemical technic itself. For example, the irregular degree of hydrolysis of creatine phosphate resulted in greater variation between duplicate aliquots than in the other fractions. Because of these factors no difference was considered statistically significant unless the $p$ value was less than 0.01.

**Results**

Table 2 presents the results of attempts to produce arterial hypertension and cardiac hypertrophy in 151 rats. The animals are grouped according to the method used to produce hypertension and according to the number in which hypertension and cardiac hypertrophy were obtained. Severe hypertension was demonstrated in 66 animals. The duration of hypertension ranged from more than 1 month in 48 animals to more than 5 months in 13. Severe hypertension was not observed in 4 animals whose hearts were hypertrophied, 2 each in groups A and B. In each of these animals there was difficulty in obtaining satisfactory blood pressure readings. Of the 48 animals that survived to time of biochemical analysis, cardiac hypertrophy was found in 43.

It is apparent from table 2 that the ease with which arterial hypertension is produced in rats varies with the procedure used. Of the 3 methods employed, the DOCA pellet procedure proved the most satisfactory. The surgery involved in placing latex capsules about the kidneys was attended by a high postoperative mortality rate. The manipulation and trauma accompanying the repeated DOCA injections resulted in a greater mortality rate than in the pellet-treated group. Pellet implantation requires a single, simple operative procedure, performed under light ether anesthesia, with minimal subsequent manipulation of the animals. Although small differences in the chemical levels were noted among the 3 groups, comparable differences were noted in the simultaneous control analyses. None of these differences was statistically significant.

The results of biochemical analyses for the myocardial levels of phosphate fractions in 80 control and 45 hypertrophied rat hearts are summarized in table 3. From these data it appears that cardiac hypertrophy is accompanied by a statistically significant decrease in the ventricular concentration of creatine phosphate, adenosine triphosphate, inorganic phosphate, and in total TCA-extractable phosphate. Such a decrease in all phosphate fractions suggest dilution of phosphates by increased interstitial tissue or edema. However, if the total TCA-extractable phosphate is used as an index of the dilution effect, it will be noted that the per cent decrease in total TCA-extractable phosphate (12.2 per cent) is greater than the per cent decrease in adenosine triphosphate (8.2 per cent) and...
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inorganic phosphate (8.8 per cent), but is much less than the per cent decrease in creatine phosphate (46.7 per cent). This indicates that there is a statistically significant decrease in the creatine phosphate fraction alone, whereas the decreases in the adenosine triphosphate, inorganic phosphate, and total TCA-extractable phosphate fractions are not statistically significant.

The use of dry ash weights for determination of phosphate fractions per Gm. dry weight would eliminate dilution by edema. This procedure was not possible in our experiments because the extreme lability of the compounds being measured necessitated quick freezing of tissue and rapid extraction and separation in the cold to prevent breakdown of high-energy phosphate bonds. In addition, the small size of the hearts did not permit measuring the dry weight/wet weight ratio in duplicate samples.

Discussion

These experimental observations demonstrate that cardiac hypertrophy in rats is accompanied by a decreased concentration of ventricular creatine phosphate without significant change in the levels of adenosine triphosphate and free inorganic phosphate. The question immediately arises whether such a finding is due to the hypertrophy or is a result of some other factor. A decrease in creatine phosphate has been found in a variety of other conditions: asphyxia, high output failure, and slow cardiac rate. None of these was present in our experiments.

Although desoxycorticosterone has been shown to result in necrosis of myocardial fibers, there is no evidence that it directly affects myocardial levels of creatine phosphate or adenosine triphosphate. Furthermore, in 10 animals, hypertension and cardiac hypertrophy were produced by the bilateral application of latex kidney capsules, and a decrease in creatine phosphate was observed in all but 2 of these animals.

The decrease in creatine phosphate therefore appears to represent an actual diminution in concentration due to cardiac hypertrophy. Such a conclusion has important implications in relation to the biochemistry of cardiac muscle contraction. Since the activity of cardiac muscle is dependent upon a steady supply of high-energy phosphate from adenosine triphosphate and creatine phosphate, any physical alteration in the myocardium, such as hypertrophy, should affect both of these compounds. Yet we observed a decrease in creatine phosphate alone. This selective reduction of creatine phosphate may be explained, in part, by the sparing of adenosine triphosphate by cardiac muscle. Since adenosine triphosphate takes part in the reaction between the actual contractile elements of muscle, actin and myosin, maintenance of adequate amounts of this substance is a necessity for normal muscular contraction. Creatine phosphate is not an integral part of the contractile process, but provides a reserve supply of energy; on hydrolysis it liberates high energy phosphate bonds for prompt re-synthesis of adenosine triphosphate. The principal sources of energy for the formation of adenosine triphosphate are the oxidative processes of the tricarboxylic acid cycle, which normally can keep pace with the demand for high-energy phosphate. However, if the myocardium requires energy at a rate that exceeds that ability of the tricarboxylic acid cycle, it would require an additional supply of high-energy phosphate from creatine phosphate.

Table 2

Results of Attempts to Produce Hypertension and Cardiac Hypertrophy in 151 Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Total No.</th>
<th>Total No. with Hypertension</th>
<th>Total No. for chem. analysis</th>
<th>Total Phosphoryl groups</th>
<th>Total Phosphoryl groups with hypertrophy</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>66</td>
<td>8</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>B</td>
<td>24</td>
<td>9</td>
<td>11</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>C</td>
<td>61</td>
<td>49</td>
<td>27</td>
<td>24</td>
<td>22</td>
</tr>
<tr>
<td>TOTAL</td>
<td>151</td>
<td>66</td>
<td>48</td>
<td>45</td>
<td>43</td>
</tr>
</tbody>
</table>
Table 3

Mean Values of Myocardial Levels of Phosphate Fractions in Eighty Control and Forty-five Hypertrophied Rat Hearts

<table>
<thead>
<tr>
<th></th>
<th>Concentration in micromoles phosphorus per gram tissue ± s.d.</th>
<th>Per cent decrease from control</th>
<th>T value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control group</td>
<td>Hypertrophied group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatine phosphate</td>
<td>1.72 ± 0.47</td>
<td>0.92 ± 0.43</td>
<td>46.7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Adenosine triphosphate</td>
<td>6.63 ± 0.90</td>
<td>6.09 ± 0.84</td>
<td>8.2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Inorganic phosphate</td>
<td>9.46 ± 0.96</td>
<td>8.63 ± 0.90</td>
<td>8.8</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Total TCA-extractable phosphate</td>
<td>17.81 ± 1.25</td>
<td>15.64 ± 1.26</td>
<td>12.2</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Cycle to supply adenosine triphosphate with high-energy phosphate, the reserve supplies will be drawn from creatine phosphate. As this process progresses, more energy must be supplied by creatine phosphate, and its level in the myocardium falls. Theoretically, the level of adenosine triphosphate would not diminish until the creatine phosphate stores were exhausted.

A decrease in creatine phosphate may result from failure or decrease in its production or from its increased utilization by the myocardium. There is no evidence in our experiments of a defect in the production of high-energy phosphate compounds in cardiac hypertrophy, since normal levels of adenosine triphosphate were found. Creatine phosphate derives its high-energy phosphate from the same sources as adenosine triphosphate. Our data suggest that the decrease in creatine phosphate is due to increased utilization of phosphate bond energy by the hypertrophied myocardium. The larger, hypertrophied myocardial fiber requires more energy than the normal fiber and probably consumes it more rapidly.

In our experiments, cardiac hypertrophy existed without clinical or pathologic evidence of congestive heart failure, except in 1 instance, in which the creatine phosphate level was elevated. Most of the animals that succumbed had very severe hypertension, which probably caused their death, yet cardiac hypertrophy was absent. The fact that cardiac hypertrophy was present in the animals that survived with prolonged, sustained hypertension, while it was absent in those that died spontaneously, suggests that hypertrophy is a compensatory mechanism allowing the heart to perform efficiently with an increased work load. An increased work load may result in either cardiac hypertrophy or failure: if the stress is chronic, hypertrophy may develop; if a great load is imposed acutely on the heart, the heart may fail, in the absence of hypertrophy, and death may or may not occur immediately. With confirmed survival, hypertrophy may subsequently develop. Neither hypertrophy nor failure necessarily precedes or follows each other, nor does one necessarily cause the other.

Cardiac hypertrophy and failure are pathologically distinct. Our findings of decreased levels of creatine phosphate in hypertrophy and the numerous observations of normal levels in failure indicate that the biochemical processes differ as well.

Summary

Arterial hypertension was produced in 66 rats by means of constricting latex capsules about the kidneys, by the intramuscular implantation of desoxycorticosterone pellets, and by the subcutaneous injection of desoxycorticosterone. The most satisfactory method was the intramuscular implantation of desoxycorticosterone pellets. Myocardial hypertrophy was produced in 43 rats.


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Biochemical analysis of the ventricles of 80 control and 45 hypertrophied rats were performed for creatine phosphate, adenosine triphosphate, and free inorganic phosphate. Cardiac hypertrophy in rats was accompanied by a statistically significant decrease in the ventricular concentration of creatine phosphate, while levels of adenosine triphosphate and free inorganic phosphate were not significantly altered.

It is suggested that congestive heart failure in hypertrophied hearts is related to a defect in the utilization of phosphate-bond energy by the hypertrophied myocardial fiber.

**Summario in Interlingua**

Hypertension arterial esseva producita experimentalmente in 66 rattos per medio del application al rerens de constringentc capsulas de latex, del implantação intramuscular de granos de disoxyeorticosterona, e del injection subciitanoo de disoxycorticosterona. Le plus satisfaccnte motliodo esseva, le implantation intramuscular de granos do disoxyeorticosterona. Hypertension myoeardial osscva producite in 43 rattos. Le analyse biochiinic del veiitriculos de 80 rattos do controlo e de 45 rattos liypertrophiate esseva effectuate pro phospliato de ereatina, triphosphato adonosinic, e inorganic phospliato libere. Hypertrophia cardiac in rattos esseva accompaniede de statisticaiiicnte significative reductiones in le concentration ventricular do phospliato del parte del hypertrophiate fibra myoeardial.

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