Amino Acid-Activating Enzymes of the Heart Muscle

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(Received for Publication, March 6, 1962)

The enzymatic activation of amino acid was recognized and measured by the formation of hydroxamic acids in the presence of amino acids, adenosine triphosphate and hydroxylamine. Tyrosine-activating enzyme was partially purified from the cardiac muscle of dogs by the method of Schweet. The "cell-sap" fraction of the cardiac muscle of dogs formed hydroxamic acids of 18 kinds of amino acids. Tryptophan, tyrosine, cysteine, leucine, and proline were more strongly activated.

In addition of the mixture of 14 kinds of amino acids, the mean \( \mu \) moles of hydroxamic acids formed in 1.0 g of wet weight/hour was 0.031 and 0.076 respectively for the cardiac muscle and the liver of normal rabbits.

Mean activities of the enzyme of hypertrophied cardiac muscle of dogs caused by aortic valvular lesions and of thyrotoxic cardiac muscle of rabbits were higher than those of the control, while those of the starved, hypoxic and noradrenalinolone administered cardiac muscle of rabbits were lower. There was no difference between the mean value of the acute cardiac dilatation and that of the control.

It is suggested from these results that the protein synthesis from the amino acid-level is present in the cardiac muscle and that it is likely to be disturbed at the level of amino acid-activation under some pathologic conditions.

Since the first experiments of Hoagland\(^1\), the evidence has been obtained in many laboratories to indicate that the sequence of protein synthetic reactions begins with an amino acid-activation. This activation is carried out by specific enzymes and requires ATP, according to the following reaction:

\[
\text{Amino Acid } + \text{ ATP } + \text{ Activating Enzyme } \xrightleftharpoons{\text{Enzyme}} \text{AMP} + \text{Amino Acid} + \text{PP}
\]

In the course of the reaction ATP is used up, pyrophosphate is split off, and the amino acyl-adenylate complex which is formed remains bound to the enzyme\(^5\). The binding of the carboxyl group of the amino acids to AMP is considered as an activation of the carboxyl group. Magnesium appears to be essential for this reaction. The activation of amino acids is generally measured by either of the following two methods:

1. The amino acid-dependent exchange of inorganic pyrophosphate with ATP

\[
\text{ATP} + \text{PP}\text{\textsuperscript{32}} \xrightleftharpoons{\text{AA}} \text{ATP}\text{\textsuperscript{32}} + \text{PP}\text{\textsuperscript{32}}
\]

and

2. The formation of amino acid-hydroxamate when preparations are incubated with amino acids, ATP, and relatively high concentrations of hydroxylamine.

\[
\text{Enzyme-AMP } \xrightarrow{\text{COCHRNH}_2+ \text{NH}_2\text{OH}} \text{Enzyme} + \text{AMP} + \text{NH}_2\text{CHR-CO-NH}_2
\]

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Supported in part by a grant in Aid for Miscellaneous Scientific Research from the Education Ministry.

The abbreviations used are: AMP and ATP=adenosine mono- and triphosphate, PP=inorganic pyrophosphate, Tris=Tris (hydroxymethyl) aminomethane, AA=Amino Acid.
Evidences indicate that a separate enzyme exists for activating each of the amino acids, and several of these amino acid-activating enzymes e. g. those for methionine, tyrosine, valine, tryptophan, alanine, and serine have been purified. The presence of activating enzymes of amino acids was demonstrated in many animal and plant tissues and in microorganisms, in the liver, spleen, kidney, pancreas, smooth muscle, gut, and brain.

No study of amino acid-activating enzymes in the heart muscle, however, has yet been obtainable. In connection with the work on myocardial protein metabolism, which has been carried out in our laboratory, the need has been felt for the demonstration of amino acid-activating enzymes and the measurements of their activities in the heart muscle.

The present study dealt with the enzymatic activating pattern of amino acids of the dog heart muscle and the partial purification of tyrosine-activating enzyme from this organ. And the activity of "pH 5 enzyme" in various pathologic heart muscle of dogs and rabbits was also determined.

**Materials and Methods**

For the demonstration of amino acid-activating enzymes and the measurements of their activity, the cardiac muscle of apparently normal adult mongrel dogs was used. Aortic valvar lesions were produced in other dogs by the aid of aortic catheterization according to the method of Sugiyama. These dogs developed the signs of left ventricular hypertrophy as shown in Table 1.

All the rabbits used were male and 2–3 months old, and the controls were fed daily with fresh vegetables and bean-curd refuse. The animals were divided into seven groups. One group served as the controls. The animals of A group were starved completely for 10 days. Chronic liver damage was produced in B group by intramuscular administration of carbon tetrachloride in 20% olive oil solution at a dose level of 0.5 ml./kg. body weight once a week for 1-5 months. Five milligrams of Durabolin (norandrostenolone) were administered intramuscularly once a week for 4-5 months to C group animals. For the animals of D group Tyradin (thyroid extracts) was administered orally at a dose level of 0.1 G per kg. for 1-2 months. The inhalation of low oxygen tension (5% O₂) was given to E group. After fifteen minutes of the inhalation, the hypoxic heart muscle was examined. For the animals of F group, acute cardiac dilatation was made by the constriction of the ascending aorta for thirty minutes, under the open chest operation.

**Preparation of cell-sap fraction of the heart muscle.**

To obtain the heart muscle, dogs were bled while under sodium pentobarbital anesthesia, and rabbits were stunned and bled. Quickly opening the chest, the beating heart was excised. The excised heart was immediately opened, blood was washed out with cold tap water, and the heart was placed in a glass beaker surrounded by ice. After the pericardium, epicardial fat and base of the great vessels were removed by dissection, the muscles of left ventricle of dogs and both ventricles of rabbits were weighed and minced finely with scissors.

For the demonstration of the amino acid-activating pattern of dog heart muscle, the muscle was minced by the Suzuki-type juicer and two volumes of Tris-HCl buffer (pH 7.4) were added, and the mixture was stirred continuously for 30 minutes. The mixture was transferred to a 50 ml. centrifuge tube, and was spun at 14,000 r.p.m. for 40 minutes, and the supernatant was used as a

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Heart Weight of Dogs with Aortic Valvular Lesion Vs. Normal Dogs. Sex All Female</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dogs with Aortic Valvular Lesion (± S. D.)</strong></td>
<td><strong>Control (± S. D.)</strong></td>
</tr>
<tr>
<td><strong>Number of Cases</strong></td>
<td>5</td>
</tr>
<tr>
<td><strong>Total Heart Weight</strong></td>
<td>76.78 ± 18.94 (g)</td>
</tr>
<tr>
<td><strong>L. Ventricle</strong></td>
<td>36.00 ± 7.83 (g)</td>
</tr>
<tr>
<td><strong>R. Ventricle</strong></td>
<td>15.63 ± 3.03 (g)</td>
</tr>
<tr>
<td><strong>Septum</strong></td>
<td>10.64 ± 6.69 (g)</td>
</tr>
<tr>
<td><strong>Auricle</strong></td>
<td>8.51 ± 2.02 (g)</td>
</tr>
<tr>
<td><strong>H. W./B. W.</strong></td>
<td>8.57 ± 1.02</td>
</tr>
<tr>
<td><strong>L. V./R. V.</strong></td>
<td>2.31 ± 0.22</td>
</tr>
</tbody>
</table>

S.D. = Standard deviation; P = probability; H. W. = heart weight; B. W. = body weight; L. V. = left ventricle; R. V. = right ventricle; n. s. = not significant statistically.

*Japanese Circulation Journal Vol. 26, May, 1962*
enzyme preparation. If the supernatant was used without further treatments extremely high blank values were obtained presumably due largely to amino acids contained in the supernatant.

Partial purification of tyrosine-activating enzyme.

The procedure of Schweet\textsuperscript{15} with some modifications was employed for the partial purification of tyrosine-activating enzyme. The left ventricular heart muscle of dogs was defatted and 30 G. portion was homogenized for 2 minutes with 100 ml. of acetone at -15C. in the waring blender. The mixture was filtered on the Buchner funnel. The cake was homogenized the second time with 100 ml. of acetone and filtered again. The cake was homogenized for 30 seconds with 100 ml. of peroxide-free ether at -15C. The final filter cake was dried at room temperature as rapidly as possible and the fine powder obtained was stored at -20C.

Fraction 1: 5 G. of acetone powder were ground in an ice cold mortar with the addition of small volumes of 0.02 M Tris-buffer, pH 7.4. The wet slurry was suspended in a total volume of 100 ml. of the same buffer and stirred occasionally for 20 minutes. The suspension was centrifuged at 14,000 r.p.m. for 60 minutes, and the supernatant was decanted through glass wool.

Fraction 2: To the clear supernatant were added 20 ml. of the calcium-phosphate gel, which had previously been centrifuged and the supernatant discarded to minimize the dilution of enzyme extract. The slurry was adjusted to pH 7.4, stirred gently for 30 minutes, and centrifuged. After discarding the supernatant, the gel was mixed with 50 ml. of 2.0 M potassium-phosphate buffer, pH 8.1. The mixture was stirred for 30 minutes, centrifuged, and the supernatant was dialyzed with stirring for 18 hours against two changes of 1.0 L. of 0.02 M Tris-buffer, pH 7.4.

Preparation of the pH 5 fraction

The pH 5 fraction was prepared by the method of Hoagland et al.\textsuperscript{19} with some modifications. All operations were performed at 0 to 4C. Five grams of heart muscle were homogenized with 7.0 ml. of 0.05 M KCl. This homogenate was diluted with 2 volumes of 0.05 M KCl, and was spun at 14,000 r.p.m. for 40 minutes. The soluble protein fraction was drawn off with a syringe and pH was brought to 5.0 by dropwise addition of 0.1 N HCl with constant stirring. The resultant precipitate was centrifuged and was resuspended by homogenization in 0.1 M Tris-HCl buffer, pH 7.4. This solution was divided into two parts and one part served as the control. Protein concentration was determined by the direct nesslerization method.

Assay Procedures: The formation of hydroxamic acids

The enzyme preparations were incubated at 37C. for 3 hours in a final volume of 2.0 ml. with 10 \mu moles of K\textsubscript{2}ATP, 10 \mu moles of MgCl\textsubscript{2}, 20 \mu moles of KF, about 3 milimoles of salt-low hydro-

\begin{table}[h]
\centering
\caption{Hydroxamic acid-formation by heart muscle sap of dogs with various amino acids}
\begin{tabular}{|l|c|c|c|}
\hline
\textbf{Experiments} & \textbf{1} & \textbf{2} & \textbf{3} \\
\hline
\textbf{Amino Acids} & Hydroxamic Acid formed \( \mu \text{M/litre} \), mg. of protein & & \\
Alanine & 0.0121 & & \\
Glycine & 0.0109 & & \\
Tryptophan & 0.0286 & & \\
Valine & 0.0061 & & \\
Tyrosine & 0.0235 & & \\
Glutamic Acid & & 0.0170 & \\
Aspartic Acid & & 0.0056 & \\
Cysteine & & 0.1019 & \\
Lysine & & 0.0077 & \\
Leucine & & 0.0100 & \\
Histidine & & & 0.0070 \\
Phenylalanine & & & 0.0066 \\
Serine & & & 0.0092 \\
Methionine & & & 0.0092 \\
Proline & & & 0.0117 \\
Threonine & & & 0.0092 \\
Cystine & & & 0.0068 \\
Arginine & & & 0.0068 \\
\hline
\end{tabular}
\end{table}

1.0 ml. of cell-sap fraction of the dogs heart muscle was incubated with 10 \mu M of K\textsubscript{2}ATP, 10 \mu M of MgCl\textsubscript{2}, 20 \mu M of KF, 3 \text{mM} of salt-low hydroxylamine, 100 \mu M of Tris-HCl buffer and each 10 \mu M of amino acid. Total volume 2.0 ml., 37C., 1.5 hours; protein concentration 41.2 mg/1.0 ml.

xylamine prepared from hydroxylamine sulfate and barium hydroxide, 300 μ moles of Tris-HCl buffer (pH 7.4), and 10 μ moles of amino acids or 5 μ moles of each amino acid when the amino acid mixture were used according to the plan of the experiment. Controls were prepared using the above mixture except for the amino acid. The formation of hydroxamic acids was measured by the procedure of Hoagland et al. with the use of succinononohydroxamate as a standard at the wave length of 505 mμ in the spectrophotometer (Hitachi).

The complete amino acid-mixture (pH 7.4) contained glycine and the following L-amino acids: alanine, aspartic acid, cystine, glutamic acid, histidine, leucine, lysine, phenylalanine, proline, threonine, tyrosine and valine.

RESULTS

The presence of amino acid-activating enzymes in the heart muscle.

By measuring the formation of hydroxamic acid, which is a simple but rather insensitive method to determine the enzymatic activity, it was found that the heart muscle supernatant contained appreciable amounts of amino acid-activating enzymes. (Table 2)

All (18) of the amino acids tested formed the hydroxamic acids. Although there were however some differences in the rate of the hydroxamic acid formation between them, and alanine, tryptophan, tyrosine, cysteine, leucine and proline were more strongly activated than the others.

Fig. 1 shows levels of amino acid-activating enzyme in various organs when the mixture of 14 kinds of amino acids was added. No difference of the “pH 5 enzyme” activity was observed between the heart muscle of normal dog and that of normal rabbit. The mean μ moles of hydroxamic acid formed in 1.0 Gm. of wet tissue/hour were 0.031 and 0.076 respectively for the heart muscle and the liver of normal rabbit.

Partial purification of tyrosine-activating enzyme in the heart muscle.

Table 3 represents partial purification of tyrosine-activating enzyme. In the fraction 2, approximately 2.5 fold purification was obtained with a yield of 66%. Though no

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (mL)</th>
<th>Protein Concentration mg/mL</th>
<th>Specific Activity Unit/mg</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Acetone powder Extract</td>
<td>100</td>
<td>6.75</td>
<td>0.007</td>
<td></td>
</tr>
<tr>
<td>2. Calcium Phosphate Gel treated</td>
<td>30</td>
<td>6.00</td>
<td>0.018</td>
<td>66</td>
</tr>
</tbody>
</table>

1.0 ml of each extract from the dog heart muscle was incubated with 10 μM of K₂ATP, 10 μM of MgCl₂, 20 μM of KF, 2 mM of sodium hydroxide, 100 μM of Tris-HCl buffer and 10 μM of L-tyrosine. Total volume 3.0 mL. 37°C, 2 hours; 1 unit denotes 1 μM of hydroxamic acid formed/hour measured with the use of succinononohydroxamic acid as the standard.
Amino acid-activating enzymes of the heart muscle

EFFECT OF ENZYME CONCENTRATION ON ACTIVITY

![Graph showing the effect of enzyme concentration on activity.]

Hydroxamic acids of L-leucine, L-valine, L-tryptophan and glycine were formed in this stage of the enzyme preparation, those of L-tyrosine, L-alanine, L-glutamic acid and L-aspartic acid were formed.

Some of the properties of tyrosine-activating enzyme preparation.

Some properties of tyrosine-activating enzyme are shown in Fig. 2 & 3. The enzymatic activity was proportional to the protein concentration and the pH optimum for maximal hydroxamic formation was at about 7.4.

The level of amino acid-activating enzyme

![Graph showing the effect of pH on enzyme activity.]

Of the heart muscle under various pathological conditions.

The activities of the enzyme varied considerably from day to day, and from experiment to experiment and therefore each activity of various pathologic heart muscles was estimated in comparison with that of each normal heart muscle which was measured in parallel with the control. Table 4 represents the activities of the enzyme in various conditions and the analysis of variance to controls.

In the heart muscle of starved rabbit, the mean level of the enzyme activity was 0.0177 units (one unit means 1 μM of hydro-

<table>
<thead>
<tr>
<th>Conditions</th>
<th>No. of Cases</th>
<th>Controls Mean Value (Units)</th>
<th>Experimental Mean Value (Units)</th>
<th>Mean Square betw. Cont. &amp; Experi.</th>
<th>Mean Square of Remainder</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starvation</td>
<td>5</td>
<td>0.0273</td>
<td>0.0177</td>
<td>0.00002284</td>
<td>0.00000756</td>
<td>30.2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Hypertrophy</td>
<td>4</td>
<td>0.0295</td>
<td>0.0555</td>
<td>0.001352</td>
<td>0.000900</td>
<td>1.4</td>
<td>n.s.</td>
</tr>
<tr>
<td>Duramin Ad.</td>
<td>4</td>
<td>0.0464</td>
<td>0.0265</td>
<td>0.003997</td>
<td>0.001462</td>
<td>54.3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Liver Injury</td>
<td>5</td>
<td>0.0472</td>
<td>0.0419</td>
<td>0.0000708</td>
<td>0.000611</td>
<td>0.11</td>
<td>n.s.</td>
</tr>
<tr>
<td>Tyrocin Ad.</td>
<td>4</td>
<td>0.0111</td>
<td>0.0135</td>
<td>0.0000125</td>
<td>0.000040</td>
<td>3.30</td>
<td>n.s.</td>
</tr>
<tr>
<td>Anoxia</td>
<td>3</td>
<td>0.0131</td>
<td>0.0093</td>
<td>0.000011</td>
<td>0.000020</td>
<td>10.2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Acute Cardiac Dilatation</td>
<td>5</td>
<td>0.0078</td>
<td>0.0078</td>
<td>0.00000081</td>
<td>0.0000079</td>
<td>0.008</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

1 unit denotes 1 μM of hydroxamic acid formed/hour/1 Gm. of wet tissue measured with the use of succinonorhydroxamic acid as the standard.

Ad. = Administration; F = Snedecor's F.

P = probability; n.s. = not significant statistically.

xamic acid formed in 1 hour), while that of controls 0.0273 units. The difference between the starved and the control was statistically significant. \( p < 0.01 \) The mean units were 0.0555 and 0.0295 respectively for the hypertrophied cardiac muscle of dogs and for the control, but there was no statistically significant difference between them.

Between the enzymatic activity in the heart muscle of Durabolin administered rabbit heart muscle and that of the control, which are 0.0265 and 0.0464 units respectively, statistically significant difference was seen. \( P < 0.01 \)

The mean activity of the enzyme in the heart muscle of rabbits with liver injury and that in the control were 0.0419 and 0.0472 units respectively. Though the enzyme-activity in the heart muscle of Tyradin administered rabbit heart muscle (0.0135 units) was slightly higher than that in the control (0.0111 units), no statistically significant difference was observed.

The mean value of enzymatic activity of hypoxic heart muscle was 0.0093 units, while that of the control was 0.0131. This difference was statistically significant, \( P \) being less than 0.05. There was no difference between the mean value of the acute cardiac dilatation and that of the controls (both 0.0078 units).

**Discussion**

There is little information as to the origin of the proteins of muscle fibres. Moscona\(^{23}\) found the indication that cytoplasmic granules might be the precursors of myofibrils. Recently, Dreyfus et al.\(^{24}\) reported a 30 day life time for myosin of the rat skeletal muscle. McLean et al.\(^{25}\) have shown that in the muscle, mitochondria are as active as microsomes in incorporating isotopic amino acids into protein, and observed the labelling of myofibrils also.

In general, the dominant current conception of the mechanism of protein synthesis envisages activation of amino acids, transfer of the activated amino acids to s-RNA, and migration of part or all of the s-RNA-amino acid complex to the ribosomes whose RNAs act as templates determining the sequence of amino acids appropriate for the synthesis of specific protein molecules. To study the activation of amino acids is, therefore, the first step to solve the problems on protein synthesis. Pennington\(^{26}\) reported the presence of amino acid-activating enzymes in the skeletal muscle, and also presented a pattern of amino acid-activation. His report showed that in the presence of the complete mixture of amino acids, the muscle preparation formed 0.05 \( \mu \)M of hydroxamic acid/mg of protein/hour. Novelli\(^{27}\) reported the rate of hydroxamic acid formation, as catalyzed by the individual amino acids, in several different extracts. These data showed that, in guinea pig liver, aspartic acid, asparagine, glutamic acid, glutamine, glycine, lysine, proline, serine, tryptophan, histidine and tyrosine were more strongly activated. It is interesting to compare those results with the results of the heart muscle reported in this paper. The fact that various amino acids are present in activated forms, and also the presence of tyrosine-activating enzyme in the heart muscle, together indicates that the protein synthesis from the amino acid level might take place in the heart muscle. The activation of amino acids appears to indicate a chain of reactions which eventually lead to peptide formation in the microsome.

It would be interesting to determine the level of amino acid-activating enzymes in the heart muscle in pathologic conditions. There have been some attempts to determine the activity of amino acid-activating enzymes in unusual conditions. According to McCrquo-dale and Müller\(^{13}\), the activity of amino acid-activating enzyme in the uterine homogenate of rat increased rapidly over the ensuing 24 hours period in response to a single administration of estradiol. Pennington\(^{26}\) reported that the muscle cell-sap from mice with hereditary muscular dystrophy showed, in average, less incorporation per/mg of protein. Holley et al.\(^{26}\) reported that potassium deficiency in intact rats had little effect on the activity of the enzyme in the preparation from liver. Recently Bucovaz\(^{27}\) et al. reported a trend of increasing activity in the preparations from mammary tissue of rats from no detectable exchange of \( P^{26} \) to a maximal response in rats.
in the early stage of lactation. The level of exchange appeared to be lower in glands in late lactation than in those in early lactation.

Although there were very large variations from day to day in the measurement, by the treatments of analysis of variances, statistically significant difference was obtained between the starved rabbit heart muscle and control. Manchester reported that in the absence of insulin, incorporation of C\textsuperscript{14}-glycine into the protein in diaphragm from Wistar and hooded rats starved for 24 hr. was slightly (13–12 per cent) reduced in comparison with diaphragm from non-fasting animals. It could be easily imagined that, in starvation, the protein synthesis would be decreased.

There was also a statistically significant difference between the Durabolin administered rabbit heart muscle and controls. After the administration of Durabolin, which is said to stimulate protein synthesis, the value of amino acid-activating enzyme was decreased. The reason for this decrease is not clear. The hormone might stimulate protein synthesis at other point.

The hypertrophied heart muscle showed higher hydroxamic formation than that of controls though statistically not significant. Meeson and Zayats reported that in the heart of rabbits with experimentally produced aortic stenosis protein synthesis is double in hypertrophied stage. During the second stage, referred to stable hyperfunction, the rate of protein synthesis returned to normal. During the third stage referred to as that of cardiac decompensation, the protein synthesis decreased. These observations are very interesting, because the hypertrophied muscle in the present experiment was in the first or second stage.

Sokoloff et al. reported that thyroxine pretreatment in vivo or addition in vitro increased the rate of amino acid-incorporation into protein by cell-free rat liver homogenates, and that the increased amino acid-incorporating activity had been found to be associated with the mitochondrial fraction. In my experiment slight increase of the level of amino acid activating enzyme was observed but it was not statistically significant.

The activity of enzyme in liver-injury rabbits did not differ from the controls.

As for acutely induced conditions, the hypoxic heart muscle and the dilated heart muscle were observed. The hypoxic heart muscle showed a decrease in the enzymatic activity. On the other hand, the enzymatic activity of the heart muscle was not influenced by the acute dilatation. It is recognized that the activity of various energy producing enzymes were decreased in hypoxic heart muscle. It would be interesting to compare this observation with the results which obtained in the amino acid-activating enzymes in hypoxic heart muscle. They would suggest that the protein synthesis in the heart muscle might be disturbed in hypoxic state.

The available evidences suggest that the protein synthesis from the amino acid-level is present in the cardiac muscle and that it is likely to be disturbed at the level of amino acid-activation under some pathologic condition. Because the studied cases in this experiment were relatively few, much more work would be needed in order to elucidate the enzymatic activity under various pathologic conditions, though statistically significant results were obtained.

**Summary**

1. The soluble protein fraction from the dog heart muscle contained several amino acid-activating enzymes which catalyse amino acid hydroxamate formation.
2. Tyrosine-activating enzyme was partially purified from the dog heart muscle and some of its properties were investigated.
3. The activities of amino acid-activating enzyme in the heart muscle, skeletal muscle, and in liver of rabbits were compared.
4. The level of amino acid-activating enzymes of the heart muscle under various pathologic conditions were determined and their significance in protein synthesis was discussed.

**Acknowledgment**

Grateful acknowledgment is made to Prof. S. Aoyama and Asst. Prof. H. Matsumura for their constant interest and guidance in this investigation.
REFERENCES


