Hypertrophied Non-Failing Rat Heart
PARTIAL BIOCHEMICAL CHARACTERIZATION
By Charles H. Dart, Jr., M.D., and John O. Holloszy, M.D.

ABSTRACT
Cardiac hypertrophy was induced in rats by constructing an arteriovenous fistula. Heart weights approximately doubled in 7.5 weeks. The levels of activity, expressed per gram of heart, of a variety of enzymes of the mitochondrial respiratory chain and of the citric acid cycle, as well as the concentrations of cytochrome c and of mitochondrial protein, were the same in the hypertrophied and control hearts. Similarly, the capacity of whole heart homogenate to oxidize pyruvate was unaltered in hypertrophy.

There was also no change in the levels of activity of cytochrome oxidase and malate dehydrogenase, or in the concentration of cytochrome c in early hypertrophy (3 to 10 days after construction of an A-V fistula). These results suggest that cardiac mitochondria increase in parallel with the other components of the myocardial cell.

The levels of activity, per gram of heart, of creatine phosphokinase and adenylate kinase, and of the rate-limiting enzymes of glycolysis and glycogenolysis, were the same in the hypertrophied and the control hearts.

The above findings suggest that the capacity, per gram of heart, for regenerating ATP, both aerobically and anaerobically, is unchanged in the non-failing, hypertrophied heart.

ADDITIONAL KEY WORDS

- cell fractions
- protein concentrations
- water content
- cell growth

Recent studies have provided much new information on the events early in the development of cardiac hypertrophy. Meerson (1) and Gudbjarnason et al. (2) measured incorporation of radioactively labeled amino acids into ventricular protein and found that the rate of protein synthesis is increased during the early phase of hypertrophy. Schreiber et al. (3), using an isolated heart preparation, have shown that acute cardiac overload augments protein synthesis by cardiac muscle microsomes as early as 1 hour after onset of the stress. Further studies provide evidence that this early response to an increased workload is due to increased formation of messenger RNA (4).

Relatively little work has been done to characterize the new protein synthesized during development of cardiac hypertrophy. Badeer (5) has speculated "that the major outcome of the process of hypertrophy in the myocardium is the laying down of increased numbers of myofibrils along the length of the muscle fibers." However, there is evidence that other components of the cardiac cell are also affected (1, 6).

Our initial interest in this area stemmed from the finding that in skeletal muscle a chronic increase in work load can result in a twofold increase in the levels of activity of the enzymes of the mitochondrial electron transport chain (7). This is associated with a doubling in the capacity for the oxidation of pyruvate and for the aerobic generation of ATP (7). It appeared possible that a similar response might occur in heart muscle subjected to an increased work load. The
published results of studies relating to this question are conflicting (1, 6, 8-15). A considerable portion of the present study was, therefore, devoted to obtaining biochemical information, using a number of independent approaches, regarding the response of heart mitochondria to cardiac hypertrophy. In addition, the responses of a number of the cytoplasmic enzymes involved in energy metabolism were also investigated.

In our studies, cardiac hypertrophy was induced by an arteriovenous fistula. This method was chosen because it results in the rapid development of massive, biventricular hypertrophy (16, 17).

**Materials and Methods**

**Animal Care and Surgical Procedure**

Male Sprague-Dawley (Holtzman) rats were kept in individual cages and maintained on a diet of Purina chow and water. At the time of surgery they weighed 400 to 450 g.

Animals were anesthetized with ether, and anesthesia was maintained with sodium pentobarbital, 4 mg/100 g body weight, ip. After opening the abdominal cavity through a midline incision, the posterior peritoneum was opened from the level of the renal artery to the lumbar vessels. The vena cava and aorta were temporarily occluded. A 2- to 3-mm incision was made in the medial aspect of the vena cava just anterior to the renal vessels and at a point 1 cm distally. The arteries and veins entering this 1-cm segment were also temporarily occluded. A 2- to 3-mm incision was made in the medial aspect of the aorta. A no. 70 double-armed suture (Taper BV-1 needle) was used to sew the posterior lip of the aortic incision to a double thickness of the wall of the vena cava. A 2-mm incision was made in the medial aspect of the vena cava just anterior to the suture line. The anterior lip of this incision was then approximated to the anterior lip of the aortic incision by a continuation of the superior and inferior portions of the suture line. Patency of the arteriovenous fistula was evidenced by a thrill at the anastomosis, by an increase in heart rate, and by arterIALIZATION of the venous blood cephalad to the fistula. To obtain optimal visualization of the vessels, the surgeon wore a loupe that provided a twofold magnification.

**Tissue Preparation and Assay Methods**

Animals were decapitated approximately 7.5 weeks after surgery. The heart was excised and the atria and great vessels were trimmed away. The ventricles were cut open, rinsed free of blood in ice-cold Ringer's solution, blotted, weighed, chopped into a fine mince, and homogenized in a glass Potter-Elvehjem homogenizer. The tubes were immersed in ice water during the procedure.

Mitochondria for spectrophotometric assays of enzyme activity (other than ATPase), and for studies of $O_2$ uptake were prepared as described previously (7) from homogenates made in 175 mM KCl containing 0.1 mM EDTA. Mitochondria for determination of mitochondrial ATPase activity were prepared by centrifuging the homogenate for 15 minutes at 700 $\times$ g; the supernatant fluid was decanted and twice centrifuged for 10 minutes at 1,000 $\times$ g. The supernatant fluid was then centrifuged for 15 minutes at 8,000 $\times$ g. The mitochondrial pellet was washed once by resuspension in 175 mM KCl and recentrifugation at 8,000 $\times$ g. The mitochondria for the ATPase assay were suspended in 250 mM sucrose and vigorously homogenized (40 complete passes of the tube) in a Potter-Elvehjem homogenizer with a tightly fitting pestle. The mitochondrial preparation was then aged for 2 hours at room temperature before the ATPase assay.

$O_2$ uptake was measured in a Gilford differential respirometer, at 30°, with air as the gas phase. Respiration was measured in duplicate as described previously (7, 18), using whole homogenates instead of isolated mitochondria to avoid possible differences in mitochondrial yield. P-0 ratios were determined on isolated mitochondria as described earlier (7).

Succinate oxidase and cytochrome oxidase activities were measured manometrically as described by Potter (19). $O_2$ uptakes are expressed as milliliters or microliters of dry $O_2$ under standard conditions.

Spectrophotometric assays were performed in a Gilford model 240 spectrophotometer with a thermostated cell compartment in 1-ml cuvettes of 1-cm light path at 30°. Readings were taken at 15- or 30-sec intervals. Initial reaction rates were determined from a segment of the linear portion of the change in absorbance and corrected for the rates of any nonspecific activity. Assays were performed under conditions in which the reaction rate was proportional to enzyme concentration. Enzyme activities are reported as $\mu$moles of substrate utilized per minute.

Aconitase activity was determined by Anfinsen's method (20). The homogenization medium contained 175 mM KCl and 2 mM sodium citrate. The mitochondrial pellet was resuspended in the same medium and frozen and thawed twice prior to the measurement of aconitase activity. Formation of cis-aconitate was followed at 240 m$\mu$.

TPN-dependent isocitrate dehydrogenase was measured by the method of Plaut and Sung (21) on mitochondria that had been frozen and thawed twice.
DPN-dependent isocitrate dehydrogenase activity was assayed by a modification of the method of Plaut and Aogachi (22). The assay mixture contained in a final volume of 1 ml: 30 mM tris buffer, pH 7.2, 2 mM MnCl₂, 2 mM ADP, 0.33 mM DPN, 2 mM sodium amytal, and 4 mM DL-isocitrate. The medium used for muscle homogenization contained 100 mM KPO₄ buffer, pH 7.2, 2 mM ADP, 2 mM EDTA, and 10 mM reduced glutathione.

Succinate dehydrogenase activity was assayed by the method of Bonner (23), using whole heart homogenates.

Malate dehydrogenase activity was measured by following the rate of oxidation of reduced DPN (DPNH) in the presence of excess oxaloacetate. The reaction mixture contained 50 mM KPO₄ buffer, pH 7.4, 2.4 mM oxaloacetate, and 0.2 mM DPNH. Mitochondrial and cytoplasmic malate dehydrogenases were distinguished as described by Shonk and Boxer (24).

Mitochondrial ATPase activity was determined in the presence of an ATP regenerating system (25) to avoid the inhibitory effect of ADP. The reaction mixture contained in a final volume of 1 ml: 50 mM tris HC1 buffer, pH 7.4, 4 mM MgCl₂, 4 mM ATP, 2 mM phosphoenolpyruvate, 0.2 mM DPNH, 0.5 mM dinitrophenol, 32 μg pyruvate kinase, 16 μg lactate dehydrogenase, and when oligomycin sensitivity was being determined, 2 μg oligomycin. DPNH oxidation was followed at 340 μm.

Adenylate kinase activity was assayed as described by Markland and Wadkins (26), except that a final concentration of 0.3 mM DPNH was used. Assays were performed on whole heart homogenates.

Creatine kinase activity of whole heart homogenate was measured in a system coupled with pyruvate kinase and lactate dehydrogenase. The assay mixture contained in a final volume of 1 ml: 50 mM tris buffer, pH 9, 5 mM MgCl₂, 4 mM ATP, 0.8 mM phosphoenolpyruvate, 0.15 mM DPNH, 60 mM creatine, 0.15 μg of pyruvate kinase, and 50 μg of lactate dehydrogenase. The reaction was started by adding creatine after a preincubation period of approximately 5 minutes, during which contaminating ADP and pyruvate were used up.

Hexokinase activity was measured in a system coupled with glucose-6-phosphate dehydrogenase, as described by Uyeda and Racker (27), using whole heart homogenates.

Phosphorylase activity of whole heart homogenates was determined by the method of Brown and Brown (28).

Phosphofructokinase activity was measured as described by Mansour (29). Homogenates for assay of phosphofructokinase were prepared in a medium containing 100 mM K₂HPO₄, 5 mM MgSO₄, 5 mM mercaptoethanol, 0.5 mM ATP, and 30 mM NaF (cf. refs. 30, 31).

Pyruvate kinase was measured in a system coupled with lactate dehydrogenase. The reaction mixture contained in a final volume of 1 ml: 60 mM TrisCl, pH 7.6, 8 mM MgCl₂, 75 mM KCl, 0.8 mM phosphoenolpyruvate, 4 mM ADP, 0.15 mM DPNH, and 50 μg of lactate dehydrogenase.

Lactate dehydrogenase activity was determined as described by Pesce et al. (32). Protein was measured with the biuret method (33). The cell fractions were prepared for protein assay by extraction with 95% alcohol followed by precipitation in 5% trichloroacetic acid (34).

The cytochrome c content of heart muscle was determined as described by Rosenthal and Drabkin (35).

**Materials**

TPN, DPN, DPNH, cytochrome c (type II), ATP, ADP, AMP, Tris (reagent grade), and hexokinase (type III) were obtained from Sigma. Lactate dehydrogenase, pyruvate kinase, aldolase, triosephosphate isomerase, α-glycerophosphate dehydrogenase, and glucose-6-P₄ dehydrogenase were obtained from Calbiochem. Silk suture material was obtained from Ethicon.

**Results**

Animals with functioning arteriovenous (A-V) fistulas developed massive cardiac hypertrophy involving both ventricles. Animals with
TABLE 2
Oxygen Consumption and P-O Ratios during Uncontrolled Respiration with Pyruvate plus Malate as Substrate

<table>
<thead>
<tr>
<th>Group</th>
<th>Oxygen uptake* (liters O_2/min/g wet wt)</th>
<th>P-O ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-V fistula</td>
<td>100 ± 0(5)</td>
<td>2.6 ± 0.2(5)</td>
</tr>
<tr>
<td>Control</td>
<td>106 ± 0(10)</td>
<td>2.6 ± 0.2(5)</td>
</tr>
</tbody>
</table>

* Measured on whole heart homogenates.
† Determined on isolated mitochondria.

The oxygen uptake of whole heart homogenates during uncontrolled respiration (i.e., in the presence of nonlimiting amounts of ADP and P_i), with pyruvate plus malate as substrate, was the same in the hypertrophied and control hearts (Table 2). This was true whether the results were expressed per gram of heart or per milligram of cardiac protein. Coupling of oxidation to phosphorylation, as reflected in the P-O ratios obtained on isolated mitochondria, was also unchanged in the hypertrophied hearts (Table 2).

These findings suggest that the respiratory capacity of cardiac muscle increases in proportion to cardiac mass. Further evidence for this interpretation is provided by the observation that the levels of activity of cytochrome oxidase, succinate oxidase, and succinate dehydrogenase were not significantly different in the hypertrophied and control hearts (Table 3). The concentration of cytochrome c in ventricular muscle was similarly unaffected by hypertrophy, averaging 19 ± 1.8 μmoles/g of heart for 10 animals with functioning fistulas, compared to 20.2 ± 1.7 μmoles/g for 10 controls.

In keeping with the finding that the efficiency of oxidative phosphorylation and the total oxidative capacity per gram of heart was unaltered, mitochondrial ATPase activity was the same in the hypertrophied and control hearts (Table 4). To minimize the contribution of contaminating myofibrillar and microsomal ATPases, the values for the oligomycin-sensitive component of the mitochondrial ATPase activity are given in Table 4. Total mitochondrial ATPase activity per gram of heart was also the same in the two groups.

Like the respiratory enzymes, mitochondrial ATPase is a component of the mitochondrial cristae. Mitochondria do not necessarily turn over as a unit, and can undergo changes in composition (36, 37). It appeared of interest,
therefore, to also investigate the response of some of the enzymes of the mitochondrial matrix to hypertrophy. There was no significant difference between the hypertrophied and control hearts in the levels of activity of the four mitochondrial citric acid cycle enzymes measured in the present study—aconitase, the TPN-dependent form of isocitrate dehydrogenase, the DPN-dependent form of isocitrate dehydrogenase, and malate dehydrogenase (Table 5).

It is of interest that the level of cytoplasmic malate dehydrogenase was also unaltered in cardiac hypertrophy (Table 5). The response of a number of other enzymes located in the cytoplasm was investigated. These included hexokinase, phosphorylase, phosphofructokinase, pyruvate kinase and lactate dehydrogenase. As shown in Table 6, the levels of activity of these enzymes were not significantly altered in the hypertrophied hearts.

Because of their roles in the regeneration of ATP, the responses of adenylate kinase and creatine kinase to hypertrophy were studied. As with all other enzymes measured, no significant difference was found between the control and the hypertrophied hearts.

The finding that the levels of activity, per gram of heart, of a wide variety of mitochondrial and cytoplasmic enzymes remain constant in cardiac hypertrophy, suggested the possibility that the process of hypertrophy may involve all the protein components of the heart uniformly. Further evidence for this possibility is provided by the finding that the concentrations of protein in whole heart homogenates and in three crude subcellular fractions obtained by differential centrifugation were not significantly different in hypertrophied and control hearts (Table 7).

Meerson et al. (14, 15) have reported that early in the development of hypertrophy ("First Stage") the concentration of mitochondrial protein, and the area occupied by mitochondria in electron microscopic sections, approximately double. No evidence for such an overshoot in the formation of mitochondria early in hypertrophy was found in the present study. Hearts were studied 3 to 10 days following the creation of an arteriovenous fistula. Although there was a significant increase in heart weight, no change occurred either in the levels of cytochrome oxidase and cytochrome c, which were used as markers for

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**TABLE 5**

Activity of Certain Enzymes of the Citric Acid Cycle (μmoles/min/g wet wt)

<table>
<thead>
<tr>
<th>Group</th>
<th>Mitochondrial</th>
<th>Cytoplasmic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aconitase</td>
<td>TPN-linked isocitrate dehydrogenase</td>
</tr>
<tr>
<td>A-V fistula</td>
<td>3.5 ±0.8(7)</td>
<td>14.6 ±2.0(6)</td>
</tr>
<tr>
<td>Control</td>
<td>3.5 ±0.3(7)</td>
<td>13.4 ±1.2(6)</td>
</tr>
</tbody>
</table>

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**TABLE 6**

Activity of Certain Enzymes of Carbohydrate Metabolism (μmoles/min/g wet wt)

<table>
<thead>
<tr>
<th>Group</th>
<th>Phosphorylase</th>
<th>Hexokinase</th>
<th>PFK</th>
<th>Pyruvate kinase</th>
<th>Lactate dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-V fistula</td>
<td>79 ±4(3)</td>
<td>5.3 ±0.7(6)</td>
<td>16.7 ±1.4(6)</td>
<td>126 ±6(6)</td>
<td>487 ±35(6)</td>
</tr>
<tr>
<td>Control</td>
<td>80 ±7(3)</td>
<td>5.1 ±0.4(11)</td>
<td>16.7 ±2.7(6)</td>
<td>130 ±12(11)</td>
<td>502 ±40(11)</td>
</tr>
</tbody>
</table>

* Phosphofructokinase.
TABLE 7
Concentrations of Protein in Whole Heart Homogenates and Three Subcellular Fractions of Hypertrophied and Control Hearts (mg protein/g heart)

<table>
<thead>
<tr>
<th>Group</th>
<th>Whole heart homogenate</th>
<th>700 X g Precipitate</th>
<th>8,000 X g Mitochondria</th>
<th>14,000 X g Supernatant fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-V fistula</td>
<td>178 ± 0(20)</td>
<td>89 ± 5(5)</td>
<td>8.2 ± 0.6(10)</td>
<td>79 ± 3(6)</td>
</tr>
<tr>
<td>Control</td>
<td>184 ± 5(20)</td>
<td>91 ± 3(5)</td>
<td>8.7 ± 0.5(10)</td>
<td>80 ± 4(6)</td>
</tr>
</tbody>
</table>

TABLE 8
Malate Dehydrogenase, Cytochrome Oxidase, and Cytochrome c in Early Cardiac Hypertrophy

<table>
<thead>
<tr>
<th>Group</th>
<th>Heart wt (g)</th>
<th>Range (g)</th>
<th>Malate dehydrogenase (mmoles/min/g)</th>
<th>Cytochrome oxidase (ml O₂/min/g)</th>
<th>Cytochrome c (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early hypertrophy</td>
<td>1.71 ±0.06*</td>
<td>1.56-1.81</td>
<td>1066</td>
<td>2.34</td>
<td>20.0</td>
</tr>
<tr>
<td>Control</td>
<td>1.17 ±0.02</td>
<td>1.04-1.23</td>
<td>1082</td>
<td>2.42</td>
<td>18.6</td>
</tr>
</tbody>
</table>

* Early hypertrophy vs. control, P < 0.001.

Discussion

There is considerable controversy in the literature regarding what happens to heart mitochondria in cardiac hypertrophy (1, 6, 8-15). Wollenberger and his co-workers (8-10) have reported that there is a decrease in the ratio of mitochondrial to myofibrillar area, a 50% disappearance of mitochondrial cristae, a significantly reduced capacity to carry out oxidative phosphorylation, and a diminished level of malate dehydrogenase activity in hypertrophied, non-failing canine hearts. In contrast, other workers have reported increases in the levels of succinate dehydrogenase (11) and cytochrome c (12) in the hearts of animals with cardiac hypertrophy.

Recently, Stoner et al. (13) reported “abnormally high ADP rates of O₂ consumption in the presence of malate-pyruvate” by mitochondria from the hearts of animals with long-standing aortic stenosis or aortic insufficiency without congestive failure. However, these investigators found no difference between the normal and stressed hearts with respect to the yield of mitochondria, expressed as milligram of mitochondrial protein per gram of heart.

McCallister and Brown (6), in an electron microscopic study, found no significant difference between normal and hypertrophied rat hearts in the percentage of cytoplasm occupied by mitochondria. They did find an increase in the mean area of individual mitochondria, as well as in the size of the inner structures of the mitochondria, in hypertrophied hearts (6).

Meerson (1), in a review in English of previous work published in the Russian literature, stated that there is an increase “in the activity of the oxidation-reduction enzyme systems of the myocardium . . .” and that “it acquires its greatest significance when the hypertrophy of the heart becomes marked. . .” He also states that succinate dehydrogenase (determined histochemically) increases
with cardiac hypertrophy (1). In contrast, Meerson and associates (14, 15) have more recently reported that the cell area occupied by mitochondria and the concentration of mitochondrial protein are normal in stable hypertrophy. However, they state that in early hypertrophy the concentration of mitochondrial protein and the cell area occupied by mitochondria, as visualized under the electron microscope, are increased twofold (14, 15).

In the present study, the status of the mitochondria of massively hypertrophied, non-failing hearts was investigated by several independent approaches.

Information on the respiratory chain enzymes of the mitochondrial cristae was obtained from whole heart homogenates by measuring total respiratory activity under conditions of uncontrolled respiration. The levels of activity of succinate oxidase, cytochrome oxidase, succinate dehydrogenase, and mitochondrial ATPase, as well as the concentration of cytochrome c served as additional markers for the mitochondrial cristae. With the exception of the ATPase determination, all the above assays were performed on whole heart homogenates to avoid possible differences in sampling or in the percentage yield of mitochondria.

Mitochondrial aconitase, DPN-dependent isocitrate dehydrogenase, mitochondrial TPN-dependent isocitrate dehydrogenase, and mitochondrial malate dehydrogenase were used as markers for the mitochondrial matrix, while the concentration of mitochondrial protein served as an indicator of the yield of mitochondria per gram of heart.

No significant differences were found between the hypertrophied and control hearts in the levels of any of the above mitochondrial constituents, either when the results were expressed per gram of heart or per milligram of cardiac protein. Taken together, these observations, which in many cases involve independent variables, provide strong evidence that cardiac mitochondria are increased in proportion to the other protein components of the hypertrophied cell. Furthermore, the increase in mitochondria in the hypertrophy-

As the hearts of the animals with the arteriovenous fistulas weighed approximately twice as much as those of the controls, the total mass of the mitochondria in the hypertrophied hearts must have roughly doubled. Our biochemical data do not indicate whether this increase resulted from a doubling in the number or in the size of the mitochondria. On the basis of the electron microscopic findings of McCallister and Brown, it would appear that the response involves an increase in size.

In the present study, hypertrophy was produced by means of a "volume overload." In the other studies discussed earlier (1, 6, 8-15), a "pressure overload" was used to produce hypertrophy. It is our current working hypothesis that the biochemical events involved in the development of cardiac hypertrophy, including the response of the mitochondria, are the same regardless of the mechanical stimulus used to increase the workload. However, in view of the controversy in the literature regarding the response of the cardiac mitochondria to a pressure overload (1, 6, 8-15), and our results, relative to the response of mitochondria in the early stages of hypertrophy, further work comparing the response to pressure and volume overloads is needed.

The levels of activity of pyruvate kinase, lactate dehydrogenase, phosphorylase, hexokinase, and phosphofructokinase were the same in the hypertrophied and control hearts. Since the latter three are the rate-limiting enzymes for glycolysis and glycogenolysis, it seems likely that the capacity, per gram of
heart, for regenerating ATP via the glycolytic system was unaltered by hypertrophy. The specific activities of creatine kinase and adenylate kinase were also the same in the hypertrophied and control hearts.

Thus it appears, on the basis of the foregoing evidence, that the capacity for regenerating ATP, both aerobically and anaerobically, expressed either per gram of heart or per milligram of myocardial protein, is unchanged in the non-failing, hypertrophied heart.

Further detailed studies involving specific proteins will have to be done to firmly establish whether all the proteins of the myocardial cell increase in parallel in cardiac hypertrophy. However, our finding that the levels of activity, per milligram of whole homogenate protein, of a large number of mitochondrial and cytoplasmic enzymes remain constant in the hypertrophied heart certainly favors this possibility. Further evidence for this interpretation comes from the observation that the concentrations of protein in the crude subcellular fractions obtained by differential centrifugation were the same in the hypertrophied and control hearts.

On the basis of the above information, it appears warranted to suggest that the process leading to the development of cardiac hypertrophy involves the derepression of all the genetic material normally represented in the heart cell. In other words, hypertrophy seems not to result from the induction of specific proteins, such as actin and myosin or mitochondrial enzymes, but, instead, seems to involve the reactivation (or acceleration) of growth of the whole cardiac cell.

Acknowledgments

The authors wish to express their appreciation to Mrs. May Chen for technical assistance and to Mrs. Celeste Amitin for assistance in the preparation of this manuscript.

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