Depletion of Cardiac Norepinephrine during Two Forms of Hemolytic Anemia in the Rat

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SUMMARY Knowledge of the status of cardiac norepinephrine (NE) during anemia could lead to a better understanding of the role the sympathetic nervous system plays in cardiac function during anemia. Rats were made anemic by treatment with phenylhydrazine (PHZ). After the rapid onset of anemia, 60% of the stored NE in the heart was lost within 48 hours after treatment. Associated with the loss of cardiac NE was an increase in the wet weight of the heart, which reached a value 40% above control 48 hours after treatment. PHZ itself probably does not directly mediate this depletion of NE, since the vas deferens, brain, and spleen had a normal store of NE at 48 hours. This contention was supported when rats, treated with PHZ, were transfused with normal rat red blood cells. This transfusion resulted in PHZ-treated rats which were not anemic. The hearts of these rats were not depleted of NE, but the hearts of the nontransfused, PHZ-treated controls were. Anemia also was induced by treating rats with anti-rat red blood cell serum. The hearts of these rats also were depleted of NE. These experiments show that during two forms of anemia there is a loss of NE from the sympathetic neurons innervating the heart. The effect of this on regulation of cardiac function remains to be determined.

AN INCREASE in cardiac output during anemia has been observed consistently in both clinical and experimental studies.1,2 This increase appears to be due to an increase in heart rate or stroke volume, or both. Over the past 20 years many laboratories have attempted to determine the physiological mechanism underlying these changes in cardiac function. Although some progress has been made, many questions are far from resolved. Since an activation of the sympathetic nervous system can mediate increases in cardiac output, several investigators have pursued the possibility that the sympathetic nervous system mediates the changes in cardiac function which occur during anemia. The results of these experiments have been contradictory;3 while some workers suggest that the sympathetic nervous system may play a significant role in causing the increase in cardiac output in anemia,4 others propose that it probably has little or no role.4,5

During the past 15 years a great deal of information has been gathered on the mechanism of synaptic transmission of the postganglionic sympathetic neuron.6,7 These studies have led to an understanding of the alterations in the metabolism of norepinephrine (NE) in cardiac sympathetic nerves which occur during increased nerve activity. In the experiments reported here we studied the NE in the nerves that innervate the heart to determine whether alterations in metabolism of cardiac NE result from anemia and are similar to changes which occur when the cardiac sympathetic nerves are activated. Our approach permitted sympathetic function to be examined in intact unanesthetized animals. We used two forms of experimental hemolytic anemia for this study, anemia caused by phenylhydrazine (PHZ) and anemia induced by an anti-rat red blood cell antibody. PHZ anemia has been used in the past as a model system for some clinically observed drug-induced hemolytic anemias, such as those resulting from various genetically mediated enzyme deficiencies (e.g., glucose-6-phosphate dehydrogenase) in the red blood cell.6 The experimental immune anemia has been used rarely in the study of the physiology of anemia6 but holds great promise as an experimental tool because drug-related side effects are minimal.

Methods

Male rats (Charles River, CD strain) were made anemic with phenylhydrazine HCl (Eastman) administered intraperitoneally. To study the time course of the response of the blood and cardiac NE to PHZ two types of experiments were performed. In the first, rats were given PHZ (125 mg/kg) 48 hours after treatment. In the second, the same dose was given 4, 12, 90, 168, and 408 hours before they were killed. To determine the effects of PHZ anemia on the NE content of the vas deferens, spleen, and brain, we treated the rats with PHZ (125 mg/kg) 48 hours before they were killed. We studied the effects of different degrees of anemia on cardiac NE content by treating rats with various doses of PHZ (35, 70, 85, and 125 mg/kg). These rats were killed 48 hours after treatment. In these experiments rats weighed 250–325 g at the time of sacrifice.

A transfusion experiment was performed in the following manner. Male rats (Charles River, CD strain) were anesthetized with ether and bled by puncture of the abdominal aorta. Blood was centrifuged and plasma and white blood cells were aspirated. The red blood cells (RBC's) were washed three times in 2 vol of saline (0.9% NaCl) with centrifugation and aspiration of the supernatant fluid after each wash. After the third wash the RBC's were diluted in saline to provide an 80% suspension. Rats of the same strain and sex as the donor were transfused by intraperitoneal injection of this RBC suspension. A dose of 7 ml of an 80% suspension was given twice to each of two groups of rats: a PHZ-treated group and a normal group. The third and fourth groups in this experiment were composed of animals given distilled water instead of PHZ, and saline in place of the RBC suspension (normal group), and animals given.

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To produce an experimental immune anemia, we prepared an anti-rat RBC rabbit serum. Blood was obtained from rats by puncture of the abdominal aorta. The RBC's were washed three times with 25 vol of saline in the manner outlined above. Subsequently the red cells were adjusted to a 10% suspension with saline. A dose of 2 ml of this red cell suspension was injected intravenously into the external marginal ear vein of male rabbits weighing 2.5–2.9 kg (New Zealand albino, Janes Rabbitry), 10 such injections being given during a 17-day period, as recommended by Campbell et al. Blood (20–25 ml) was obtained from rabbits by cardiac puncture 14, 18, and 22 days after the initial injection of the rat RBC suspension. The blood was allowed to clot and serum was obtained after centrifugation. The different samples of serum were then assayed for their ability to lyse RBC's in vitro, by the method of Campbell et al. The most potent sera, in terms of ability to lyse RBC's, were used to induce anemia in rats. In this experiment, we produced anemia in rats weighing 100–140 g through the tail vein. Control rats were given 0.2 ml of normal rabbit serum intravenously at the same intervals. Rats were killed 84 hours after the initial treatment with antisera.

We monitored the degree of anemia by taking 20 μl of blood from the tail at various times before they were killed. Blood was assayed for hemoglobin by the cyanomethemoglobin spectrophotometric assay. Rats were killed by cervical dislocation, and the organ to be assayed for NE was quickly removed from the animal, cleaned of adhering tissue, placed in 10 ml of cold 10% trichloroacetic acid, weighed, and frozen for storage. Tissue content of the blood decreased by 40%. This was followed by a gradual decline in hemoglobin concentration over the next 2 weeks. After thawing, the tissue was homogenized with a VirTis homogenizer at 4°C. The homogenate was then filtered. To the filtrate we added 1.0 ml of 1.0 M tris(hydroxymethyl)-aminomethane (Tris) buffer (pH 8.4), 0.5 ml of 10% Na2-ethylenediaminetetraacetic acid (EDTA), and 0.1 ml of freshly prepared 5% sodium metabisulfate. The filtrate was then titrated to pH 8.4; 5 N NaOH was used to bring pH to 7.5, then 0.5 N NaOH to reach pH 8.4. The filtrate was added immediately to 0.4 g of alumina (Woelm neutral activity grade 1) in a 30-ml stoppered centrifuge tube. The alumina had previously been washed with acid and dried according to the method of Anton and Sayre. Before the filtrate was titrated, the 0.4 g of alumina had been suspended in 10 ml of 0.1 M Tris buffer (pH 8.4). This buffer was aspirated just before the addition of the filtrate. After the addition of the filtrate to the centrifuge tube containing the alumina, the tube was shaken for 5 minutes and the supernatant fluid was aspirated. The alumina was then washed with distilled water three times and the supernatant fluid was aspirated and discarded each time. The alumina was then shaken with 5 ml of 0.2 N acetic acid. The supernatant fluid, containing eluted NE, was then assayed fluorometrically by the method of Crout except that reversed blanks were used instead of faded blanks. Samples were read on an Aminco Bowman spectrophotofluorometer. For each sample a reversed blank and an internal standard were assayed. The efficiency of isolation of NE on alumina was 75–85%.

For statistical analysis we used Student's t-test and the linear regression test, according to the methods described by Snedecor and Cochran.

Results

Figure 1 is a summary of the results of the two experiments in which we studied the time course of hemoglobin loss, increase in heart weight, and cardiac NE depletion. Within 4 hours after administration of PHZ the hemoglobin content of the blood decreased by 40%. This was followed by a gradual decline in hemoglobin concentration over the next 44 hours and then a gradual return to normal by 168 hours.

![Figure 1](https://example.com/figure1.png)
When the hearts of these rats were assayed for NE, there was a gradual decline in NE concentration which reached the lowest level (approximately 40% of control when based on data expressed in units of micrograms per heart) 48 hours after treatment. This was followed by a gradual return to normal values 168 hours after treatment. An increase in heart wet weight mirrored the time course of NE depletion and anemia; weight exceeded control by approximately 40% 48 hours after treatment and returned to normal at 168 hours. Because the increase in heart weight reduces the apparent NE concentration in the heart when this value is expressed in units of micrograms of NE per gram of heart, the data on cardiac NE are expressed as percent of control computed from data expressed in units of micrograms of NE per heart.

When the spleen, vas deferens, and brain were assayed for NE 48 hours after PHZ treatment, the concentration of neurotransmitter was normal (Table 1). There was a highly significant correlation between the severity of anemia and degree of cardiac NE depletion (Fig. 2).

Changes in circulating hemoglobin caused by both PHZ treatment and transfusion are shown in Figure 3. Within 12 hours after PHZ treatment there was a sharp drop in circulating hemoglobin, from approximately 16 g/100 ml of blood to 8 g/100 ml of blood. After the transfusions of the RBC suspension (given 12 and 24 hours after PHZ treatment), the concentrations of hemoglobin increased and returned to normal 48 hours after PHZ treatment. During this period, rats given PHZ but not transfused remained anemic, normal rats maintained their level of circulating hemoglobin, and the normal transfused group became polycythemic. When the rats were killed, we found that transfusion of animals made anemic by PHZ had prevented the depletion of cardiac NE and increase in heart weight which were seen during PHZ anemia. Transfusion alone did not alter cardiac NE concentration or heart weight of normal rats (Table 2).

When rats were treated with anti-rat RBC rabbit serum, there was a gradual decline in the circulating hemoglobin concentration during the 80 hours following the initial treatment. This decline was followed by a gradual return of hemoglobin to normal levels by 160 hours. The control group, treated with normal rabbit serum, maintained a normal hemoglobin concentration during the course of the experiment (Fig. 4). Rats were killed 84 hours after initial treatment. Rats treated with anti-rat RBC rabbit serum showed a decreased store of cardiac NE and a small increase in heart wet weight (Fig. 5).

**Discussion**

The experimental method used in this study offers several advantages in the examination of the physiology of anemia. Anemia can be induced quickly, easily, with high reproducibility, and on a large scale through the use of PHZ or rabbit antisera. Molecular events occurring during anemia can be studied in intact unanesthetized animals. In addition, chronic as well as acute experiments can be performed.

A disadvantage to the study of PHZ anemia is that observed changes could be due to the presence of PHZ itself. The results of several experiments argue against this with respect to depletion of cardiac NE. If PHZ, or a metabolite of PHZ, directly mediates the loss of NE from cardiac

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**Table 1** Effects of Phenylhydrazine (PHZ) Anemia on Norepinephrine (NE) Content of Spleen, Vas Deferens, and Brain

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Spleen</th>
<th>Vas deferens</th>
<th>Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hb (g/100 ml)</td>
<td>NE (µg/spleen)</td>
<td>Hb (g/100 ml)</td>
</tr>
<tr>
<td>Control</td>
<td>15.5 ± 0.19</td>
<td>0.173 ± 0.012</td>
<td>15.4 ± 0.29</td>
</tr>
<tr>
<td>PHZ</td>
<td>6.56 ± 0.38*</td>
<td>0.169 ± 0.013†</td>
<td>7.15 ± 0.37*</td>
</tr>
</tbody>
</table>

Data, expressed as mean ± SEM, from three separate experiments in which rats were treated with PHZ (125 mg/kg) and killed 48 hours later. Splenic NE is expressed as µg NE/spleen, because of spleen enlargement during anemia. N = 6; Hb = hemoglobin.

* P < 0.001 when compared to control.
† P > 0.05 when compared to control.

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**Figure 2** Correlation between the severity of anemia and cardiac norepinephrine (NE) depletion. Triangles present data for animals from a single experiment in which phenylhydrazine in doses of 35, 70, or 85 mg/kg was given. Circles, inverted triangles, and diamonds present data for animals from three other experiments and their controls in which phenylhydrazine (125 mg/kg) was given. Results for control animals in the four experiments are identified by the appropriate symbol. Rats were killed 48 hours after treatment.
sympathetic nerves, then noradrenergic nerves which are present in other organs should be similarly affected, because adrenergic neurons in the various organs they innervate appear to be identical in terms of NE synthesis, storage, and release. Since only the heart, and not the vas deferens, spleen, or brain, was depleted of NE 48 hours after PHZ treatment, PHZ probably did not directly induce the depletion of cardiac NE. The high degree of correlation between the severity of anemia and cardiac NE depletion (Fig. 2) is evidence supporting the contention that anemia and not PHZ plays a major role in causing the loss of cardiac NE.

Transfusion of rats made anemic by PHZ 12 and 24 hours after PHZ treatment provided nonanemic PHZ-treated rats. If PHZ alone produces the loss of cardiac NE, then these rats should show as much depletion as the nontransfused animals given PHZ. However, the transfusion prevented cardiac NE depletion. This experiment shows that PHZ does not directly mediate cardiac NE loss during PHZ anemia, and that anemia (that state characterized by a decrease in the number of functional RBC's in the circulation) plays a major role in cardiac NE depletion during PHZ anemia. However, one cannot conclude that anemia acts alone to produce the loss of cardiac NE, since anemia could be functioning synergistically with some other factor (e.g., PHZ itself) to produce this NE depletion.

In order to determine whether PHZ, a PHZ metabolite, or a by-product of PHZ treatment other than anemia plays a role in cardiac NE depletion, we performed experiments using an experimental immune anemia. When hearts of immune anemic rats were assayed for NE, we found a decreased store of the neurotransmitter. From these results we concluded that cardiac NE depletion is associated with the state of hemolytic anemia and is not dependent in any way on the treatment of rats with PHZ. In addition, this experiment supports the conclusion, drawn from the transfusion experiment, that anemia plays a major role in causing the loss of NE from the heart.

It would not be reasonable to deduce from these experiments that cardiac NE is depleted during all forms of anemia. Some factor associated with the loss of RBC's during hemolytic anemia (e.g., increase in plasma bilirubin) may act in concert with the decrease in number of RBC's to deplete cardiac NE. Since this factor may not be present during other forms of anemia, depletion of cardiac NE may not occur. A study of cardiac NE stores during a nonhemolytic anemia would demonstrate whether or not cardiac NE is depleted during forms of anemia other than that caused by hemolysis.

It would seem unlikely that the depletion of cardiac NE is induced by an increase in the rate of release of the neurotransmitter that is due to an increase in the frequency of spike trains in the neurons. Under various experimental conditions, when the activity of the sympathetic nervous

### TABLE 2 Effect of Red Blood Cell (RBC) Transfusion on Cardiac Norepinephrine (NE) and Heart Weight during Phenylhydrazine (PHZ) Anemia

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hb (g/100 ml)</th>
<th>NE (μg/heart)</th>
<th>Heart, wet wt (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>16.5 ± 0.23</td>
<td>0.431 ± 0.030</td>
<td>0.777 ± 0.022</td>
</tr>
<tr>
<td>Normal-transfused</td>
<td>27.8 ± 0.52*</td>
<td>0.452 ± 0.080</td>
<td>0.807 ± 0.020</td>
</tr>
<tr>
<td>PHZ</td>
<td>6.86 ± 0.21*</td>
<td>0.181 ± 0.018*</td>
<td>0.981 ± 0.020*</td>
</tr>
<tr>
<td>PHZ-transfused</td>
<td>19.6 ± 2.84</td>
<td>0.373 ± 0.031</td>
<td>0.827 ± 0.020</td>
</tr>
</tbody>
</table>

Rats were treated with PHZ or its vehicle, and 12 and 24 hours later they were transfused. The time course of alterations in circulating hemoglobin (Hb) is illustrated in Figure 3. Animals were killed 48 hours after the initial treatment. Results are expressed as mean ± SEM. N = 6.

* P < 0.001.
system is increased, the concentration of NE in these nerves remains remarkably constant. Even though there is an increase in the rate of loss of NE from the nerve terminals, the released NE is replaced as a result of NE uptake by these terminals and an increased rate of NE synthesis. The presence of such a system to maintain a steady state for NE has been demonstrated in cardiac sympathetic nerves. However, it is conceivable that during anemia the rate of release of NE could be increased to such an extent that these compensatory mechanisms might not be able to maintain a normal store of NE.

Another factor that might mediate the depletion of cardiac NE is hypoxia. Table 1 shows that the vas deferens, spleen, and brain were not depleted of NE during PHZ anemia. If hypoxia mediates NE depletion, then the level of hypoxia in these organs must not be severe enough to deplete NE. Accordingly, if hypoxia mediates cardiac NE loss, then the heart must be more hypoxic than these other organs.

Large increases in coronary blood flow have been reported during anemia. The magnitude of this increase depends on the severity of the anemia. The increases in coronary blood flow appear to be unable to compensate for the decrease in oxygen availability. Bhatia et al. found a significant decrease in myocardial oxygen consumption during anemia even though there was an increase in workload on the heart. Considering the decrease in oxygen available and the possible increase in its utilization, it would appear likely that the heart is hypoxic during anemia.

In the past, there have been reports on a few experiments on the effects of hypoxia on NE stores in different tissues. Debijadi et al. reported a reduction of 50% in hypothalamic NE in the cat exposed to hypobaric hypoxia. Davis and Carlsson showed that hypoxic hypoxia can inhibit the synthesis of NE in the brain by inhibiting tyrosine hydroxylase; however, they did not observe NE depletion. Goldman and Harrison studied the effects of hypoxic hypoxia on cardiac NE and found no loss of NE after 4 hours of exposure. This experiment does not rule out hypoxia-mediated depletion of NE during hemolytic anemia, because the duration of exposure to hypoxia was shorter than the time interval preceding the observation of NE depletion during PHZ anemia.

A second condition that might be involved in the loss of NE during anemia is enlargement of the heart. During the course of this study, whenever NE was depleted there was an increase in the size of the heart. The increase in size is, at least in part, due to growth of some type, since there is an increase in the dry weight of the heart in anemic rats (unpublished observation). The rapidity of the increases in heart weight reported here is surprising. However, under a variety of experimental conditions that cause increased cardiac work, relatively large increases in heart weight have been observed within several days.

Two laboratories have reported NE depletion in conjunction with cardiac hypertrophy. Fisher et al. have shown cardiac NE depletion associated with cardiac hypertrophy during aortic constriction in the rat. Their studies on the status of cardiac NE suggest a decrease in the NE-binding capacity of the nerves. Chidsey and Braunwald, in their studies of cardiac NE depletion during congestive heart failure (with associated hypertrophy), indicate that in association with congestive heart failure there is a decrease in the number of sympathetic nerve terminals in the heart. A...
finding supporting this contention is a marked drop in the content of tyrosine hydroxylase in the heart during congestive heart failure. Whether the mechanism that underlies the depletion of cardiac NE during anemia is similar to that in aortic constriction or congestive heart failure awaits further study.

The experiments reported here indicate that cardiac NE is depleted during hemolytic anemia. A question of physiological consequence is: Does a depletion of NE mean that the sympathetic nerves are not able to modify cardiac function during anemia? Gaffney et al. report that cardiac sympathetic nerves are able to elicit normal cardiac responses when only 15% of the normal store of NE is present in nerve terminals. However, in an experiment by Covell et al. hearts responded to a smaller extent to sympathetic nerve stimulation when these nerves were depleted of 75% of their NE. At the present time it is difficult to conclude whether a reduction in the NE content of cardiac sympathetic nerves will or will not diminish the response of the heart to increases in sympathetic tone.

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