THE PHOSPHATASES OF HUMAN ERYTHROCYTES

By Helen Quincy Woodard, Ph.D.

VARIOUS investigators have reported that erythrocytes exhibit phosphatase activity at pH values between 4 and 6, and some believe that this is due to two acid phosphatases with maximum activities at about pH 4.2 and 5.6. Reports on alkaline phosphatase activity have been less numerous.

In the course of an investigation of the glycerophosphatase activity of human erythrocytes the present author encountered difficulties with the experimental method which led to the discovery that erythrocytes contain a labile phosphorus compound which decomposes spontaneously as soon as the cells are hemolyzed. The decomposition is probably enzymatic, but, as its exact nature is not yet clear, it is here described only by the general term "autolysis." It is probable that some of the variations in phosphatase activity reported by previous authors were, in reality, due to variations in the degree of autolysis which takes place under different experimental conditions. For instance, Behrendt found considerable alkaline glycerophosphatase activity, but little alkaline phenylphosphatase activity. Since phenyl phosphatase is determined by measuring the amount of phenol liberated, the findings would not be confused by the presence of phosphate liberated by autolysis, as they would in the determination of glycerophosphatase. In the present report some of the properties of the autolytic process are described, and evidence is presented which suggests that the amount of the substance which undergoes autolysis may be increased in certain diseases.

METHOD

The early work was done on cells washed from clots, no anticoagulant being used. The blood was centrifuged, and the serum and clots were removed. The loose cells were then washed twice with physiologic saline with brief centrifuging, the buffy coat being removed as far as possible. They were then suspended in physiologic saline in graduated centrifuge tubes and packed by being centrifuged for fifteen minutes at about 1000 times gravity.

Because of the difficulty of obtaining sufficient cells when no anticoagulant was used, the effect of heparin was studied. Heparin-Lederle in solution suitable for intravenous use was found to be free of significant amounts of inorganic phosphate, and to be without significant effect on erythrocyte phosphatase or on autolysis in concentrations 60 times those likely to be encountered when it is used as an anticoagulant. In its behavior toward heparin, erythrocyte phosphatase thus differs from the acid phosphatases of human prostate and of rat liver, which are markedly inhibited by this agent. Much of the present work was done with erythrocytes obtained from blood in which coagulation had been prevented by the addition of 0.10 cc. of Heparin-Lederle to 1 cc. of blood.

When a specimen could not be used immediately, the washed erythrocytes were kept in a packed condition in the presence of a small amount of saline in the ice box for periods up to twenty-four hours. Immediately before an experiment was begun, the saline was pipetted off, distilled water equal in volume to the volume of the packed cells was added, the tube was closed with a rubber stopper and shaken vigorously for a few seconds, and the hemolysate was used as soon as the bubbles had risen.

Glycerophosphatase was determined by the author's modification of the method of Bodansky. The hemolysate was handled like serum except that, for inorganic phosphate determination, it was di-
luted with only three volumes of trichloracetic acid, instead of nine volumes. Hexose phosphatase determinations were made in a substrate containing 800 mg./liter of a calcium hexose diphosphate preparation obtained from Schwartz Laboratories. The method was the same as for glycerophosphatase, except that the blanks and correction curves were different. Autolysis was determined in the same way as glycerophosphatase or hexose phosphatase, except that dilute water solutions of hydrochloric acid or sodium hydroxide were substituted for the substrate solutions. Results were calculated in milligrams of inorganic phosphorus or Bodansky units of phosphatase activity per 100 cc. of packed cells.

The inorganic phosphate concentration of the filtrates obtained on precipitation of some of the preparations with trichloracetic acid increased about 2.5 per cent on standing for twenty-four hours in the ice box. Hence, all readings were made within two hours of precipitation. Because of the high viscosity of the hemolysates, their tendency to flocculate irregularly when added to substrates at pH values between 5 and 6, and the difficulty of obtaining homogeneous precipitates with trichloracetic acid, the experimental error was considerably higher than that usually encountered in determinations of serum phosphatase. Errors of the order of ± 10 per cent were difficult to avoid, and larger ones were sometimes seen in values which were obtained by difference.

RESULTS

The inorganic phosphate of freshly prepared hemolysates of fresh cells was usually between 0.5 and 2.0 mg./100 cc., although values up to 4.5 mg./100 cc. were seen occasionally. Small and irregular fluctuations in inorganic phosphate content took place in washed cells which were kept unruptured in the ice box for twenty-four hours. As stated in the introduction, it was observed early in the work that, as soon as the cells were ruptured, the inorganic phosphate of the preparation began to increase very rapidly. The increase was detectable in a few minutes at room temperature, and reached five-fold in one hour at 37 C. In the determination of phosphatase by the Bodansky method it is necessary to subtract the inorganic phosphate which is originally present in the preparation from the total observed after incubation with a substrate in order to obtain the amount which is liberated from the substrate. In order to determine the phosphatase activity of a hemolysate on a substrate such as sodium β glycerophosphate, it is therefore also necessary at the same time to allow another portion of the hemolysate to autolyze in distilled water of the same volume, pH and temperature as the substrate. The amount of inorganic phosphate liberated by autolysis plus that originally present is then subtracted from that found in the mixture of hemolysate and substrate after incubation, in order to find the amount liberated from the substrate. In the course of developing the technic for this procedure a comparison was made of the effect of various experimental conditions on autolytic and phosphatase activities.

Effect of pH. The relation of autolytic activity to pH appears to be variable. In the majority of preparations the maximum activity occurs at pH 9, as is shown in figure 1. The data in this curve were obtained from five preparations. Three were cells from the heparinized blood of 2 normal males and one normal female, one was from the heparinized blood of a female with osteitis deformans, and one was a pool of cells obtained from the clotted blood of 6 patients with neoplastic disease. As the absolute activities of the different preparations differed somewhat, all the activities were calculated in terms of that at pH 9.0 as 100 per cent. While the autolytic activities of these selected preparations exhibited a fairly consistent relationship to pH, certain other specimens which were examined behaved differently. In
these, autolytic activity occurred about equally over a broad range between pH 5 and pH 8, with or without a secondary maximum at pH 9. The difference did not appear to be related to the sex or diagnosis of the donor of the erythrocytes, nor to whether or not an anticoagulant (heparin) had been used, and its cause is not apparent at present.

The relation of glycerophosphatase activity to pH is shown in figure 2. The data are derived from twelve experiments. In six of these, pooled erythrocytes washed from clots were employed. Each pool was obtained by combining the cells from about six blood samples from patients with various neoplastic diseases. In the remaining experiments, erythrocytes from the heparinized blood of 2 normal males, 2 normal females, and 2 males with osteitis deformans were studied separately. Each point represents the difference between the total inorganic phosphate found after incubation in a mixture of hemolysate with sodium β glycerophosphate and that found after incubation in a mixture of hemolysate with water adjusted to the same pH as that of the serum-substrate mixture. In each experiment the activities at other pH values were calculated in terms of that at pH 6.0 as 100 per cent. It is evident that the erythrocyte preparations exhibited a rather sharp maximum of glycerophosphatase activity between pH 5.5 and 6.2, and was low at pH 4 and pH 9. There appeared to be no consistent difference between the cells from heparinized and clotted blood. It is probable that the scatter of the individual points is due to
experimental error rather than to true differences in the behavior of the different specimens.

A similar but less extensive study was made of the relation of the hexose phosphatase activity of erythrocytes to pH. The activity with respect to this substrate also showed a rather sharp maximum at pH 6.

*Effect of Temperature.* After incubation for one hour at pH 6.0 at various temperatures the autolytic activities of hemolysates did not show a clear-cut temperature maximum, but increased irregularly up to the boiling point. The poor reproducibility was probably due to the variable amount of coagulation of protein encountered at the higher temperatures. It was evident, however, that autolysis did not cease at temperatures high enough to inactivate most enzymes.

![Graph](image)

**Fig. 2.—Relation of Glycerophosphatase Activity to pH. (Symbols same as in figure 1.)**

The β glycerophosphatase of erythrocytes has a maximum activity at about 57°C. The approximate activities at various temperatures after incubation for one hour at pH 6.0 are shown below.

<table>
<thead>
<tr>
<th>Temperature (degrees C.)</th>
<th>Relative Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.5</td>
</tr>
<tr>
<td>37</td>
<td>1.0</td>
</tr>
<tr>
<td>57</td>
<td>1.5</td>
</tr>
<tr>
<td>80</td>
<td>0.8</td>
</tr>
</tbody>
</table>

The effect of temperature on calcium hexose diphosphatase was not studied.

*Effect of Time of Incubation.* Figure 3, G shows the amount of phosphate liberated
from sodium β glycerophosphate at 37 C. and pH 6.0 after various times of incubation, calculated in terms of the amount liberated in one hour as unity. It is seen that the rate of decomposition of the substrate increases up to one hour, and remains approximately constant between one hour and four hours. Times of incubation longer than four hours were not studied, as the concentration of phosphate became too high to determine colorimetrically.

Figure 3, A shows the amount of phosphate liberated by autolysis at 37 C. and pH 6.0, calculated in terms of the amount liberated in one hour as unity. The rate of autolysis is seen to be essentially constant during the first four hours. The rate later decreases, and the process ceases after about twenty hours of incubation. This is shown in figure 4, in which the amount of phosphate liberated by autolysis at 37 C. and pH 6.0 in various times is calculated in terms of the amount liberated in twenty-five hours as unity. If the incubation is carried out at pH 9, the maximum is reached somewhat sooner, but the total amount of phosphate liberated is the
same. The cessation of activity after twenty to twenty-five hours of incubation is probably due to exhaustion of the naturally occurring substrate, although the possibility of the development of some type of inhibition has not been excluded.

Effect of Leukocytes. It is evident that any specimen of erythrocytes is likely to contain a few leukocytes even though every effort is made to remove the buffy coat. Hence it is important to have some idea of the magnitude of the effect of such contamination. The technics employed in this study of erythrocytes cannot be used readily for leukocytes. This is partly due to the impossibility of obtaining adequate volumes of normal leukocytes, and partly due to the tendency of myelocytes, and especially lymphocytes, which may be obtained from patients with leukemia, to form an almost unmanageable jelly as soon as lysed. Nevertheless, sufficient work has been done with leukocytes obtained from patients with leukemia to show that both lymphocytes and myelocytes exhibit autolytic activities over the entire range from pH 3.5 to pH 10.0. The activity is somewhat greater in the acid range, and amounts to 10 to 20 units per 100 cc. of packed cells per hour. Both lymphocytes and myelocytes decompose sodium β glycero phosphate vigorously at pH values between 4 and 6, with a possible secondary maximum at pH 9.5. The activity of lymphocytes appears to be about 50 units per 100 cc.; that of myelocytes between 300 and 400 units per 100 cc. As these values are considerably above the
corresponding ones for erythrocytes, it is evident that incorrect readings for erythrocyte phosphatase may be obtained for patients with leukemia. However, no serious error due to the unavoidable presence of leukocytes is likely to occur in individuals with normal white counts, or those with mild degrees of leukocytosis.

Effect of Diagnosis. Individual differences in erythrocyte phosphatase have been studied in a small series of cases. The results are summarized in table 1. All determinations were made on heparinized blood at pH 5.9 to pH 6.1. Incubation periods for glycerophosphatase and hexose phosphatase were one hour. Autolysis was determined after incubation for one hour and for twenty-three to twenty-five hours.

The normal individuals, 14 males and 3 females, were mostly blood donors, and all had normal hemoglobin readings. The anemic patients had hemoglobin values of 5 to 10 Gm. per 100 cc. They included 2 cases of pernicious anemia and one case of macrocytic anemia with free hydrochloric acid in the gastric contents; 3 cases each of myelogenous leukemia and lymphatic leukemia; and one case each of lymphosarcoma, Hodgkin’s disease, Cooley’s anemia, and carcinoma of the breast with bone metastases. Seven were males and 6 were females. The last group consisted of 8 female patients with carcinoma of the breast who had developed a polycythemia with 15 to 20 Gm. per 100 cc. of hemoglobin while under therapy with testosterone propionate. The white counts of the patients with leukemia ranged from 38,000 to 156,000 per cu. mm.; the white counts of the other subjects were normal.

Under the experimental conditions used, the phosphatase activity of normal erythrocytes with respect to calcium hexose diphosphate averaged about 90 per cent of that with respect to sodium β glycerophosphate. The glycerophosphatase activities of erythrocytes from anemic blood appeared to be somewhat above normal, and those of erythrocytes from polycythemid blood somewhat below normal, although there is so much overlapping between the groups that the differences are

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of cases</th>
<th>Glycerophosphatase units/100 cc./hr. Average</th>
<th>Range</th>
<th>Hexosephosphatase units/100 cc./hr. Average</th>
<th>Range</th>
<th>Autolysis mg./100 cc./hr. Average</th>
<th>Range</th>
<th>Autolysis mg./100 cc./24 hrs. Average</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>17</td>
<td>15.0</td>
<td>10.4-18.4</td>
<td>13.5</td>
<td>9.6-15.0</td>
<td>2.5</td>
<td>1.4-3.6</td>
<td>12.8</td>
<td>9.0-16.9</td>
</tr>
<tr>
<td>Anemia with leukemia</td>
<td>6</td>
<td>18.5</td>
<td>11.2-30.1</td>
<td>13.5</td>
<td>9.6-15.0</td>
<td>6.7</td>
<td>2.3-10.5</td>
<td>14.6</td>
<td>17.0-32.0</td>
</tr>
<tr>
<td>Anemia without leukemia</td>
<td>7</td>
<td>17.9</td>
<td>13.6-23.7</td>
<td>13.5</td>
<td>9.6-15.0</td>
<td>4.4</td>
<td>1.5-8.6</td>
<td>10.5</td>
<td>14.3-24.3</td>
</tr>
<tr>
<td>Testosterone-induced polycythemia</td>
<td>8</td>
<td>11.4</td>
<td>7.6-15.0</td>
<td>13.5</td>
<td>9.6-15.0</td>
<td>2.8</td>
<td>1.9-3.3</td>
<td>12.7</td>
<td>10.6-15.1</td>
</tr>
</tbody>
</table>

* The author is indebted to Dr. Paul Reznikoff of New York Hospital for the opportunity to examine these patients.
of doubtful significance. The differences in the autolytic activities in the different groups are more conspicuous. The erythrocytes from the majority of the anemic bloods liberated considerably more phosphate by autolysis both in one hour and in twenty-four hours than did either normal erythrocytes or those from patients with polycythemia. While the figures for leukemic bloods may be elevated a little by the unavoidable inclusion of some leukocytes in the preparation, the differences between the anemic groups with and without leukemia are not great. Since the amount of phosphate liberated by autolysis in twenty-four hours probably represents the total amount of substrate originally present in the cells, there is thus a suggestion that the concentration of this substance is increased in erythrocytes from various types of anemia.

Summary

Hemolysates of human erythrocytes begin to liberate inorganic phosphate as soon as they are lysed. In most cases the process takes place most rapidly at pH 9, but some preparations are about equally active over the entire range from pH 5 to pH 9. The process ceases after incubation for twenty-four hours at 37 C.

Hemolysates of human erythrocytes decompose sodium $\beta$ glycerophosphate and calcium hexose diphosphate about equally. For both substrates there is a well defined maximum of activity at pH 6.0. No other maxima were observed over the range from pH 4 to pH 9.

There is some evidence that erythrocytes from patients with various types of anemia liberate more inorganic phosphate when lysed than do those from normal individuals.

References

THE PHOSPHATASES OF HUMAN ERYTHROCYTES

HELEN QUINCY WOODARD