The Cholinesterase of Erythrocytes in Anemias

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In 1940 it was found that the cholinesterase activity of the erythrocytes was greater than normal in various anemias; in pernicious anemia in relapse the enzymic activity was normal or low, and during treatment rose to high values like those of other anemias, then fell gradually as the blood picture approached normal. The findings in pernicious anemia were confirmed in 1948 by Meyer and co-workers. Similar observations were made in 1948 by Cline and co-workers in macrocytic anemia of pregnancy responding to anti-pernicious anemia therapy. In 1948, Pritchard found high cholinesterase activity in the red cells of rats made anemic by repeated bleeding, and furthermore showed experimentally that the elevated enzymic activity was localized in the reticulocytes and young cells.

The primary purpose of the present investigation was to determine whether or not the increase in cholinesterase activity found in anemias represents solely an increase in the concentration of the same enzyme (or mixture of enzymes) found in normal red cells.

CLINICAL MATERIAL†

Since the changes in enzymic activity occurring in anemias have been well established, it was evident that an intensive study of a few clinically clearcut cases would answer the main question. Two groups of patients were selected for this purpose: (1) 4 cases of anemia from uncomplicated hemorrhage, and (2) 6 cases of pernicious anemia of the addisonian type. Because of the suggestion contained in the earlier data and substantiated by Pritchard's work that high titers of cholinesterase activity in the erythrocytes are associated specifically with hyperactive, orderly hematopoiesis, a third group was added. This was composed of 4 miscellaneous cases of severe anemia secondary to cachectic diseases in which the hematopoietic function was presumably suppressed.

Case histories are given briefly by groups, since individual histories contribute little or nothing to the interpretation of the data.

Anemia from hemorrhage: Four cases, all otherwise healthy men, aged 24–76, were hospitalized for acute hemorrhage from peptic ulcer. All responded well to supportive therapy and all were in very good clinical condition at the time the samples for the present work were obtained, from five to twelve days after the onset of symptoms. The red cell counts

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(RBC) were 2.5-3.6 million; hematocrits (VPC) 24-33; and hemoglobin (Hbg.) 6.1-9.0 grams.

Pernicious anemia: Three men and 3 women aged 56-77, all had macrocytic, hyperchromic anemia, megaloblastic marrow, achlorhydria, glossitis and neurologic involvement varying in degree from loss of vibratory sense to severe ataxia. Admission RBC were 1.0-2.9 million, VPC 11-33, Hbg. 3.4-11.1 grams, mean corpuscular volume (MCV) 110-150 cubic micra, reticulocytes 0-3.5 per cent. All had good reticulocyte responses to injected liver extract, and all improved clinically.

Miscellaneous anemias: In 4 cases, severe anemia was secondary to acute myelogenous leukemia, cirrhosis of the liver with jaundice (moribund), sarcoma with a long history of extensive x-ray therapy, and sarcoma under treatment with nitrogen mustard. All were in poor clinical condition and all died within a few weeks of the time the samples were taken. RBC were 1.8-2.7 million, VPC 17-25. The two sarcoma cases were also leukopenic, with white cell counts 2500 and 2700 respectively.

EXPERIMENTAL METHOD

Blood samples (5 or 10 ml.) were drawn from the antecubital vein and placed in bottles prepared by evaporating to dryness 1.0 ml. of a 2.4 per cent solution of sodium citrate. Three or 5.0 ml. of blood was centrifuged, the plasma drawn off, the buffy coat removed with a glass hook and the cells washed three times in 0.9 per cent saline. No visible hemolysis occurred at any stage. The washed cells were then diluted with approximately twice their volume of distilled water and stored temporarily at 4 C.

From the remainder of the sample preliminary determinations were made, using 3 × 10⁻⁴ M quinidine sulfate as a selective inhibitor of the plasma enzyme to determine the quantity of red cells which would give the most suitable reaction rates. The washed cells were then further diluted with distilled water to a volume such that 1.0 ml. contained from 7 to 14 µl. of cells. The high dilution was made to reduce errors in sampling and to ensure complete hemolysis for the sake of uniformity. Cells handled in the manner described were found to require dilution with 15 parts of water for complete hemolysis as judged by the end-point of the fragility test and by examination in the counting chamber. A glass bead was added and the preparation shaken vigorously to ensure uniform suspension of cell fragments, since investigators differ as to the distribution of the enzyme between the stroma and the supernatant fluid. These dilute preparations tended to lose their activity after a few weeks, and were discarded as soon as they exhibited less activity than that shown in the preliminary determinations.

In order to ascertain whether any substance capable of affecting the results had diffused into or out of the cells during the necessary delay of two to three hours between the drawing of the sample and the completion of the processing, activity-pS curves (defined in the following section) were determined using whole blood from each of the clinical conditions, with quinidine to inhibit the plasma enzyme. The curves so obtained were indistinguishable from the corresponding ones for washed red cells.

Determinations of the rates of hydrolysis of acetyl choline iodide (ACh) were made with Barcroft differential manometers at 37 C. The basic procedure was as previously described. 1.0 ml. of red cell hemolysate was placed in the main chamber of the manometer flask. The Ringer-bicarbonate for the main chamber was made up in sufficiently increased concentration to give final salt concentrations in the 3.0 ml. reaction mixture equal to those of Krebs and Henseleit.

Special procedures were used for various supplementary experiments and these will be described along with the results.

THEORETIC BASIS OF THE PRESENT WORK

Under controlled conditions the enzymic activity (initial velocity at any chosen concentration of the substrate) is proportional to the amount of enzyme present. When samples
from different sources are compared, however, there is a possibility that such an interpretation might be in error. There are two major types of cholinesterase, one predominant in normal red cells, the other in the plasma of most mammals including humans, and the enzyme-substrate relationships of the two types are quite different. Qualitative differences in the enzyme can be detected by investigating the apparent kinetic properties of the two samples.

The enzyme-substrate relationship of the cholinesterase of normal human erythrocytes was analyzed in 1948. A more comprehensive investigation of the enzyme of horse and cow erythrocytes, and of the same type of enzyme from other tissues is included in Augustinsson's recent monograph on cholinesterases. The enzyme from all of these sources was found to form its complexes with the substrate in accordance with Haldane's equation:

\[
V = \frac{V(S)}{K + (S)^n/K^2}
\]

(Equation 1)

where \(v\) is the initial velocity at substrate concentration \((S)\), \(V\) the theoretic limiting velocity, \(K\) the apparent dissociation constant of the active enzyme-substrate complex \((ES)\) and \(K_2\) that of the inactive complex \((ES_2)\), with \(n\) in this case equal to 2. The type of cholinesterase found in human plasma and various tissues does not form the inactive complex and \(V\) approaches \(V\) asymptotically as \((S)\) is increased. Also its affinity for the substrate \((1/K)\) is only about one-tenth that of the red cell type, so that maximal activity is observed at much higher concentrations of ACh, concentrations at which the red cell enzyme would be almost completely inactivated by the formation of \((ES_2)\).

If the experimental values of \(K_2\), \(K_2\) and \(n\) are the same in two samples, this provides evidence that the same enzyme (or mixture of enzymes in the same proportions) is present in both samples. It also rules out the possibility that one sample contains a competitive inhibitor of the enzyme while the other does not. The possible presence of activators or noncompetitive inhibitors has to be tested in other ways, as will be discussed later. If all of these possibilities are ruled out, \(V\) may be considered as proportional to the concentration of enzyme.

The experimental data from which the constants of Equation 1 are evaluated can conveniently be plotted and referred to as the activity-\(pS\) curve (cf. fig. 1), where \(pS = \log (1/S)\). In the following discussion, \(v\), the initial velocity, and \(V\), the limiting velocity, are expressed as micro-mols (mols \(\times 10^{-6}\)) of ACh hydrolyzed per minute per 100 microliters (mm.\(^3\)) of red cells.

**TREATMENT OF DATA**

1. **Estimation of initial velocity:** The evaluation of the constants of Equation 1 requires measurements of initial velocity at substrate concentrations so low that the velocity falls off rapidly with time when the manometric method is used. The experimental error and the error or interpretation are large. There is no really satisfactory way of estimating initial velocity, and the procedure used is described only to give evidence that the problem was met with a relatively satisfactory expedient. It is not recommended as a method, for subsequent experimental work has made it clear that the problems of low substrate concentration can be met more satisfactorily if the experimental method is continuous titration.

It was decided that the minimal requirements would be met if a relatively objective procedure for estimating initial velocity could be found, which gave consistent results on repeated analysis of the same data, which made use of a maximum number of experimental points and which did not give increasing apparent values of \(K\), with increasing amounts of

*The derivation of Equation 1 and the theoretic interpretation are given by Haldane. The equation and the kinetics of inhibition of this type of enzyme are discussed by Augustinsson.*
enzyme, as does any method which tends to use an average velocity for initial velocity. When such a method was found, it seemed desirable to push the determinations to as low substrate concentrations as possible.

At suboptimal concentrations of ACh the individual reactions fitted the "monomolecular" or "die-away" equation, which can be written: \( \frac{x}{a} = 1 - e^{-at} \), where \( a \) is the amount of substrate present at the beginning of the reaction and \( x \) is the amount hydrolyzed in time \( t \). It was necessary to extrapolate the origin to about 45 seconds after tipping.

At concentrations of ACh around the optimal, however, the reactions were more nearly linear than the equation. (Integration of Equation 1 gave an equation which was more nearly linear than the reactions.) Various arbitrary modifications of the "monomolecular" equation were tried, and it was found empirically that the experimental curves fitted the equation: \( \frac{x}{a} = 1 - e^{-at} + bkt \), where \( b \) is the fraction of enzyme combined as \( ES \), calculated from Equation 1 for each value of \( a \). Below about \( 6 \times 10^{-4} \) M ACh, \( b \) is negligible and the equation becomes equivalent to the "monomolecular" equation. Above about \( 4 \times 10^{-2} \) M ACh, the equation is linear for practical purposes, as were the experimental curves. Templates were used to draw the best curve through the experimental data to the extrapolated origin, and initial velocity \((ka + bka - \text{spontaneous hydrolysis})\) was read from a nomogram.

No theoretic significance is attached to the equation used, for although the added term \((a)kt\) is not completely meaningless, it has not been possible to interpret the equation as a whole.

2. Estimation of \( V, K_1, K_2 \) and \( n \): The experimental values of these constants were estimated by the method of Lineweaver and Burk until it became evident that 2.0 as a value of \( n \) would satisfy all of the data as well as the values from 1.9 to 2.1 yielded by the analysis. The meaning of \( n \) makes it reasonable to regard the integer as the proper value. Subsequently a rapid mechanical method was employed, by which the data were fitted directly to theoretic curves.
Results

Apparent kinetic properties of the enzyme in anemias: In figure 1 are shown the data of two representative experiments from which the experimental values of the constants of Equation 1 were derived. Curve N is that of normal erythrocytes. Curve A is that of anemia from hemorrhaging peptic ulcer on the eleventh day of treatment. The theoretic curves drawn through the experimental points were both calculated with the same values of the apparent dissociation constants and \( n \): \( K_1 = 1.9 \times 10^{-4} \); \( K_2 = 1.0 \times 10^{-2} \); \( n = 2.0 \). The difference between the two curves is in the value of \( V \). In Curve N, \( V = 1.55 \); in Curve A, \( V = 2.38 \). The dotted curves represent the limits of the normal range for six individuals (\( V = 1.24-1.77 \)), with deviations of \( \pm 20 \) per cent, in agreement with previous reports.\(^1\)\(^2\) Similar experimental curves for the other three cases of anemia from hemorrhage gave values of \( V = 1.88, 2.25 \) and 2.45. The cases of pernicious anemia during the third to fifth weeks of treatment had values very similar to those of the hemorrhage cases, \( V = 2.39-2.56 \). In all of these, and in the 4 cases of secondary anemia discussed in a later section, it was clear that the data would fit theoretic curves calculated with the values of \( K_1, K_2 \) and \( n \) used in figure 1 as well as with the slightly varying values yielded by the analysis. The apparent kinetic properties of the enzyme were the same in all cases.

In the 6 cases of pernicious anemia in relapse, the values of \( V \) were 0.74–2.20, with 5 cases in the low-to-normal range and one high. The experimental values of \( K_1 \) were from 2.0 to 4.5 \( \times 10^{-4} \), with approximately normal values for \( K_2 \). In 3 of these cases the data appeared to be quite incompatible with the normal value of \( K_1 \). This suggests the presence of a competitive inhibitor (or mixed competitive and noncompetitive) in the cells of relapse. However, the error of measuring initial velocities at low substrate concentrations with the manometric method is sufficiently large that this apparent abnormality cannot be regarded as established by the present work.

Folic acid and liver extract: To determine whether the increased apparent concentration of enzyme in pernicious anemia during treatment could be attributed to direct activation of the enzyme by the therapeutic agent, and whether the low values of relapse could be attributed to a deficiency of such an activator, folic acid and liver extract were added to the reaction mixtures with relapse cells and with normal cells. Amounts up to 0.1 mg. of folic acid and 0.1 U.S.P. unit of injectable liver extract had no effect on the velocity of hydrolysis, whether or not the mixtures were incubated at 37 C. for one and a half hours. This is in agreement with Hawkins.\(^13\) When larger amounts of either agent were used, inhibition was observed.

Heat-inactivated cell preparations: To detect a possible heat-stable activator or inhibitor of the enzyme in the abnormal cells, preparations of low-activity cells and high-activity cells from pernicious anemia in relapse and during treatment were incubated at 60 C. to inactivate the enzyme. The former had no effect on the activity-pH curve of normal cells; the latter had no effect on relapse cells.

Mixtures of cells: To detect a heat-labile agent, appropriate quantities of high-
and low-activity cells were mixed with each other and with normal cells. The mixtures gave velocities within ±5 per cent of the sum of the velocities determined separately. This experiment was feasible only at optimal or higher substrate concentrations; consequently it was not possible to prove or disprove by this means the suggested presence of a competitive inhibitor in the cells of relapse. With this possible exception, it can be concluded that the cells of pernicious anemia do not differ from normal in whatever content they may have of activators or inhibitors of the enzyme.

The experimental values of $V$ are proportional to the concentration of active, normal enzyme in the cells.

**High relative concentration of enzyme in young cells:** Pritchard's procedure for separating the cells into reticulocyte-rich and -poor fractions by centrifugation was followed, using normal cells and the cells of pernicious anemia at the height of the reticulocyte response. His results, obtained with the cells of normal rats and of rats made anemic by repeated bleeding, are duplicated by the results with human cells. These are given in table 1. In each case the activity was higher in the reticulocyte-rich (top) layer, and the difference was greatest when the reticulocyte count was highest. The normal case shows that the large young cells continue to have a high content of enzyme after the reticulum has disappeared.

In view of these findings, which explain the fact that high enzymic activity persists during the treatment of pernicious anemia long after the reticulocytosis has disappeared, it can be concluded that the relative concentration of enzyme in the red cells is a more sensitive indicator of increased hematopoietic activity than the reticulocyte count.

**Miscellaneous anemias:** It follows from the foregoing that if hematopoiesis is not hyperactive the enzymic concentration in the red cells can be expected to

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be normal or possibly lower than normal. A patient with anemia caused by severe
generalized impairment of bone marrow function would be expected to show little
or no increase in enzymic titer.

Eight cases of severe anemia secondary to conditions in which suppression of
hematopoiesis is characteristic have been studied, 4 in 1940 and 4 in the present
series.* Enzyme titers and the essential hematologic data are given in table 2.
Since the previous experimental conditions† and those of the present work were
quite different, discussion in terms of absolute values would be cumbersome. For
this reason relative enzymic concentration "E" is expressed as the ratio of the
patient's enzyme titer to the average normal value for the series to which he

| Diagnosis                                      | RBC | VPC | Reticu-
|                                               |     |     | locytes % | "E"
| Chronic lymphoid leukemia† (Case 3)           | 1.3 | 14  | 0.2       | 1.48 |
| Chronic myelogenous leukemia† (Case 4)        | 1.8 | 16  | 0.3       | 1.43 |
| Sarcoma; leukemia (x-ray therapy)             | 2.7 | 25  | 0.5       | 1.11 |
| Hodgkin's lymphoma (moribund)† (Case 5)      | 2.0 | 20  | 0.3       | 1.10 |
| Aleukemic leukemia (moribund) (1940, unpub-
  lished)                                      | 1.5 | 13  | 0.4       | 1.00 |
| Cirrhosis of liver; jaundice (moribund)      | 2.2 | 20  | 0.3       | 1.00 |
| Sarcoma; leukopenia (nitrogen mustard ther-
  apy)                                         | 1.8 | 17  | 0.2       | 0.76 |
| Acute myelogenous leukemia                    | 2.1 | 20  | 0.7       | 0.72 |
| Pernicious anemia, relapse 8 cases, including†
  (Cases 13, 14 and 15)                         | 0.9–2.9 | 11–33 | 0–3.5 | 0.47–1.22 |
| 1 case                                         | 2.7 | 32  | 0.5       | 1.40 |
| Normals (12)                                  |     |     |           | 0.8–1.2 |

* Clinical data are given on p. 152.
titers as low as half the normal value are found in patients whose clinical condition and general nutrition are good. When the group is extended to include pernicious anemia, the outstanding feature in common is suppression or malfunction of the bone marrow.

In leukemias the enzyme titers varied from low to high. The two high values may be taken as evidence that areas of increased erythrocytic activity such as are frequently found in leukemia at autopsy were sufficiently extensive to maintain high proportions of young cells in the peripheral blood. This is not reflected by the reticulocyte counts or by the degree of anemia. The lowest titer in the group was found in acute myelogenous leukemia. Although of course nothing can be proved by a single case, it may be noted that acute leukemias are seldom benefited and often harmed by x-ray therapy.

One pernicious anemia patient had a high titer in relapse. This indicates that his bone marrow function was more nearly physiologic than is usual, and suggests that the biochemical abnormalities of relapse were not fully developed in this patient.

It is hoped that further investigation may result in a useful test of bone marrow function when reticulocytosis is slight or absent. At present only one clinical application is suggested. If an anemic patient is a candidate for treatment with agents which suppress bone marrow function and has a high cholinesterase titer, he will probably tolerate such therapy better than one who has a normal or low titer, even though the degree of anemia may be the same in both. When hyperactivity of the hematopoietic system is progressively reduced, as in leukemia, a fall in cholinesterase titer from high toward normal is expected to appear sooner or later. Whether or not this can be made use of as a criterion for discontinuing marrow-suppressing therapy is uncertain, for it has not been demonstrated that the falling titer would precede the appearance of the usual indications for discontinuing such therapy.

**SUMMARY AND CONCLUSIONS**

1. Activity-pS curves were determined experimentally for the cholinesterase of the erythrocytes in various human anemias.
2. The data were analyzed to give experimental values for the apparent dissociation constants, $K_s$ of the active enzyme-substrate complex ($ES$) and $K_i$ of the inactive complex ($ES_i$), and the theoretic limiting velocity $V$.
3. The analysis showed that the enzyme in the cells of anemia is the same as that in normal cells. The abnormally high enzymic activity (initial velocity at a single substrate concentration) known to occur in various anemias and the change from low activity in pernicious anemia in relapse to high activity during treatment are attributable to changes in concentration of the enzyme.
4. The enzyme in the red cells of pernicious anemia in relapse was not activated by folic acid or liver extract.
5. It was shown experimentally that the reticulocytes and young cells of normal blood and of pernicious anemia during treatment contain much higher concentrations of enzyme than old cells. The increased concentration persists for some time after the reticulum has disappeared.
6. The relative concentration of enzyme in the red cells is a more sensitive indicator of hyperactive hematopoiesis than the reticulocyte count.

7. Evidence is presented that failure of elevation of enzymic concentration in severe anemia is associated specifically with suppression or malfunction of the hematopoietic system.

8. It is believed that the relative concentration of enzyme can be used as a test of bone marrow function in severe anemias. It is hoped that further investigation may result in a useful test of wider application.

REFERENCES

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