Determination of Plasma Amine Oxidase Activity

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In recent years clinical medicine has increasingly called upon enzymologists to help solve diagnostic problems. Thus, LaDue et al. (1) and Walsh and collaborators (2), among others, have used the quantitative estimation of serum transaminase activity as a means of differentiating certain cardiac, hepatic, and pulmonary diseases. Other investigators, among them Zimmerman (3), have utilized variation of the serum lactic acid dehydrogenase activity for similar purposes. Recently, Leach et al. (4), reported that a plasma oxidase, "ceruloplasmin," is involved in the oxidation of adrenalin and that a qualitative difference may exist in this enzyme system between normal and schizophrenic patients. Akerfeldt (5) found that N, N-dimethyl-p-phenylenediamine (DPP) can be used as a convenient substrate for the estimation of plasma amine oxidase. He also found differences in activity of this enzyme system between normal individuals and those suffering from certain mental disorders, particularly schizophrenia. As the result of his preliminary study Akerfeldt raised the possibility that his test might be of value in differentiating schizophrenics from other patients. Abood et al., (6) using a different substrate and a somewhat different method, conclude that the enzymatic measurement of "ceruloplasmin" activity of serum has little, if any, value as a diagnostic test for any disorder, including schizophrenia. In view of these diverging estimates of the possible diagnostic value

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of the plasma oxidase test in mental disorders it was felt a detailed study should be carried out to determine the various factors influencing this test. The present report describes the results of these studies which led to the development of an improved method for the estimation of amine oxidase activity in plasma.

REAGENTS

The reagents used in the test as finally adapted as a standard procedure, are listed as follows:

Molar acetate buffer, pH 6.0: Dissolve 136 Gm. of sodium acetate, trihydrate in about 800 ml. of water. Adjust pH to 6.0, using HCl. Dilute to 1000 ml.

\( N,N\)-dimethyl-p-phenylenediamine: Dissolve 100 mg. of \( N,N\)-dimethyl-p-phenylenediamine (DPP) monohydrochloride in about 90 ml. of double distilled water. Add sufficient HCl (0.6 ml. of 1N) to form the dihydrochloride and dilute to 100 ml. This reagent is usable for at least one day.

Versene solution, 10 per cent: Dissolve 10 Gm. of the disodium salt of ethylenediamine tetracetic acid in water and dilute to 100 ml. As anticoagulant use 0.1 ml. for 10 ml. of blood.

METHOD

Since other constituents besides amine oxidase were to be determined in the patients, 10 ml. of blood were drawn routinely by venipuncture and gently transferred to tubes containing versene as anticoagulant. The samples were kept in an ice bath until centrifuged in a refrigerated centrifuge. The plasma was separated and kept cold until used.

In the routine method as finally adopted, the following test system was set up by adding the reagents in the order given.

<table>
<thead>
<tr>
<th>Sample cell</th>
<th>Reference cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.3 ml.</td>
<td>Buffer</td>
</tr>
<tr>
<td>0.2 ml.</td>
<td>Plasma</td>
</tr>
<tr>
<td>1.0 ml.</td>
<td>DPP</td>
</tr>
</tbody>
</table>

The cells were quickly placed into the recording spectrophotometer (Beckman Model DK-2 equipped for reaction rate studies). The rate of oxidation of DPP was followed at 552 m\(\mu\) for exactly 6 minutes after the end of the lag period (Fig. 1).

This figure shows a typical normal tracing. It should be remembered that the pen carriage of the DK-2 moves from the right to the
left. The instrument is adjusted to have the pen travel 1 inch per 2 minutes, starting at point 0. The line OA which is parallel to the abscissa is referred to in this report as the lag period (LP) (Akerfeldt refers to it as the plateau). The reaction is allowed to proceed until the distance AB equals 3 inches (6 minutes). A perpendicular is dropped from C to B and the slope of AC is calculated by dividing the change in absorbance (CB) by the time in minutes (AB). Since only 0.2 ml. of plasma is used in the determination, this ratio is multiplied by 500 to express the activity of 100 ml. of plasma. Hence,

$$\frac{\Delta \text{Abs.}}{6} \times 500 = \text{Apparent "Ceruloplasmin" Activity (ACA).}$$

(Equation 1)

RESULTS

In the original method Akerfeldt (5) carried out the reaction at the approximate pH of serum, while Abood (6) adjusted his system to pH 6.8. The results of an experiment carried out to determine the optimum pH for plasma oxidase activity are shown in Fig. 2. All buffers used in this study were made up to contain 1 M. acetate, the pH being adjusted by the addition of varying amounts of HCl. It can be readily seen from this figure that the optimum pH range for the oxidase activity is pH 5.5-6.0. On the basis of these results the choice of pH 6.0 for the routine determination of plasma oxidase activity seems to be well justified.
In order to evaluate the quantitative aspects of the proposed method, a study was carried out in which varying amounts of plasma were carried through the standard procedure, keeping the final volume of the system constant through compensatory adjustments of the buffer.
added. The serum and plasma used were obtained from two patients of the neuropsychiatric wards. The results listed in Table 1 show a satisfactory relationship between enzyme concentration and activity measured by this method.

In a recent lecture Akerfeldt (7) implicated glutathione and sulfhydryl (—SH) groups as inhibitors of the plasma amine oxidase. Abood (6) lists ascorbic acid as one of the more potent inhibitors of "ceruloplasmin" activity by his method, while Leach et al. (4) discuss the inhibitory effect of albumin in their test system for "ceruloplasmin" activity. Therefore, it became of interest to see how these and other factors influence the proposed method. One of the several differences between Akerfeldt's test system and the proposed method is the concentration of plasma used. He used 1.5 ml. of plasma in a total volume of 3.0 ml. In the present method, 0.2 ml. of plasma are diluted to a total volume of 3.5 ml. Thus the albumin concentration in the proposed method is approximately one tenth of that present in the Akerfeldt system. An experiment, the results of which are shown in Fig. 3, brings out rather strikingly the inhibitory effect of albumin on the plasma oxidase activity. Increasing amounts of 3 per cent bovine albumin in normal saline were added to the standard test system, and the final volume kept constant by reducing the amount of buffer used. It can be seen from Fig. 3 that when the amount of albumin is approximately doubled (Curve 4) the ACA is reduced to one-third the activity of the original plasma. It should be pointed out that all the curves obtained are straight lines, whereas Akerfeldt reported curves of diminishing slope in both his normal and abnormal plasma samples.

It has been suggested (5, 7) that the inhibitory effect of albumin is due to the presence of free sulfhydryl groups. If that be the case,
then one would expect that reduced glutathione would react in much the same way. Figure 4 shows that this is the case. When increasing amounts of reduced glutathione were added to the standard system and the change in absorbance recorded over a 6-minute period, a definite inhibitory effect became apparent. In this particular case the reference cell contained only distilled water. In Fig. 5 the experiment was repeated, using the plasma of a different patient and adding much smaller increments of glutathione. The results again show an inhibition of oxidase activity, but without changing the shape of the curve in the lower concentration. Thus, glutathione affects the plasma oxidase activity in much the same manner as albumin. If added in lower concentration to the test system both reduce the slope of the activity curve. In higher concentration they not only reduce the slope but also alter the shape of the curve. The inhibition pro-
duced by hydroxylamine hydrochloride is qualitatively similar to that produced by glutathione.

The effect of albumin and of glutathione could be explained either in terms of their reducing properties or in terms of their ability to form copper mercaptides and thus reduce the activity of "ceruloplasmin." Versene, on the other hand, has no significant reducing properties, but is a powerful chelating agent, not only for calcium but for heavy metal ions as well. In order to determine if Versene has any inhibitory effect on plasma oxidase activity, hog serum was carried through the standard procedure. The test was then repeated, adding Versene in increments as shown in Table 2. The results indicate that the first increment of Versene added significantly decreased the ACA of the serum, but further increments have little, if any further effect. These findings can best be interpreted in terms of suppression of auto-oxidation of DPP by the chelating of the heavy metal ions. It would seem, therefore, that if an accurate expression of the plasma oxidase activity is desired, plasma obtained by using Versene as anticoagulant should be used, rather than serum.

According to Abood (6) ascorbic acid is a potent inhibitor of
Fig. 5. Inhibitory effects of small quantities of glutathione on plasma oxidase activity.

"ceruloplasmin" activity. Akerfeldt (5), on the other hand, reports no inhibitory effect of ascorbic acid, but only an increase in the plateau (lag period). The results obtained in the present investigation fully support the findings of Akerfeldt. Plasma and serum

| Table 2. Effect of Versene Upon the Apparent Amine Oxidase Activity of Serum |
|-----------------|---|---|
| Versene (mg. )  | AOA |
|-----------------|---|---|
| Hog serum       | 0.0 | 74  |
|                 | 0.1 | 56  |
|                 | 0.25 | 56  |
|                 | 0.50 | 51  |
|                 | 1.00 | 48  |
| Human serum     | 0.0 | 49.8|
|                 | 1.00 | 37.4|
|                 | 2.00 | 34.5|
|                 | 3.00 | 36.5|
were carried through the standard procedure and the lag period (if any) and the ACA determined. The serum or plasma samples were then fortified with ascorbic acid, dissolved in 0.6 per cent metaphosphoric acid, in amounts equivalent to 0.0, 1.25, 2.5, 3.75, and 5 mg. per cent. The LP and the ACA were determined on each of these specimens, and after correcting for the possible effect of metaphoric acid the ACA values were compared.

In 9 samples of hog serum the activity in the presence of ascorbic acid was 97.5 per cent (range 92-107) of that found in its absence. In 14 samples of human origin the activity was 101 per cent (range 92-115) of that found in the absence of ascorbate. In all cases, addition of ascorbic acid increased the duration of the lag period proportional to the amount added.

The standard procedure was used to evaluate the plasma levels obtained from 77 normal individuals (freshman medical students). The mean ACA value obtained was 8.0 units ± 2.3 (Std. Dev.). Assuming that the range equals the mean value ± 2 (Std. Dev.), the normal range as determined by the proposed method would be 2.5-13.6 units. This group showed a mean lag period of 1.3 minutes and a mean ascorbic acid level of 1.02 mg. per cent.

**DISCUSSION**

Various methods have been employed for the quantitative determination of "ceruloplasmin" activity, since this substance was first isolated by Holmberg and Laurell, in 1948 (8). Some authors have used a manometric method to follow the rate of oxidation of the substrate (9, 10), and others have used spectrophotometric means of following the enzyme activity (4, 5, 6). Among these latter methods the one introduced by Akerfeldt is particularly effective and, when modified as proposed in the present report, it is capable of giving reproducible results in a minimum of time and with only 0.2 ml. of plasma.

The present study has shown that the optimum pH for "ceruloplasmin" activity lies between 5.5-6.0 (Fig. 2). Akerfeldt does not mention the final pH of his test system, but presumably it is around pH 7.0. In the present study, the lower pH was chosen in preference to the more physiologic milieu, because the multifold increase in enzyme activity at pH 6.0 greatly reduces the interference of nonspecific oxidation of DPP (9). This increased enzyme activity at pH 6.0 also makes it possible to reduce the amount of plasma used for the test,
which is highly desirable in order to diminish the inhibitory effect of albumin shown in Fig. 3. It will be noticed that while this inhibitory effect is quite pronounced the response obtained with the amounts used in Fig. 3 is still linear. However, with higher concentration of added albumin this no longer holds, and a flattened curve, such as Akerfeldt describes for his system, is obtained. The fact that glutathione reacts qualitatively similar to albumin strongly suggests that the reducing properties, such as may be due to the sulfhydryl groups, of the albumin molecules are responsible for the inhibiting effect of albumin.

On the surface it may be difficult to visualize why reducing substances should act as inhibitors in this reaction, when DPP itself is a powerful reducing agent. The reactions involved in the determination are shown in Fig. 6. In reaction 1 oxygen is activated by "Ceruloplasmin" to oxidize DPP. In the presence of an extraneous reducing agent some, or all, of the oxidized DPP may be reduced back to DPP. If reaction 2 is sufficiently fast, as it is in the case of ascorbic acid, then no appreciable amounts of oxidized DPP can be detected in the system as long as any reduced ascorbic acid is present. In this case, if the over-all reaction is followed at sufficiently frequent intervals and the results plotted, a lag period or plateau at the beginning of the reaction will be seen, the duration of which will be a direct function of the amount of ascorbic acid present. After all the ascorbic acid has been oxidized to dehydroascorbic acid by reaction 2, the products of

![Reaction Diagram]

**Fig. 6. Reactions involved in determining "ceruloplasmin" activity.**
reaction 1 will accumulate and their rate of formation will be a direct expression of "cerulophasmin" activity. If, instead of measuring this rate of reaction, one measures only the amount of substrate formed in a fixed time interval without considering the lag period (6) then the effect of ascorbic acid might easily be misinterpreted as inhibitory. For during the lag period no accumulation of oxidized substrate occurs so long as ascorbic acid is present.

A somewhat different situation is obtained when reaction 2 proceeds at a slow rate relative to reaction 1. According to the law of mass action the rate of reaction is proportional to the active concentration of the reacting substances, that is:

\[ V = K [\text{oxidant}] [\text{reductant}] \]  

(Equation 2)

However, at the beginning of the reaction the concentration of oxidized DPP is very low, hence the velocity of reaction will also be low and very little inhibition will be apparent. But as the over-all reaction proceeds, the concentration of oxidized DPP will rapidly increase due to the higher velocity of reaction 1. This increase in concentration of oxidized DPP will in turn accelerate reaction 2 according to Equation 2. The net effect on the over-all reaction, as measured by the present method, is an apparent decrease in the reaction rate, which is indicated by a bending of the reaction rate curve. Such curves are obtained when albumin, glutathione, or hydroxylamine are added to the test system in sufficient amounts (Fig. 5, Curve 4). The figure also shows, however, that with smaller amounts of glutathione added (Curves 2 and 3) no bending of the response curve can be detected, yet the slopes are definitely reduced. This suggests that reducing agents such as sulfhydryl compounds, along with their effect on reaction 2, also inhibit reaction 1.

Another type of inhibition is observed when a substance capable of chelating heavy metal ions, such as versene, is added to the system (Table 2). The effect of versene can best be explained in terms of suppression of the heavy metal catalyzed oxidation of DPP. It can be seen from Table 2 that the sequestering agent, when added in sufficient amounts to chelate all the heavy metal ions, causes a significant reduction in ACA. Further additions of versene seem to have no further action. However, if left in contact with plasma or serum long enough (24 hours) versene will cause a further reduction in ACA, presumably due to removal of some of the copper from ceruloplasmin. Since versene thus suppresses a nonspecific oxidation of DPP it
would seem to be highly desirable to have this substance present when
determining the ACA.

The ACA value of a group of 77 normal individuals was found to be 8.0 ± 2.8 units. In two cases of Wilson’s disease no detectable amounts of "ceruloplasmin" were observed. In certain mental diseases, in carcinoma cases, and in pregnancies, elevated ACA values were found. The results obtained in various diseases will be treated in detail in a future report.

SUMMARY

A detailed study of the Akerfeldt test for plasma oxidase activity has been carried out and it has been found that:

1. The optimum activity of the enzyme system involved occurs at pH 5.5-6.0.
2. The amount of plasma needed for the test at pH 6.0 can be reduced to 0.2 ml.
3. The reduced amount of plasma removed the inhibitory influence of albumin and a linear response was always obtained.
4. The inhibitory effect of albumin and glutathione is due in part to inhibiting the enzyme system, and in part to the reduction of oxidized DPP.
5. The inhibiting influence of Versene appears to be due to the chelating and inactivation of heavy metal catalysts.
6. The effect of ascorbic acid is solely one of increasing the lag period without inhibiting the enzyme system.

As a result of this study a modification of the Akerfeldt test is proposed. With this proposed method it was determined that the plasma oxidase activity, ACA, of 77 normal individuals was 8.0 ± 2.8 units.

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REFERENCES