The Horseradish Peroxidase-catalyzed Oxidation of 3,5,3',5'-Tetramethylbenzidine
FREE RADICAL AND CHARGE-TRANSFER COMPLEX INTERMEDIATES*

P. David Josephs†, Thomas Eling‡, and Ronald P. Mason∥
From the †Laboratory of Pulmonary Function and Toxicology and the ‡Laboratory of Environmental Biophysics, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709

Benzidine and related compounds are well known substrates for horseradish peroxidase/H₂O₂ oxidation. Typically, two different colored products are formed. In this paper, we study the oxidation of 3,5,3',5'-tetramethylbenzidine. The first colored product is a blue charge-transfer complex of the parent diamine and the diimine oxidation product. This species exists in rapid equilibrium with the radical cation. The radical was observed by ESR spectroscopy, and hyperfine splitting constants were determined. Addition of equimolar hydrogen peroxide yields the yellow diimine, which is stable at acid pH. At less than equimolar peroxide, all four species (diamine, radical cation, charge-transfer complex, and diimine) exist in equilibrium. A theoretical analysis of this redox system is presented, including a determination of the extinction coefficients and equilibrium constant for the nonradical species.

Benzidine is carcinogenic in animals and man (1). It is also mutagenic to bacteria following activation by hepatic microsomes in the Ames test (2).

Peroxidases catalyze the oxidation of a wide variety of substrates, including benzidine and other aromatic amines. Mammalian tissues contain peroxidases; the peroxidatic activity of blood is the basis for the use of benzidine as a reagent for the detection of occult blood. A positive "benzidine test" is indicated by the formation of a blue oxidation product of benzidine (3).

The chemical hazard presented by the use of benzidine as a clinical reagent prompted a search for a less hazardous substitute (4). Holland et al. (5) synthesized the compound 3,5,3',5'-tetramethylbenzidine in which the amino groups are protected by methylation of the adjacent ring positions. This compound proved to be much less hazardous than benzidine itself and even more sensitive as an analytical reagent (5).

The oxidation of aromatic amines by peroxidases has been studied for many years. Saunders (6) has presented a review of this work. Horseradish peroxidase, an enzyme frequently used in these studies, is believed to be capable of both one-electron and two-electron oxidations, depending on the substrate employed. Saunders suggested that the blue oxidation product of benzidine is either its one-electron oxidation product (cation free-radical) or a complex of its two-electron oxidation product (diimine) with the parent diamine. Both of these products are at a net oxidation state halfway between the diamine and diimine, but they are chemically distinct.

The oxidation of o-dianisidine (3,3'-dimethoxybenzidine) by horseradish peroxidase/H₂O₂ has been examined (7). Attempts to detect a free radical intermediate using electron spin resonance spectroscopy were unsuccessful. The authors suggested "a direct two-electron oxidation mechanism for horseradish peroxidase acting on o-dianisidine ..." and held that "oxidation of dianisidine does not proceed via any detectable semiquinone radical intermediate." This conclusion contrasts with earlier polarographic evidence (8) for the formation of radical intermediates during the electrochemical oxidation of benzidine and dianisidine.

In this paper, we examine the oxidation of the benzidine derivative, 3,5,3',5'-tetramethylbenzidine, by horseradish peroxidase/H₂O₂. ESR was used to detect and identify the 3,5,3',5'-tetramethylbenzidine semiquinone-imine cation free radical formed during the oxidation. Optical spectroscopy was used to detect the charge-transfer complex. (See Scheme 1 for structures.)

** MATERIALS AND METHODS
3,5,3',5'-Tetramethylbenzidine was obtained from Sigma. (This compound should not be confused with the related benzidine derivative, N,N,N',N'-tetramethylbenzidine.) H₂O₂ (30%) was obtained from Fisher Scientific. Horseradish peroxidase, D₂O, and o-dianisidine-2HCl were obtained from Sigma. 3,5,3',5'-Tetramethylbenzidine stock solutions were prepared by
dissolving the compound in 0.2 N HCl; H₂O₂ and enzyme stock solutions were prepared in H₂O. Incubation mixtures were prepared in 0.2 M acetate buffer, pH 5.0, unless noted otherwise.

Optical spectra were recorded with an Aminco DW-2A spectrophotometer. ESR spectra were recorded with a Varian E-109 or E-104 spectrometer, using an aqueous flat cell and TM110 cavity. Further details are given in the figure legends. All experiments were performed at room temperature.

RESULTS

The oxidation of 3,5,3',5'-tetramethylbenzidine by the horseradish peroxidase/H₂O₂ system yields colored products. Incubations turn blue, pass through a green stage, and finally become yellow. The green solution is simply a mixture of the initial blue product and final yellow product. This reaction sequence was studied by optical spectroscopy.

After addition of enzyme to a solution of 3,5,3',5'-tetramethylbenzidine and H₂O₂, optical spectra were recorded using the auto scan mode of the spectrophotometer (Fig. 1). The UV chromophore of 3,5,3',5'-tetramethylbenzidine (λmax = 285 nm) decreases to zero during the reaction, and no new UV peaks appear (data not shown). In the visible region, two new peaks appear initially, at 370 and 652 nm. These peaks grow to a maximum and then decay; they are replaced by a peak at 450 nm. The later stages of the reaction are characterized by growth and decay of each peak.

In a further experiment, H₂O₂ was titrated into a solution of 3,5,3',5'-tetramethylbenzidine and enzyme, and the absorbance at 700 nm (blue product) was measured. The resulting titration curve (Fig. 2) shows the formation and subsequent destruction of the blue product. The curve is nearly symmetrical about the midpoint, which corresponds to ½ mol of peroxide/mol of substrate. At the endpoint, reached at approximately equimolar peroxide and substrate, no blue product remains. Beyond this point, the concentration of the yellow product does not increase with additional peroxide. We concluded that the blue product is a one-electron oxidation product of 3,5,3',5'-tetramethylbenzidine, and that the yellow product is the two-electron oxidation product (diimine).

Two chemically distinct one-electron oxidation products of 3,5,3',5'-tetramethylbenzidine can be envisaged: the 3,5,3',5'-tetramethylbenzidine radical cation and the charge-transfer complex of the diimine (electron donor) and the diimine (electron acceptor). The chemical structures of these species are shown in Scheme 1. ESR experiments (reported below) demonstrate the presence of a free radical, but this radical is not necessarily the same as the species responsible for the optical absorption bands since the radical and charge-transfer complex could both exist in equilibrium. Charge-transfer complexes are distinguished by spectral shifts upon dilution caused by the re-equilibration among the electron donor, the electron acceptor, and the charge-transfer complex (9). 3,5,3',5'-Tetramethylbenzidine was oxidized with enzyme and sufficient peroxide to yield the green stage. Buffer (1 ml) and this mixture (1 ml) were placed in the two chambers of a Yankee-cuvette. Spectra were recorded before and after mixing (Fig. 3). The distinct change in the spectrum indicates that the equilibrium has shifted from the blue product to the yellow product. This suggests that the blue product is indeed a complex of the parent compound and its imine oxidation product.

ESR experiments were performed, using a ratio of ½ mol of H₂O₂/mol of 3,5,3',5'-tetramethylbenzidine. The ESR signal developed in a few seconds and was stable for hours. The ESR spectrum observed with the full system. No spectrum was observed in the absence of peroxide, 3,5,3',5'-tetramethylbenzidine, or enzyme. Horseradish peroxidase, denatured by boiling for 1 h immediately before use, had almost no activity.

To our knowledge, the ESR spectrum of the 3,5,3',5'-tetramethylbenzidine cation radical has not been reported. The analysis of these spectra was performed with the aid of a computer program for simulation of spectra. The spectra of the cation radicals of benzidine and N,N,N',N'-tetramethylbenzidine (prepared by iodine oxidation in acetonitrile) have been reported (10). These spectra were used as a starting point in our analysis. A summary of these data is given in Table I.

Fig. 1. Oxidation of tetramethylbenzidine by horseradish peroxidase/H₂O₂: optical spectra. The incubation mixture contained 10 µM 3,5,3',5'-tetramethylbenzidine and 15 µM H₂O₂ in acetate buffer, pH 5.0. At zero time, horseradish peroxidase was added (10 ng/ml) and repetitive scanning started (5 nm/s). The first six scans are shown in A and the subsequent scans in B. Arrows indicate the growth and decay of each peak.

Fig. 2. Oxidation of tetramethylbenzidine by horseradish peroxidase/H₂O₂: titration curve. The incubation mixture contained 3,5,3',5'-tetramethylbenzidine (100 nmol) and horseradish peroxidase (1 mg) in acetate buffer, pH 5.0; 2 ml. H₂O₂ (5 mM) was added in 1-μl aliquots, and absorbance at 700 nm was measured after each addition. The resulting titration curve shows the formation and subsequent destruction of the charge-transfer complex. (See also Fig. A-2.)

The 3,5,3',5'-tetramethylbenzidine cation radical has four distinct types of magnetic nuclei, each characterized by a hyperfine splitting constant. These are: the amine N nuclei (two), the
amine protons (four), the protons at the 2 and 4 positions of the rings (four), and the methyl protons (12). Thus, the maximum number of lines possible is $5^2 \times 13 = 1625$. In fact, only about 31 lines can be resolved in Fig. 4. This implies considerable accidental degeneracy.

We considered, first, the amine N and amine H hyperfine splitting constants. If we assume that these are equal, then a 25-line pattern is reduced to a 9-line pattern. Of course, any such assumption can be justified only by a successful analysis of the complete spectrum. The two remaining (proton) hyperfine splitting constants are expected to be much smaller than the amine H and N hyperfine splitting constants. The spacing of the observed lines is remarkably regular. We assumed that this repetitive spacing (about 1.1 gauss) was equal to these two smaller hyperfine splitting constants; the regular spacing can be maintained throughout the spectrum if these hyperfine splitting constants are both close to $\frac{1}{n}$ times the large hyperfine splitting constant, with $n$ an integer. The most promising pattern arises from the assumption that $n = 3$, giving $a_{\text{N,H}} = a_{\text{N,H}} = 3 \times a_{\text{H}}$, and $a_{\text{D}} = a_{\text{H}}$. This yields a stick spectrum of 41 equally spaced lines. The wing lines of this spectrum are exceedingly weak relative to the central lines and are likely to be missed. Computer simulations of this set of hyperfine splitting constants gave reasonable simulations of the observed spectrum, and slight refinement produced an excellent agreement (Fig. 5). Finally, the deuterium-exchanged spectrum was simulated, using the gyromagnetic

**TABLE I**

Hyperfine splitting constants of cation radicals of benzidine and derivatives  

<table>
<thead>
<tr>
<th></th>
<th>$a_{\text{N,H}}$ (G)</th>
<th>$a_{\text{H}}$ (G)</th>
<th>$a_{\text{D}}$ (G)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td>3.39</td>
<td>4.81</td>
<td>3.09</td>
</tr>
<tr>
<td>(b)</td>
<td>3.52</td>
<td>4.70</td>
<td>3.33</td>
</tr>
<tr>
<td>(c)</td>
<td>1.44</td>
<td>1.65</td>
<td>1.08</td>
</tr>
<tr>
<td></td>
<td>1.10</td>
<td>0.76</td>
<td>1.08</td>
</tr>
</tbody>
</table>
Free Radical and Diimine Metabolites of Benzidine

![ESR spectrum and computer simulation of deuterated tetramethylbenzidine cation radical](image)

Fig. 6. ESR spectrum and computer simulation of deuterated tetramethylbenzidine cation radical. A, experimental spectrum. Conditions were as in Fig. 4A, except that all solutions were prepared in D₂O. B, computer simulation. Hyperfine splitting constants were: \( a_{N} = 3.09 \text{ G} \); \( a_{H} = 0.52 \text{ G} \); \( a_{\text{ring}} = 1.08 \text{ G} \); and \( a_{\text{extra}} = 1.08 \text{ G} \). Line width = 0.4 G.

ratios of H and D. This simulation (Fig. 6) is also in close agreement with the observed spectrum and confirms the assignment of the amino N and H hyperfine splitting constants. However, the ring proton (2, 6, 2', and 6' positions) hyperfine splitting constants cannot be assigned unambiguously. For example, if these constants are set to zero (leaving 12 rather than 16 protons with \( a_{\text{ring}} = 1.08 \text{ G} \)), the resulting simulations are almost identical with those of Figs. 5 and 6. We prefer the assignment of \( a_{\text{ring}} = 1.08 \text{ G} \) because related benzidines have 2,6,2',6' ring proton hyperfine couplings on this order (Table I). We concluded that the ESR signal corresponds to the 3,5,3',5'-tetramethylbenzidine radical cation.

We examined the relationship between the concentrations of the blue product (detected optically) and the free radical (detected by ESR). A series of incubations was prepared, with 3,5,3',5'-tetramethylbenzidine concentrations ranging from 50 to 500 µM. In each incubation, H₂O₂ was added to give a ratio of \( 1/2 \) mol of peroxide/mol of 3,5,3',5'-tetramethylbenzidine. The ESR spectrum of each incubation was recorded, using a modified Gilford rapid sampler system so that the flat cell could be filled without removing it from the cavity. Relative ESR signal strength was measured by the peak to peak amplitude of a given line in the spectrum. A second aliquot of each sample was added to an optical cuvette, and the absorbance at 700 nm was measured. Fig. 7 shows the relationship between the two signals; clearly, the radical concentration is proportional to the square root of the concentration of the blue product.

The effect of horseradish peroxidase concentration was also examined. In this experiment (Fig. 8), the concentrations of 3,5,3',5'-tetramethylbenzidine and H₂O₂ were fixed, and enzyme concentration was varied. The formation of the charge-transfer complex was measured as a function of time following addition of enzyme by recording absorbance at 700 nm. In each case, the final absorbance was the same. Thus, the enzyme affects the rate of the reaction, but not the concentrations of the products formed.

The results shown in Fig. 7 suggest that the blue product (diimine/diimine charge-transfer complex) is in rapid equilibrium with the radical cation. If we represent these species by \( AA^2+ \) and \( A^- \), respectively, then we may write this equilibrium as:

\[ 2A^- \rightleftharpoons AA^2+ \]

Thus, the radical concentration varies as the square root of the charge-transfer complex concentration, as observed. The equilibrium constant could, in principle, be determined, using quantitative ESR to measure the absolute concentration of the radical. This was not attempted; however, in the following discussion, we will show that the ratio of radical concentration to charge-transfer complex concentration is small (in the concentration regime studied).

![ESR spectrum and computer simulation of deuterated tetramethylbenzidine cation radical](image)

Fig. 7. Relationship between ESR signal strength and optical absorbance at 700 nm. A series of incubations was prepared, with 3,5,3',5'-tetramethylbenzidine concentrations ranging from 50 to 500 µM and horseradish peroxidase 1 ng/ml. In each case, H₂O₂ concentration was one-half of the 3,5,3',5'-tetramethylbenzidine concentration. From each incubation mixture, an ESR spectrum and an optical spectrum were recorded. ESR signal strength was measured as peak to peak height of a particular line divided by receiver gain. The figure shows a plot of ESR signal strength squared versus absorbance at 700 nm.

![Effect of enzyme concentration on 3,5,3',5'-tetramethylbenzidine oxidation](image)

Fig. 8. Effect of enzyme concentration on 3,5,3',5'-tetramethylbenzidine oxidation. 3,5,3',5'-Tetramethylbenzidine (TMB) (250 µM) and H₂O₂ (75 µM) were prepared in acetate buffer, pH 5. This solution (3 ml) was added to a cuvette fitted with a plunger for addition of enzyme. Absorbance at 700 nm was recorded as a function of time; at zero time, the plunger was depressed to start the reaction. The amount of horseradish peroxidase (HRP) added (dissolved in 20 µl of buffer) is indicated.
The charge-transfer complex is, itself, in equilibrium with the diamine (A) and diimine (\(A^2\)):

\[ A + A^2 \rightleftharpoons AA^2 \]

In this case, the equilibrium constant expression is:

\[ K_{eq} = \frac{[AA^2]}{[A][A^2]} \]

This equilibrium constant was determined experimentally. The titration experiment of Fig. 2 was repeated, and complete spectra were recorded at each step in the titration. Each of the three absorbing species has an absorption peak substantially free of overlap from the other two species: the diamine parent (\(\lambda_{max} = 285 \text{ nm}\)), the diimine product (\(\lambda_{max} = 450 \text{ nm}\)), and the charge-transfer complex (\(\lambda_{max} = 652 \text{ nm}\)). (The charge-transfer complex absorption peak at 370 nm is less useful due to overlap of the diimine peak.)

The extinction coefficient of each species was determined. The initial concentration of 3,5,3',5'-tetramethylbenzidine is known, and the absorbance at 285 nm was measured as \(\epsilon_{285} = 2.1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}\). The initial concentration of the diimine (following addition of excess peroxide) is equal to the initial concentration of 3,5,3',5'-tetramethylbenzidine, assuming 100% yield. This allows the determination of the extinction coefficient of the diimine, \(\epsilon_{285} = 5.9 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}\).

At each stage of the titration, the concentrations of the diamine and diimine were determined from the absorbances at 285 and 450 nm. If we assume that the radical concentration is negligible, then:

\[ (A) + (A^2) + 2(AA^2) = A_0 \]

where \(A_0\) is the initial concentration of substrate. The concentration of the charge-transfer complex is given by:

\[ [AA^2] = \frac{1}{2}[A_0 - (A) - (A^2)] \]

Thus, from an intermediate stage in the titration, the extinction coefficient of \(AA^2\) may be determined: \(\epsilon_{285} = 3.9 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}\).

Using these values of \(\epsilon\), we calculated the sum: \((A) + (A^2) + 2(AA^2)\) for each of the first seven steps in the titration, covering a 5-fold range of diamine concentration. Beyond this point, the absorbance due to the diamine became too small for accurate determinations. The sum of the three terms was within 4% of \(A_0\) for each step; this confirms our assumption that the radical concentration may be neglected and gives an estimate of the accuracy of our determination of \(\epsilon_{285}\).

Finally, the equilibrium constant was determined. In Fig. 9, we plot the concentration of the charge-transfer complex (\(AA^2\)) versus the product of the concentrations of the diamine and diimine (\(A)(A^2)\). This plot yields a straight line through the origin, with slope \(K\). Thus, we determined \(K_{eq} = 2.8 \times 10^5 \text{ liters mol}^{-1}\).

In the Appendix to this paper, we derive an algebraic expression for the concentration of the charge-transfer complex as a function of the number of oxidizing equivalents added per mol of substrate. This expression is based on the law of mass action, and the assumption that the radical concentration is negligible compared to the concentration of the complex. The equilibrium constant \(K_{eq}\) appears as a parameter in this expression, in the form \(K_c = 1/(A_0 - K_{eq})\). The parameter \(K_c\) determines both the shape of the titration curve and the maximum concentration of the charge-transfer complex (at the midpoint of the titration). Using the value of \(K_{eq}\) calculated above and the value of \(A_0\) for the experiment shown in Fig. 2, we obtain \(K_c = 0.07\); the theoretical expression given in the Appendix yields a predicted maximum concentration of 17 \(\mu\text{M}\) for the complex. Experimentally, we measured

\[ A_{200} = 0.38 \text{ at the midpoint of Fig. 2, which gives a concentration of 18 \(\mu\text{M}\), in excellent agreement with the prediction. In Fig. A-2, the data of Fig. 2 are shown superimposed on the theoretical curve for } K_c = 0.07.\]
Free Radical and Diimine Metabolites of Benzidine

We have also studied the oxidation of o-dianisidine. In contrast to the earlier report (7), we observed a transient ESR signal during the oxidation of this compound by horseradish peroxidase/H₂O₂ (Fig. 10). The ESR signal was observed in the olive green solution obtained immediately after addition of enzyme. Within a few minutes, the solution turned orange-brown, and the signal disappeared. We believe that the green product is a charge-transfer complex analogous to the blue product of 3,5,3',5'-tetramethylbenzidine. Again, the charge-transfer complex is in equilibrium with the cation-free radical. However, one or more of the dianisidine oxidation products is unstable. The spectra of the dianisidine radical in H₂O and D₂O (Fig. 10, A and B) are very similar. Each has a repetitive spacing of about 0.57 gauss. This result can be explained as follows. If ΔνM₀ = 0.57 gauss, then ΔνM₀ = (γH₂/γH) × 0.57 gauss = 3.7 gauss. This value is close to that expected for a substituted benzidine cation radical (Table I). Apparently, the accidental equivalence of the proton splitting (or reduction) product (radical) K, or a charge-transfer complex of the parent compound and final product, AA. The two analyses proceed similarly, and will be carried out in parallel.

\[ A + A' \rightarrow AA' \]  
\[ A + A' \rightarrow AA' \]

It is noted that the total number of nuclei of A, 
\[ [A] + [A'] - [AA'] + [A'] \]  
\[ [A] + [A'] - [AA'] + [A'] \]

The equilibrium constants are 
\[ k_0 = [A]^2/A[A'] \]  
\[ k_0 = [A]^2/A[A'] \]

where \( k_0 \) is dimensionless, and \( k_0 \) has dimensions: \( 1 \) molecule⁻¹. Let \( k \) be the number of equivalents of oxidation added per mole of A.

Then:
\[ [A'] + [A] = kA \]  
\[ [A'] + [A] = kA \]

Substituting (5) into (1) gives:
\[ [A'] + [A] = kA \]  
\[ [A'] + [A] = kA \]

and substituting (5) into (3) gives:
\[ [A'] + [A] = kA \]  
\[ [A'] + [A] = kA \]

Now, let \( [A'] = x' A \) 
\[ [A'] = x' A \]

and
\[ x' = kA \]  
\[ x' = kA \]

Then, substituting the expressions for [A] in (7) into equation (6) yields the quadratic equation:
\[ x'^2 + 2kA + 1 = 0 \]  
\[ x'^2 + 2kA + 1 = 0 \]

These expressions may be solved to give the ratio of the intermediate concentration to the total substrate concentration, as a function of \( x' \). It is convenient to set \( x' = 1 \) which runs from 0 to 2, by 1, at which \( t = +1, \) thus, \( t \) runs from -1 to -1. The solutions are then:
\[ 1 - x'^2 \]  
\[ 1 - x'^2 \]

and
\[ x' = \sqrt{1 - x'^2} \]  
\[ x' = \sqrt{1 - x'^2} \]

where the signs of the derivatives are fixed by the condition that \( x' = 0 \) for \( t = +1 \). Clearly, the solutions are symmetric about the point \( t = 0 \).

Or for any \( k \), the maximum value of \( x' \) occurs at \( t = 0 \), i.e., halfway from A to A'. If we denote this value as \( x'A \), then:

\[ x' = \frac{1}{2} \]  
\[ x' = \frac{1}{2} \]

Fig. A-1. Theoretical titration curves showing formation and destruction of an intermediate in the two-step oxidation (or reduction) of a substrate. The curves plot relative concentration of the charge-transfer complex (left) or free radical (right) intermediate, normalised to 1 at the midpoint, as a function of the number of equivalents of oxidation (or reduction) added. The parameter \( K \) is equal to \( A_k - K_r \) (charge-transfer complex) or \( K \) (radical), and is the reciprocal of the parameter \( K \) used in the "App-Index." Derivation of the expressions for these curves is given in the text.
Free Radical and Diimine Metabolites of Benzidine

FIG. A-2. Comparison of theoretical titration curve and experimental results. The data points are the same as those in Fig. 2. The theoretical curve was calculated as in Fig. A-1 (left). The value of $K_c$ was determined as explained in the text; this value, and the calculated extinction coefficient of the charge-transfer complex, determine the shape and height of the curve. The width (horizontal scale) was fixed by the assumption of one-to-one stoichiometry (end-point at 100 nmol of $H_2O_2$/100 mol of tetramethylbenzidine). Thus, no correction factors have been applied to the calculated curve.

In principle, both the type of intermediate involved in an oxidation, and the value of $K_c$ may be determined from a plot of relative concentration of the intermediate (as measured optically or by ESR) as a function of equivalents added. The more general case, $A + A'' + 2A' + 2A'' ightarrow 2A''$, yields a quartic equation in place of equation (8).

REFERENCES

The horseradish peroxidase-catalyzed oxidation of 3,5,3',5'-tetramethylbenzidine.
Free radical and charge-transfer complex intermediates.
P D Josephy, T Eling and R P Mason


Access the most updated version of this article at [http://www.jbc.org/content/257/7/3669](http://www.jbc.org/content/257/7/3669)

**Alerts:**
- When this article is cited
- When a correction for this article is posted

[Click here](http://www.jbc.org/content/257/7/3669.full.html#ref-list-1) to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
[http://www.jbc.org/content/257/7/3669.full.html#ref-list-1](http://www.jbc.org/content/257/7/3669.full.html#ref-list-1)