Ternary Coordination Complex between Human Serum Albumin, Copper (II), and L-Histidine*

SHOW-JY LAU AND BIBUDHENDRA SARKAR

From The Division of Biochemistry, The Research Institute of the Hospital for Sick Children, Toronto, and The Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada

SUMMARY

Physicochemical studies of the human serum albumin-Cu(II)-l-histidine (HSA-Cu(II)-L-His) ternary complex at pH 7.5 indicate several interesting features.

1. The dissociation constants of the HSA-Cu(II)-L-His ternary complex and the HSA-Cu(II) binary complex are 1.38 × 10^{-12} and 6.61 × 10^{-13}, respectively.

2. The absorption spectrum of the ternary complex system has characteristics similar to those of the NH₂-terminal peptide (1-24) of bovine serum albumin in the presence of Cu(II) and L-His. The computed spectrum of HSA-Cu(II)-L-His has a λ_{max} at 540 nm, a shift of 15 nm toward red from that of HSA-Cu(II) (λ_{max} = 525 nm).

3. The calculated moles of protons liberated in the formation of HSA-Cu(II)-L-His from HSA and Cu(II)-L-His₃ are 0.28. While this may originate from an α-amino group, the number is much less than what would be expected if a peptide nitrogen of HSA were involved in the binding.

4. Cu(II) in the ternary complex is most likely bound to both HSA and L-His. The possible binding mode changes from all nitrogen, as shown in HSA-Cu(II), to either a mixture of nitrogen and oxygen or an additional involvement of imidazole nitrogen.

5. Equilibria, existing in the ternary complex system, suggest that the ternary complex may play an important role in the biological transport of Cu(II).

EXPERIMENTAL PROCEDURE

Materials—Crystalline HSA was obtained from Hoechst Pharmaceuticals Company and used without further purification. The assumed molecular weight was 69,000. The Asp fragment of BSA was a generous gift from Dr. Theodore Peters, Jr. The radioisotope ⁶⁷Cu(II), specific activity at least 0.5 mCi of ⁶⁷Cu(II) per µg of Cu(II), was prepared (25) by irradiation of natural zinc with Bremsstrahlung from the linear accelerator at the University of Toronto. L-[³⁵C]His was obtained from Schwarz BioResearch. L-His free base ([α]_{D}^{25} + 39.9° ± 0.5°) was obtained from Nutritional Biochemicals. Dialysis membrane was obtained from Visking Company. All other reagents were of analytical grade and the water was deionized.

Equilibrium Dialysis—The extent of binding was measured by the equilibrium dialysis technique. The dialysis cells were made of rigid acrylic plastic. Six separate chambers provided for si-

It is known that a fraction of Cu(II) in human blood plasma is bound to human serum albumin (2, 3). This fraction is in rapid equilibrium with Cu(II) in tissues (2) and is considered to be the transport form of Cu(II) in blood. There is also an amino acid bound fraction of Cu(II) in serum consisting mostly of Cu(II)-l-His complex which is in equilibrium with the fraction bound to human serum albumin; BSA, bovine serum albumin; Asp fragment, NH₂-terminal peptide (1-24) of BSA; l-Thr, L-threonine. See also "List of Symbols" in Reference 17.

HSA (3, 4). The evidence has recently been presented for the existence of a ternary coordination complex between HSA, Cu(II) and L-His (5). The importance of ternary coordination complexes has long been recognized in biological chemistry and many investigators have contributed significantly in this area. Klotz and Ming (6) and Gur (7) have demonstrated that metal ions may mediate the binding of small molecules to proteins. Malmström (8) later studied the ternary complex formation between enolase, metal ion, and 1,2-phosphoglycerate. Since the original proposal of a bridge structure between enzyme, metal, and substrate by Hellerman and Stock (9), this hypothesis has been put forward to explain the mechanism of action of many enzymes (10-12). Recently, important contributions have been made by Mildvan and Cohn (13) to our understanding of the formation of the enzyme-metal-substrate bridge structures. In order to understand fully the ternary complexes involving enzymes and proteins, several laboratories have begun to study the low molecular weight ternary complexes of amino acids and peptides (14-23).

This communication deals with the physicochemical studies of HSA-Cu(II)-L-His by equilibrium dialysis, spectrophotometry, and titration. The formation of the ternary complex is also shown between the 24-residue NH₂-terminal peptide of BSA (24), Cu(II), and L-His.

* Part of the data included in this paper have been presented before the XIIth International Conference on Coordination Chemistry, Sydney, 1969 (1). This work was supported by grants from the John A. Hartford Foundation of New York and The Medical Research Council of Canada.

The abbreviations used are: l-His, L-histidine; HSA, human serum albumin; BSA, bovine serum albumin; Asp fragment, NH₂-terminal peptide (1-24) of BSA; l-Thr, L-threonine. See also "List of Symbols" in Reference 17.

1 Preparation P2 was prepared by Dr. Theodore Peters, Jr., as in Reference 24. Trichloroacetic acid was removed by Sephadex G-10 rather than ether at the final step. The calculated molecular weight was 2902 on the basis of the amino acid composition determined by two analyzer runs with 24-hour hydrolysis.
multaneous dialysis experiments within one unit. Each half-cell had a capacity of 5 ml, and was provided with an opening for introducing or withdrawing of solutions. The equilibrium dialysis was carried out in 0.1 N-ethylmorpholine-HCl buffer at pH 7.53 and ionic strength 0.16. Three different stock solutions were prepared: (a) 1% HSA in buffer, (b) CuCl₂ in 10⁻³ M HCl, and (c) L-His in buffer. The dialysis cells were assembled with 4 ml of solution on both sides. One half-cell contained Solution b mixed with ¹⁷⁷CuCl₂ and Solution a. The molar ratio of Cu(II) to HSA was kept approximately 1. The other half-cell contained various known amounts of Solution c and L-[¹⁴C]His. The total molar ratio of L-His to HSA ranged from about 0.3 to 10.0. Similar experiments were carried out involving HSA and L-His alone with a molar ratio of L-His to HSA ranging from 0.01 to 4.0.

Equilibrium was obtained by using an automatic shaker for 4 days at 30°. Radioactivity of ¹⁷⁷Cu(II) and ¹⁴C was measured both before and after dialysis. A well type scintillation counter equipped with the pulse height analyzer was used for the assay of ¹⁷⁷Cu(II) activity. The counts were corrected for decay on the basis of the 60-hour half-life of ¹⁷⁷Cu(II). After allowing sufficient time for ¹⁷⁷Cu(II) to decay, the radioactivity of ¹⁴C was determined in a liquid scintillation counter.

Absorption Spectra—The visible absorption spectra of the Cu(II) complexes were obtained with a Cary model 15 recording spectrophotometer thermostated at 25°. The cell path was 1 cm. Spectra were corrected for the slight visible absorption of HSA. The absorption spectra of the Cu(II)-HSA solution, prepared in 0.16 M NaCl, and adjusted to the required pH value, was added either CuCl₂ in 10⁻³ M HCl or Cu(II)-L-His solution at pH 7.50. The protons released were then titrated with 0.01 N NaOH in a sealed system. The molar ratio of CuCl₂ or Cu(II)-L-His to HSA remained approximately 1.

RESULTS AND DISCUSSION

Dissociation of HSA-Cu(II)-L-His and HSA-Cu(II)—The dissociation of the ternary and the binary complexes can be formulated as:

\[ K_{DHSA-Cu(II)-L-His} = \frac{[HSA-Cu(II)][L-His]}{[HSA-Cu(II)-L-His]} \] (1)

\[ K_{DHSA-Cu(II)} = \frac{[HSA][Cu(II)]}{[HSA-Cu(II)]} \] (2)

Therefore,

\[ K_{DHSA-Cu(II)-L-His} = \frac{[HSA-Cu(II)][L-His][HSA][Cu(II)]}{[HSA-Cu(II)-L-His]} \] (3)

and

\[ K_{DHSA-Cu(II)} = \frac{[HSA][Cu(II)]}{[HSA-Cu(II)]} \] (4)

The K₉ values were determined from a set of equations with the derived parameters from the equilibrium dialysis data utilizing ¹⁷⁷Cu(II) and L-[¹⁴C]His double labeling. Let:

Total Cu(II) concentration = [Cu(II)]ₜ
Total HSA concentration = [HSA]ₜ
Total L-His concentration = [L-His]ₜ
Counts of ¹⁷⁷Cu(II) before dialysis = a
Counts of ¹⁷⁷Cu(II) after dialysis (aqueous portion) = b
Counts of ¹⁷⁷Cu(II) after dialysis (protein portion) = c
Counts of L-[¹⁴C]His before dialysis = d
Counts of L-[¹⁴C]His after dialysis (aqueous portion) = e
Counts of L-[¹⁴C]His after dialysis (protein portion) = f

Then, the following equations can be set up:

\[ [Cu(II)] + [Cu(II)-L-His] = [Cu(II)-L-His] \]

\[ [HSA-Cu(II)] = \frac{b}{a} \] (5)

\[ [HSA-Cu(II)-L-His] = \frac{c - b}{a} \] (6)

\[ [HSA-Cu(II)-L-His] = \frac{f - e}{d} \] (7)

Then,

\[ [HSA-Cu(II)] = \frac{c - b}{a} \] (8)

\[ [HSA] = \frac{c - b}{a} \] (9)

\[ [L-His] = \frac{[L-His]}{[L-His]} \left[ 1 - \frac{f - e}{d} \right] - \frac{b}{a} \] (10)

and [Cu(II)] can be calculated according to the known stability constant of Cu(II)-L-His.

The equilibrium dialysis data are presented in Table I. The average value of the dissociation constant for HSA-Cu(II)-L-His is 1.38 x 10⁻¹² and that for HSA-Cu(II) is 6.61 x 10⁻¹².

Effect of -His on Spectra of HSA-Cu(II) and Asp Fragment-Cu(II)—The spectral characteristics of HSA-Cu(II) at pH values 5.50, 6.50, 8.00, and 10.03 are shown in Fig. 1A. The spectra of the Cu(II)-L-His system taken at the same
**Table I**

Equilibrium dialysis of ternary binding system

The equilibrium dialysis was carried out in 0.1 M N-ethylmorpholine-HCl buffer at pH 7.53, 6°, and ionic strength 0.16. Total Cu(II) and HSA concentrations were kept constant at $1.20 \times 10^{-4} \text{ M}$ and $1.27 \times 10^{-4} \text{ M}$, respectively, while the L-His concentration was varied. Calculations were made according to Equations 1 to 10.

<table>
<thead>
<tr>
<th>[L-His]</th>
<th>[HSA-Cu(II)]</th>
<th>[HSA]</th>
<th>[Cu(II)-L-His]</th>
<th>[Cu(II)]</th>
<th>[HSA-Cu(II)-L-His]</th>
<th>$-\log K_{\text{HSA-Cu(II)-L-His}}$</th>
<th>$-\log K_{\text{HSA-Cu(II)}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.36</td>
<td>1.10</td>
<td>1.40</td>
<td>4.51</td>
<td>0.24</td>
<td>22.71</td>
<td>21.74</td>
<td>15.54</td>
</tr>
<tr>
<td>0.71</td>
<td>1.06</td>
<td>1.75</td>
<td>9.68</td>
<td>0.49</td>
<td>11.87</td>
<td>21.44</td>
<td>15.72</td>
</tr>
<tr>
<td>1.41</td>
<td>1.01</td>
<td>2.19</td>
<td>14.93</td>
<td>1.08</td>
<td>3.21</td>
<td>21.49</td>
<td>16.08</td>
</tr>
<tr>
<td>2.78</td>
<td>0.91</td>
<td>2.83</td>
<td>21.30</td>
<td>2.29</td>
<td>1.18</td>
<td>22.20</td>
<td>16.44</td>
</tr>
<tr>
<td>6.67</td>
<td>0.68</td>
<td>4.71</td>
<td>40.12</td>
<td>5.78</td>
<td>0.55</td>
<td>22.17</td>
<td>16.62</td>
</tr>
<tr>
<td>11.77</td>
<td>0.47</td>
<td>6.63</td>
<td>59.27</td>
<td>10.49</td>
<td>0.16</td>
<td>22.15</td>
<td>16.69</td>
</tr>
</tbody>
</table>

**Fig. 1.** The pH dependence of the visible spectra of three different systems at 25°, 1-cm cell path, ionic strength 0.16. A, HSA + Cu(II); [HSA] = [Cu(II)] = $1.31 \times 10^{-4} \text{ M}$. B, HSA + Cu(II)-L-His$_2$; [HSA] = [Cu(II)-L-His$_2$] = $1.08 \times 10^{-4} \text{ M}$. C, L-His + Cu(II); [L-His] = $2 [\text{Cu(II)}] = 2.00 \times 10^{-4} \text{ M}$.

Table II

Interpolated data of concentration of Cu(II)-binding species in 1:1 Cu(II)-L-His$_2$:HSA system at pH 7.5 and 6°

<table>
<thead>
<tr>
<th>Species</th>
<th>Concentration, $10^{-4} \text{ M}$</th>
<th>Distribution of Cu(II)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSA-Cu(II)</td>
<td>9.3</td>
<td>77.5</td>
</tr>
<tr>
<td>Cu(II)-L-His$_2$</td>
<td>2.0</td>
<td>16.5</td>
</tr>
<tr>
<td>HSA-Cu(II)-L-His$_2$</td>
<td>0.7</td>
<td>6.0</td>
</tr>
</tbody>
</table>

pH values are shown in Fig. 1C. The spectral results of the addition of L-His to HSA-Cu(II) are presented in Fig. 1B. At pH 5.5, HSA-Cu(II) has an absorption peak at 525 nm, but at the same pH the Cu(II)-L-His$_2$ and the ternary systems both show peaks at 625 nm. The absence of 525 nm peak in the ternary system may mean that the affinity of L-His to Cu(II) at this pH is high enough to compete for Cu(II) with the first Cu(II)-binding site on HSA. As the pH is raised to 6.5, a new peak appears at 575 nm, but a shoulder remains at 625 nm in the ternary system. At pH 8 and 10, a sharp peak appears at 535 nm in the ternary system. At the same pH values, HSA-Cu(II) shows a $\lambda_{\text{max}}$ at 525 nm. This may mean that around pH 8.0 most of the Cu(II) is in the form of HSA-Cu(II) and HSA-Cu(II)-L-His. This is consistent with the findings of the equilibrium distribution of species in the ternary system at pH 7.5 shown in Table II.

Peters and Blumenstock (26) reported that the absorption spectrum of the Asp fragment in the presence of the first Cu(II) ion showed a single peak above pH 5.5. Maximum absorbance was at the same wave length of 525 nm as that of BSA-Cu(II). When L-His was added to Asp fragment-Cu(II) in equal molar ratio at pH 5.5, it showed an absorption maximum at 552 nm (Fig. 2). Spectra of the same solution taken at pH values 6.9 and 9.8 show a constant absorption maximum at 542 nm. Since no peak is visible either at 640 nm for the Cu(II)-L-His$_2$ system or at 525 nm for Asp fragment-Cu(II), it is most likely that a ternary complex Asp fragment-Cu(II)-L-His is formed in this system. A comparison of the extent of the ternary complex formation in the two systems is not possible since the data for the equilibrium distribution of species in the Asp fragment-Cu(II)-L-His system are not available.
Fig. 2. The visible spectra of Asp fragment-Cu(II)-L-His as a function of pH. Conditions: 25°C, 1-cm cell path, ionic strength 0.16, [Asp fragment] = [Cu(II)] = [L-His] = 2.05 × 10^{-4} M.

**Computed Spectrum of Ternary Complex**—The computed spectrum of the ternary complex was obtained by subtracting the optical density due to all other visible light-absorbing species from that of the whole system as illustrated in the following equations:

\[ A_{\text{total}} = A_{\text{HSA-Cu(II)-L-His}} + A_{\text{HSA-Cu(II)}} \]
\[ + A_{\text{Cu(II)-L-His}} + A_{\text{HSA}} \]
\[ = \epsilon_{\text{HSA-Cu(II)-L-His}} \times [\text{HSA-Cu(II)-L-His}] \]
\[ + \epsilon_{\text{HSA-Cu(II)}} \times [\text{HSA-Cu(II)}] + \epsilon_{\text{Cu(II)-L-His}} \times [\text{Cu(II)-L-His}] + \epsilon_{\text{HSA}} \times [\text{HSA}] \]

The extinction coefficient of the ternary complex was determined from the known values of the extinction coefficient of the individual species\(^3\) and the concentrations obtained from the equilibrium dialysis method. It was calculated at 5 or 10 nm wave length intervals from 700 to 460 nm to constitute the spectrum. The computed spectrum of HSA-Cu(II)-L-His is presented in Fig. 3, along with the spectra of HSA-Cu(II) and Cu(II)-L-His, the predominant species in the Cu(II)-L-His system at pH 7.5. The ternary complex shows a \(\lambda_{\text{max}}\) at 540 nm, a shift of 15 nm toward red from that of HSA-Cu(II) (\(\lambda_{\text{max}} = 525 \text{ nm}\)).

**Proton Displacement Studies**—The number of titrable protons observed by adding Cu(II) and Cu(II)-L-His to HSA at pH 7.50 is shown in Table III. When Cu(II)-L-His is added to HSA, there are more species formed than HSA-Cu(II)-L-His. Consequently, the proton displacement data of the ternary system shown in Table III do not reflect what has originated from HSA-Cu(II)-L-His. In order to solve the number of protons displaced due only to the formation of HSA-Cu(II)-L-His, it is necessary to know the distribution of all of the species in the system. This was obtained by using the concentration data from equilibrium dialysis experiment (Table I) by means of interpolations to the conditions used in titration.\(^4\) The result is shown in Table II. This enabled us to calculate the contribution of the individual Cu(II)-binding species to the observed proton displacement data.

\(^4\) This calculation did not take into account the effect of temperature on equilibrium.

---

**Table III**

<table>
<thead>
<tr>
<th>Addition to HSA</th>
<th>Protons displaced</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu(II)</td>
<td>2.58</td>
</tr>
<tr>
<td>Cu(II)-L-His</td>
<td>0.67</td>
</tr>
</tbody>
</table>

For the formation of each mole of HSA-Cu(II)-L-His from a mixture of Cu(II)-L-His and HSA, 1 mole of free L-His is liberated. Similarly, 2 moles of free L-His are liberated in the formation of each mole of HSA-Cu(II). In Cu(II)-L-His, the amino nitrogen is bonded to Cu(II); hence, at pH 7.50 the L-His liberated from Cu(II)-L-His will take up protons to protonate the newly released amino group. Meanwhile, some protons are liberated as a result of the formation of HSA-Cu(II). The actual number of protons displaced at pH 7.50 in the formation of HSA-Cu(II)-L-His can then be calculated as illustrated below:

Moles of protons taken up by L-His due to the formation of HSA-Cu(II) = 2 \times 7.50 \times 0.06 = 1.55

Moles of protons taken up by L-His due to the formation of HSA-Cu(II)-L-His = 1 \times 0.06 = 0.06

Then the total moles of protons taken up = 1.55 + 0.06 = 1.61

But, the moles of protons released in the formation of HSA-Cu(II) = 2.58 \times 7.50 = 2.00

Therefore, the net moles of protons displaced in the formation of HSA-Cu(II)-L-His = 0.67 - (2.00 - 1.61) = 0.28

**Possible Identity of Cu(II)-binding Site in HSA-Cu(II)-L-His**—HSA apparently consists of a single polypeptide chain made up of approximately 555 amino acids and stabilized by about 17 disulfide bonds (28). It has been shown that the first Cu(II)-binding site in HSA involves the NH\(_2\)-terminal sequence: Asp-Ala-His (29). The proposed binding site includes the \(\alpha\)-amino nitrogen of aspartyl residue, two intervening peptide nitrogens, and the imidazole nitrogen of the histidine in position 3 (28, 30-32). In the absence of Cu(II), free L-His does not bind to HSA at neutral pH (5). Others have also shown that amino acids such as glycine, leucine, and phenylalanine do not bind to albumin (33). It is unlikely that the attachment of Cu(II) to the first binding site changes the structure of HSA in such a way...
as to open up an entirely new site for a free amino acid to bind to form the ternary complex. This view is consistent with the finding that the ternary complex is also formed with the 24-residue N22-terminal peptide of BSA. In view of this, it is most likely that Cu(II) in the ternary complex is bound to both HSA and L-His. Both spectral and proton displacement data are in favor of this contention.

Recent studies suggest that both L-His molecules in Cu(II)-L-His at pH 7.5 are tridentate. It is also interesting to note that ternary complex HSA-Cu(II)-L-Thr, which was isolated and crystallized in our laboratory (4) from the human serum and later subjected to X-ray analysis (18), was shown to have L-His in a tridentate form. The tridentate form of L-His in HSA-Cu(II)-L-His is a possibility, although none of the results presented here entirely rule out other modes of binding. The computed spectrum of the ternary complex shows a red shift from 525 nm (HSA-Cu(II)) to 540 nm (HSA-Cu(II)-L-His). This is consistent with the change of binding mode, either from all nitrogen to a mixture of nitrogen and oxygen (34) or to an additional involvement of imidazole nitrogen. The introduction of oxygen or nitrogen (or both) in the liquid field could be from the carboxyl or imidazole groups (or both) of L-His.

The binding of the protein part to Cu(II) in the ternary complex presents some interesting possibilities. It seems unlikely that the protein part of the ternary complex would bind Cu(II) in the same manner as it does in HSA-Cu(II) (26, 30–32), since it has also to bind L-His simultaneously. Although no information is available concerning the intrinsic pKₐ of the α-amino group of HSA, it may fall around the neutral pH range (35). The protons which are displaced at pH 7.50 (Table III) come most likely from the a-amino nitrogen of HSA in the ternary complex. The displaced protons cannot come from the imidazole group of a histidyl residue since the imidazole protons are already titrated at pH 7.50. While the participation of the imidazole nitrogen is a strong possibility, no conclusion can be drawn from the available data. The quantity of protons which were displaced at pH 7.50 is much less than what one would expect if a peptide nitrogen were involved. However, these interpretations must be followed with caution, since the intrinsic pKₐ values of the donor groups are not known.

Studies with model ternary complexes involving glycine peptides, Cu(II), and glycine suggest that, in both glycylglycine-Cu(II)-glycine and diglycylglycine-Cu(II)-glycine, the α-amino nitrogen and a peptide nitrogen are involved in Cu(II) binding, while the glycine part of the ternary complex is shown to bind Cu(II) with the carboxyl and amino group (17). What influence a side chain residue, such as an imidazole group, might have in Cu(I1) with the carboxyl and amino group (17). What influence a side chain residue, such as an imidazole group, might have in Cu(I1) binding site in HSA-Cu(II)-L-His.

Possible Physiological Significance of Ternary Complex—Human serum contains approximately 1 µg of Cu(I1) per ml. Most of this is bound to ceruloplasmin, and a small fraction is bound to both albumin and amino acids (3, 4). The Cu(I1) in ceruloplasmin is not exchangeable in vivo (36). It is the albumin- and the amino acid-bound fraction of serum Cu(I1) which is exchangeable and considered to be the physiologically important transport form of Cu(I1) (2–4). In view of the findings presented here, it seems reasonable to postulate that the following equilibria are maintained in the physiological state:

\[
\text{Copper (II) + amino acid} \rightleftharpoons \text{Cu(II)-amino acid} \\
| \begin{align*} 
\text{albumin} \\
\text{albumin-Cu(II)-amino acid} \\
| \begin{align*} 
\text{albumin-Cu(II) + amino acid} \\
\end{align*}
\]

**Scheme I**

Although L-His is the major Cu(II)-binding amino acid in serum (3, 4), other amino acids could also play a similar role in forming an intermediary ternary complex. The above mechanism could play an important role in the exchange of Cu(II) between a macromolecule and a low molecular weight substance which in turn can readily be transported across the biological membrane.

REFERENCES


*The reaction kinetics in the formation of HSA-Cu(II)-L-His at pH 7.50 and 25° was attempted by using the stopped flow technique. However, no observable rate could be obtained. This indicates that the formation of both HSA-Cu(II)-L-His and HSA-Cu(II) is very rapid and beyond the detectable time range of stopped flow or that the change in optical density is not appreciable, or both.*
Ternary Coordination Complex between Human Serum Albumin, Copper (II), and 1-Histidine
Show-Jy Lau and Bibudhendra Sarkar


Access the most updated version of this article at http://www.jbc.org/content/246/19/5938

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here 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/246/19/5938.full.html#ref-list-1