THE EFFECT OF LITHIUM PERIODATE ON CRYSTALLINE BOVINE SERUM ALBUMIN

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(Received for publication, January 21, 1949)

In a recent report it was shown that the biological activities of two proteins, crystalline ribonuclease and the immune globulin of Type III antipneumococcus horse serum, are destroyed when the proteins are subjected to the action of dilute lithium periodate at physiological pH values (1). The nature of the chemical changes involved are ill understood, but that they are considerable is evident not only because of the loss in biological activity which occurs, but because marked changes in the absorption spectrum are observed following the oxidation. The present paper describes a continuation of the work. In order to gain further insight into the mode of action of the periodate ion on native proteins, a study has been made of its action on crystalline bovine serum albumin. It will be shown that the physical, chemical, and immunological properties of this protein are radically altered on contact with the reagent and that these changes are accompanied by an alteration or destruction of certain amino acids.

Materials and Methods

Crystalline bovine serum albumin was purchased from The Armour Laboratories, Chicago. Buffered lithium periodate was prepared by dissolving 2.28 gm. of HI04·2H2O in 50 ml. of water; 10 ml. of 0.5 M H3PO4 was added followed by the addition of 20 ml. of carbonate-free 1 N LiOH. The solution was then diluted to 100 ml. The pH of this solution is 7.2.

The cystine and cysteine content of the native and oxidized proteins was determined by the method of Kassel and Brand (2). The acid hydrolysis of the various proteins was carried out as described by Brand (3). Twice distilled 6 N HCl was used, and the hydrolysis was performed in the presence of urea, and in an atmosphere of CO2 at 130-35° for 8 hours. The sample was diluted to a known volume and aliquot portions were used for the determination of the cystine and cysteine. Tyrosine analyses were performed on alkaline hydrolysates of the proteins (4) by the procedure of Lugg (5). Tryptophane analyses were carried out directly on the intact unhydrolyzed protein as recommended by Sullivan (6). Standard curves for the absorption values of the colors developed were constructed, using in each instance the pure amino acid as standard. All determinations were made in a Beckman model DU spectrophotometer. The color developed in the cystine method was read at 850 mµ; in the tyrosine method 354 mµ; and in the tryptophane procedure 600 mµ. In all instances duplicate analyses were performed.

Electrophoretic Technique.—The electrophoretic experiments were carried out at 0.5° in the apparatus described by Longsworth (7). Samples of the native and oxidized proteins at concentrations of approximately 1.0 per cent were used. Prior to electrophoresis, the solutions were dialyzed against several portions of the same buffer as that used in the experiments.
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The mobilities were computed from the descending patterns, using the bisecting ordinate of the refractive index gradient curve, and referred to 0°C.

Oxidation of Bovine Serum Albumin by Lithium Periodate.—4.0 gm. of crystalline bovine serum albumin was dissolved in 200 ml. of water; 100 ml. of 0.1 M lithium periodate buffer solution described above was then added. After standing at room temperature for 72 hours the excess periodate was decomposed by the addition of 3 ml. of 50 per cent glucose solution. The solution of the oxidized protein was thoroughly dialyzed in a rocking device against running distilled water until free of electrolytes. The end product was isolated by desiccation from the frozen state. 3.8 gm. of material was recovered. The substance was obtained as a beige-colored fluffy powder.

FINDINGS

Properties of Oxidized Bovine Serum Albumin.—When bovine serum albumin was oxidized with lithium periodate at pH 7.2, the solution gradually became yellow in color, and no visible precipitate was formed. The oxidized protein was soluble in neutral and alkaline solutions, but if the pH was lowered to pH 5.0, the solution became turbid, and at a pH of approximately 3.5 to 4.0 the protein was insoluble. The solubility of the oxidized protein in ammonium sulfate was markedly changed following oxidation with lithium periodate. At 10 per cent saturation some of the protein was precipitated, and at 30 per cent saturation the major part came out of solution. Attempts were made to crystallize the oxidized derivative from ammonium sulfate, but without success. A solution of the protein behaved in some respects like one of the native material,—the oxidized protein was precipitated by the salts of heavy metals, and by trichloracetic acid. Solutions of the oxidized protein foamed and gave a strong biuret test. The oxidized protein was also hydrolyzed by crystalline trypsin, and to a greater degree than was the native protein when the hydrolysis was carried out under the following conditions:—

Ten ml. of a 0.1 per cent solution of native and oxidized protein was heated for 3 minutes at 70° in a 0.1 molar phosphate buffer at pH 7.6. To each solution was added 500 micrograms of crystalline trypsin, and after 18 hours at 37° an aliquot of the protein was precipitated with an equal volume of 10 per cent trichloracetic acid. Analyses of the supernates revealed that 34 per cent of nitrogen was found in that of the heated native albumin, and 67 per cent in that of the oxidized protein. If the experiment was carried out on unheated protein solutions, the differences in soluble nitrogen were approximately of the same order, but the degree of proteolysis was one-third less.

Change in Absorption Spectrum of Bovine Serum Albumin Following Oxidation with LiO₄.—0.4 gm. of crystalline bovine serum albumin was dissolved in 20 ml. of water; 4 ml. was removed and saved. The remainder was treated with 8.0 ml. of 0.1 M LiO₄-phosphate mixture and allowed to stand at 22°. At the end of 24, 48, and 72 hours, 6 ml. samples were removed and the periodate decomposed by the addition of 0.5 ml. of 50 per cent glucose solution. All the solutions were placed in cellophane bags and dialyzed against 0.1 M phosphate buffer at pH 7.6 until free of I⁻ and IO₃⁻. Each solution, including the control, was then diluted with the phosphate buffer until the concentration was 1.0 mg. per ml. The ultraviolet absorption spectra were determined and recorded (Fig. 1).
It is apparent from the results presented in Fig. 1 that profound changes have occurred in the ultraviolet absorption spectra of the protein after contact with the reagent. These changes become progressively greater the longer the protein is subjected to the action of the periodate ion. After 24 hour contact,

![Absorption spectra of bovine serum albumin before and after oxidation with 0.03 M LiIO₄](image)

**Fig. 1.** Absorption spectra of bovine serum albumin before and after oxidation with 0.03 M LiIO₄.

- ● = native bovine albumin.
- ♦ = bovine albumin oxidized 24 hours with LiIO₄.
- ○ = " " " 48 " " "
- □ = " " " 72 " " "

absorption below 280 m\(\mu\) is not only greater, but there is a shift of 10 m\(\mu\) in the maximum. At the end of 48 and 72 hours contact with the reagent the absorption becomes progressively greater throughout the entire range of wave lengths, and there is no discernible peak. This shift in absorption spectrum is comparable to that shown by the Type III pneumococcus immune globulin after oxidation with periodate.
Determination of Amino Acids.—Because of the shift and final disappearance in the point of maximum absorption of serum albumin following oxidation with periodate, it was thought advisable to analyze the oxidized protein for its tyrosine, tryptophane, cystine, and cysteine content.

Cystine and Cysteine.—Hydrolysates (2) of the native and oxidized proteins were analyzed for their content of cystine and cysteine (3). The values are recorded in Table I. It can be seen that the cystine content of the native protein was essentially the same as that reported by other investigators (8); the cysteine content, on the other hand, was considerably lower. Repeated analyses confirmed this observation and the difference probably resides in the fact that our particular sample of bovine serum albumin had actually a lower content of labile –SH groups.

When cystine and cysteine determinations were carried out on hydrolysates of the protein previously oxidized for 72 hours, the two values were considerably lower, 2.45 and 0.13 per cent, respectively. This would indicate that a marked decrease in S–S and in –SH groupings had taken place, without a comparable loss in total sulfur, for it can be seen in Table I that the total sulfur content of the native and oxidized protein was found to be 1.90 and 1.68 per cent, respectively.

Tyrosine.—A sample of native and of oxidized bovine serum albumin dissolved in 5 N NaOH containing 20 mg. of protein per ml. was hydrolyzed in a sealed tube for 24 hours at 100° (4). After removal of tryptophane, or its products of oxidation, as the mercury salt, the tyrosine was determined according to the method of Lugg (5). The native protein was found to contain 5.63 per cent tyrosine. The hydrolysate of the oxidized protein, on the other hand, gave an intensity of color corresponding to 3.28 per cent tyrosine. From the analytical result it appears that approximately 40 per cent of the tyrosine had been destroyed or altered by treatment of the protein with lithium periodate.

Tryptophane.—Both the native and oxidized protein were next analyzed for their tryptophane content (6). The native protein was found to have a tryptophane content of 0.7 per cent, whereas the oxidized protein gave no color with the reagents, indicating that the tryptophane had been destroyed. Since color development, both in the determination of tyrosine and tryptophane, appears to be dependent upon the integrity of the phenolic and indole rings, it can be assumed that the latter have in each instance suffered chemical degradation, the extent of which is unknown.

In this connection it might be said that when pure tyrosine was subjected to the action of 0.03 M LiIO₄ at pH 7.2, approximately 1 mol of the reagent was consumed over a period of 8 hours. During the course of the oxidation the solution darkened and eventually deposited a precipitate. Chromatographic adsorption of the reaction mixture on activated alumina revealed at least four bands, indicating that a complex series of chemical reactions had taken place.

Electrophoretic Properties of Native and Oxidized Bovine Serum Albumin.—In
the foregoing account it has been shown that the oxidation of bovine serum albu-
min with lithium periodate causes marked changes in the chemical and physical
properties of the protein. It seemed of interest, therefore, to investigate the
electrophoretic behavior of the protein after treatment with this reagent.

**TABLE I**

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Native bovine albumin</th>
<th>Bovine albumin after 72 hrs. oxidation with 0.033 M LiIO₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[\alpha]_{D}^{22}$</td>
<td>-53.0°</td>
<td>-69.0°</td>
</tr>
<tr>
<td>Total N, per cent</td>
<td>16.00</td>
<td>14.51</td>
</tr>
<tr>
<td>Total S, &quot; &quot;</td>
<td>1.91</td>
<td>1.68</td>
</tr>
<tr>
<td>Cystine, &quot; &quot;</td>
<td>5.65</td>
<td>2.45</td>
</tr>
<tr>
<td>Cysteine, &quot; &quot;</td>
<td>0.43</td>
<td>0.13</td>
</tr>
<tr>
<td>Tyrosine, &quot; &quot;</td>
<td>5.63</td>
<td>3.28</td>
</tr>
<tr>
<td>Tryptophane, per cent</td>
<td>0.70</td>
<td>0.00</td>
</tr>
</tbody>
</table>

* Oxidized 72 hours with 0.033 M LiIO₄.

**Fig. 2.** Electrophoretic patterns of bovine serum albumin before and after treatment with 0.03 M lithium periodate.

In the experiment recorded in Fig. 2 the electrophoresis of bovine serum albumin before and after treatment with lithium periodate was carried out in a sodium phosphate buffer at pH 7.7, an ionic strength 0.1, at a potential gradient of 6.34 volts per cm. for 9,000 seconds. The full curve represents native bovine serum albumin, the dashed and dotted curves are patterns of the same preparation after oxidation with 0.03 M lithium periodate for 24 and 72 hours, respectively. From the figure it is apparent that a qualitative difference in the patterns exists; the boundaries of the two oxidized proteins are more
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diffuse than is that of the native material. Under experimental conditions in which time and potential gradient have been kept constant, superimposition of the electrophoretic patterns permits a direct comparison of the approximate mobilities of the three protein preparations. Thus it can be seen that the mobilities of the substances are different. The actual values of \( u = -6.77 \times 10^{-5} \) and \( u = -7.33 \times 10^{-5} \) for the samples treated for 24 and 72 hours, respectively, are considerably higher than that of \( u = -6.14 \times 10^{-5} \) of the native protein.

The large boundary spreading shown by the two oxidized proteins suggests that these preparations are less homogeneous than the native albumin. It seemed advisable, therefore, to extend the electrophoretic study over a wider range of pH values. It was observed that the protein oxidized for 72 hours migrated as a single peak at all pH values studied, except in one instance where the determination was carried out in a 0.1 N sodium acetate buffer at pH 5.64. At this pH a separation of the boundary into two peaks was observed in the ascending patterns. A comparison with bovine serum albumin in the isoelectric pH region was not possible because of the insolubility of the oxidized protein in the pH range of 3.5 to 5.2.

The results of the mobility measurements over a wide pH range of normal bovine serum albumin and of the protein treated with lithium periodate for 72 hours are presented in Table II. All the experiments were carried out in monovalent buffers of 0.1 ionic strength, the composition of which is given in column 1. The mobility values obtained for native bovine serum albumin are in good agreement with those of Longsworth and Jacobsen (9). A comparison of the mobilities of the oxidized protein, column 4, with those of the native protein, column 3, shows that at all pH values below 9 the oxidized material has the more negative mobility; i.e., as a cation it migrates more slowly than the intact protein while as an anion it moves more rapidly.

In an attempt to find further differences between the two proteins, their electrophoretic behavior in a buffer containing methyl orange has also been studied. With the aid of the dialysis experiments and photometric technique of Klotz (10), it was found that the oxidized protein bound but half the quantity of methyl orange as that bound by normal bovine serum albumin. Longworth and Jacobsen (9) have shown that native bovine serum albumin has a higher mobility in a 0.1 N sodium acetate buffer at pH 5.6 containing 0.0002 M methyl orange than it does in pure acetate buffer. The difference in mobility of the oxidized protein when observed under the same conditions, \( \Delta u = 0.3 \times 10^{-5} \), when compared with that of native albumin (9), \( \Delta u = 0.6 \times 10^{-5} \), parallels the results obtained in the dialysis experiments.

Antigenicity of Oxidized Plasma Albumin.—In our hands crystalline bovine serum albumin has proved to be a good antigen. Rabbits injected intravenously with 5 mg. of protein for 6 consecutive days, followed by a rest period
TABLE II
Mobilities of Bovine Serum Albumin before and after Treatment with Lithium Periodate in Buffer Solutions of Ionic Strength 0.1

<table>
<thead>
<tr>
<th>Buffer (1)</th>
<th>pH (2)</th>
<th>m x 10^6 (3)</th>
<th>Δm (4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 N HCl — 0.5 N glycine</td>
<td>3.02</td>
<td>7.67</td>
<td>5.4</td>
</tr>
<tr>
<td>0.02 N NaAc — 0.2 N HAc — 0.08 N NaCl</td>
<td>3.62</td>
<td>5.43</td>
<td>4.67</td>
</tr>
<tr>
<td>0.02 N NaAc — 0.1 N HAc — 0.08 N NaCl</td>
<td>3.91</td>
<td>4.14</td>
<td>3.75</td>
</tr>
<tr>
<td>0.1 N NaAc — 0.1 N HAc</td>
<td>4.64</td>
<td>0.36</td>
<td>-0.28</td>
</tr>
<tr>
<td>0.1 N NaAc — 0.05 N HAc</td>
<td>4.90</td>
<td>0.96</td>
<td>0.66</td>
</tr>
<tr>
<td>0.1 N NaAc — 0.01 N HAc</td>
<td>5.64</td>
<td>-2.68</td>
<td>-4.68</td>
</tr>
<tr>
<td>0.02 N NaCac — 0.004 N HAc — 0.08 N NaCl</td>
<td>6.76</td>
<td>-4.24</td>
<td>-5.71</td>
</tr>
<tr>
<td>0.02 N NaV — 0.02 N HV — 0.08 N NaCl</td>
<td>7.84</td>
<td>-6.12</td>
<td>-6.64</td>
</tr>
<tr>
<td>0.1 N NaV — 0.02 N HV</td>
<td>8.62</td>
<td>-6.64</td>
<td>-6.90</td>
</tr>
<tr>
<td>0.1 N NaV — 0.005 N HV</td>
<td>9.22</td>
<td>-7.54</td>
<td>-7.53</td>
</tr>
</tbody>
</table>

Ac = acetate.
Cac = cacodylate.
V = diethylbarbiturate.

TABLE III
Precipitin Reactions in Sera of Rabbits Immunized with Native and Oxidized Bovine Serum Albumin

<table>
<thead>
<tr>
<th>Serum of rabbit injected with</th>
<th>Test antigen used</th>
<th>Final dilution of test antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1:2,000</td>
</tr>
<tr>
<td>A</td>
<td>A</td>
<td>++++</td>
</tr>
<tr>
<td>A_24</td>
<td>A_24</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>0</td>
</tr>
<tr>
<td>A_72</td>
<td>A_72</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>0</td>
</tr>
</tbody>
</table>

A = native bovine serum albumin.
A_24 = bovine albumin oxidized for 24 hours with 0.03 M LiIO_4.
A_72 = bovine albumin oxidized for 72 hours with 0.03 M LiIO_4.
+++ = complete precipitation with clear supernate.
± = very slight precipitation.
0 = no precipitation.

of a week and again injected during 6 days, developed antisera which precipitated strongly the homologous protein. Groups of animals which had received a similar course of immunization with the protein treated with lithium periodate over a 24 and a 72 hour period gave, in the first instance, antisera which were very weak indeed. Those which had received the protein oxidized for 72...
hours had no precipitins whatsoever (Table III), nor did they give a detectable immune response even after prolonged injection of the oxidized protein. These observations are contrary to those made with Type III pneumococcus immune globulin (1), where it was found that contact with lithium periodate for 24 hours did not destroy antigenicity.

TABLE IV

Precipitin Reactions of Oxidized Bovine Serum Albumin in Serum of a Rabbit Immunized with Native Albumin

<table>
<thead>
<tr>
<th>Test antigen used</th>
<th>Final dilution of test antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:2,000</td>
</tr>
<tr>
<td>A</td>
<td>++++</td>
</tr>
<tr>
<td>A&lt;sub&gt;24&lt;/sub&gt;</td>
<td>++++</td>
</tr>
<tr>
<td>A&lt;sub&gt;48&lt;/sub&gt;</td>
<td>+++</td>
</tr>
<tr>
<td>A&lt;sub&gt;72&lt;/sub&gt;</td>
<td>++</td>
</tr>
</tbody>
</table>

Fig. 3. Turbidimetric precipitin reaction of oxidized bovine albumin in native albumin antiserum.

Not only did the oxidized albumin fail to elicit homologous antibodies in rabbits, as can be seen in Table III, but there were no antibodies present in these sera capable of precipitating the native unaltered protein. This would indicate that no intact native protein molecules remain after the albumin has been treated with lithium periodate. It is of considerable interest, however, to note that the treated albumin is still capable of precipitating some of the antibodies in the sera of rabbits immunized with native serum albumin (Table
When a quantitative turbidimetric estimation (11) of these reactions was made, it was observed (Fig. 3) that the intensity of the reaction diminished the longer the albumin fraction remained in contact with the reagent. From the results of these experiments it appears justified to conclude that the ability of serum albumin to incite antibody formation in rabbits is destroyed when the protein is subjected to the action of lithium periodate. The reason for this probably resides in the oxidative changes which take place in certain of the amino acids; these changes are not so profound, however, as to impair the ability of the altered protein to combine with the antibodies specifically directed toward the intact native protein molecule.

**DISCUSSION**

Periodic acid is not a benign reagent but a vigorous oxidant capable of bringing about extensive chemical changes in many types of organic compounds (12). Certain monosaccharides in acid solution are rapidly and completely broken down by the reagent; polysaccharides of appropriate structure suffer a severance of the component monosaccharide molecules with the formation of polyaldehydes and other ill defined products of oxidation. In addition, amino acids of certain types are readily oxidized by periodic acid. It is not surprising, therefore, that proteins may also undergo chemical alteration when brought in contact with the reagent.

That bovine serum albumin suffers irreparable damage on prolonged contact with lithium periodate is apparent from the results presented above. The chemical changes involve a slight loss in total nitrogen, a change in specific optical rotation, and an alteration or possible destruction of all or part of certain amino acids. The total sulfur content of the protein is somewhat diminished, yet the cystine, cysteine, and tyrosine content, the measure of which is based upon color development which in turn is dependent upon a specific chemical configuration, are considerably diminished. The tryptophane appears to be completely destroyed. That these chemical alterations are progressive and are a function of time is apparent from the gradual changes which occur in the absorption spectrum, and in electrophoretic behavior of the protein. Yet these changes are not so great as to cause a loss of properties which would no longer permit the material to be classified as protein. Solutions of the product of oxidation are still precipitated by high concentrations of salts; they give positive biuret tests, are precipitable by trichloracetic acid, and are hydrolyzed by crystalline trypsin. In some respects the oxidized material resembles a denatured protein,—in absorption spectrum, in solubility in high concentration of salts, and in precipitability at acid pH values. Unlike denatured protein, the oxidized material is incapable of eliciting antibodies in rabbits. This loss of function is believed to be associated with alterations in the aromatic amino acid content, and in this respect the material is analogous to gelatin (13).
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which contains neither tyrosine nor tryptophane, and is not antigenic. That the oxidized protein is still specifically precipitated by antibodies to native albumin, can best be explained on the basis that not all those groups of the protein which are responsible for antigen-antibody combination have been destroyed, even after prolonged contact with the reagent.

Because of the limited solubility of the oxidized protein, the mobility measurements recorded in this paper were necessarily confined to pH 3.0 and to the pH range of 5.6 to 9.2. A determination of the isoelectric point of the periodate-treated protein was therefore not possible. If the mobility data presented in Table II are plotted as ordinate against pH as abscissae, Fig. 4, it is apparent that the oxidized bovine serum albumin has a higher negative net charge than the normal bovine serum albumin at any given pH. This would indicate that the isoelectric point of the oxidized protein is at a pH more acid than that of
the native albumin and might possibly be explained by the destruction of the
imidazole ring of the histidine molecule. The results of this report, however,
do not permit of any extensive discussion concerning the groups which may
have been altered during the oxidative process and which contribute to the
electrophoretic behavior of this protein.

SUMMARY

A study of the chemical, physicochemical, and immunological changes in
bovine serum albumin, brought about by oxidation with lithium periodate,
has been made. It has been shown that destruction of certain amino acids
occurs, that a change in the absorption spectrum takes place, and that the
electrophoretic behavior of the protein is altered. Prolonged contact of bovine
albumin with lithium periodate destroys its ability to incite antibodies in
experimental animals.

BIBLIOGRAPHY

    University Press, 1943, 62.