THE INHIBITION OF LIPID AUTOXIDATION BY HUMAN SERUM AND ITS RELATION TO SERUM PROTEINS AND α-TOCOPHEROL

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SUMMARY

1. The inhibitory (antioxidant) effect of serum on the autoxidation of tissue polyunsaturated fatty acids has been investigated.

2. Dialysis studies, DEAE-cellulose chromatography, gel filtration, immunoelectrophoresis and immunodiffusion experiments showed that this effect is the function of two serum protein fractions.

3. One fraction was identified as transferrin. The second fraction includes caeruloplasmin.

4. Studies with radioactively labelled α-tocopherol showed that serum tocopherol in the physiological concentration range contributes only marginally to the total antioxidant activity of serum.

5. These findings are discussed in the wider context of biological antioxidant protection.

Key words: human serum antioxidant activity, transferrin, caeruloplasmin, tocopherol.

Although the relative stability of polyunsaturated fatty acids in the body implies a highly efficient antioxidant mechanism, the nature and mode of operation of this mechanism are still uncertain. Barber (1961) reported that serum inhibited lipid autoxidation in incubated tissue homogenates and that the inhibition could be reversed by the addition of inorganic iron. Wills (1965) found that serum inhibited the metal-catalysed autoxidation of linoleic acid emulsions. The present work was undertaken to identify the serum fractions responsible for this protective action, the assay of antioxidant activity described in the preceding paper (Stocks, Gutteridge, Sharp & Dormandy, 1974) being used.

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MATERIALS AND METHODS

Reagents

Special chemicals for radioactive tracer, chromatographic, fractionation and electrophoretic studies were obtained from the following sources: d-α-[5-Me-^3^H]tocopherol (specific radioactivity 12 Ci/mmol) from The Radiochemical Centre (Amersham, Bucks.); dl-α-tocopherol and human caeruloplasmin (grade IV) from Sigma (London) Chemical Co. Ltd (London, S.W.6); dextran sulphate 2000 and Sephadex G-150 from Pharmacia (G.B.) Ltd (London, W.5); DE-52 (pre-swollen DEAE-cellulose) from Whatman Biochemicals (Maidstone, Kent); iron-free human transferrin and M-Partigen immunodiffusion plates from Hoechst Pharmaceuticals Ltd (Hounslow, Middx.); Tween 20 and scintillation-grade toluene from Koch-Light Ltd (Colnbrook, Bucks.); POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene] and PPO (2,5-diphenyloxazole) from Nuclear Enterprises (Edinburgh); Soluene 100 from Packard Instruments, La Grange, Ill., U.S.A.; malonyldialdehyde bisdiethylacetal from Schuchardt A.G. (München, W. Germany); 20 cm x 20 cm thin-layer plates precoated with silica gel G from E. Merck (Darmstadt, W. Germany); lyphogel and Triacetate metricel (PEM) ultrafiltration membranes from Hawksley and Sons Ltd (Lancing, Sussex). All other chemicals were AnalaR grade obtained from British Drug Houses Ltd (Poole, Dorset).

Preparation and storage of serum samples

Blood samples were collected from normal fasting adults and allowed to clot at room temperature. The serum was stored for up to 48 h at 4°C, or for up to 4 weeks at -20°C; it has been shown that these periods of storage do not impair serum antioxidant activity (Stocks et al., 1974). Serum was pooled before fractionation experiments.

Measurement of antioxidant activity

Antioxidant activity was measured by determining the percentage inhibition of lipid autoxidation in a standard ox-brain homogenate as described in the preceding paper (Stocks et al., 1974).

Fractionation procedures

DEAE-cellulose chromatography. Serum (35-40 ml) was dialysed for 24 h at 4°C against two changes of 500 ml 0-05 mol/l Tris–HCl (pH 8-6). The dialysed serum was applied at 4°C to a 37 cm x 5-2 cm column of DEAE-cellulose and eluted with a concentration gradient of 0-05-0-5 mol/l Tris–HCl (pH 8-6). The volume of starting buffer was 500 ml. The flow rate was maintained at 50–55 ml/h with a peristaltic pump. Constant-volume fractions (10 ml) were collected with an LKB Ultrorac 7000 fraction collector. Protein elution was monitored by measurement of absorbance at 280 nm in 5 mm cuvettes in a Pye Unicam SP.800 spectrophotometer. Samples were diluted where necessary. The fractions were dialysed against phosphate–saline buffer (40 mmol/l KH₂PO₄/K₂HPO₄, pH 7-4, in 0-142 mol/l NaCl) and the antioxidant activity of each fraction was measured as described above.

Gel filtration. Fractions obtained after DEAE-cellulose chromatography which showed antioxidant activity were pooled and concentrated by pressure ultrafiltration to a volume of 10 ml. The concentrate was dialysed against 0-05 mol/l Tris–HCl buffer (pH 8-0) in 0-5 mol/l
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NaCl. It was then applied to a 70 cm × 5.2 cm column of Sephadex G-150 and eluted at a flow rate of 30 ml/h. The column was cooled during fractionation by pumping iced water through a water jacket. Constant-volume (10 ml) fractions were collected. The fractions were dialysed against phosphate-saline buffer. Antioxidant activity in the fractions was measured as described above.

Analysis of fractions

Gel electrophoresis and Laurell immunoelectrophoresis were used (a) to monitor the homogeneity of the fractions and (b) to identify the components of the fractions by reference to pure substances.

Electrophoresis. Polyacrylamide discs were prepared from 7.5 g/100 ml gel made from Cyanogum 41 (95% acrylamide+5% bisacrylamide). The tank buffer was 50 mmol/l Tris -39 mmol/l glycine (pH 8.6). Where necessary samples were concentrated with lyphogel and then mixed with 2 vol. of 1·1 mol/l sucrose containing 1% Bromophenol Blue. Samples (20 μl containing 20-30 μg of protein) were applied to gel tubes. Electrophoresis was carried out at 2 mA per tube until the Bromophenol Blue was eluted. Gels were stained with 0·7 g/100 ml Naphthalene Black in 1·18 mol/l acetic acid and destained for 48 h in 1·18 mol/l acetic acid. Densitometric tracings of the gels were made in a Joyce-Loebl Chromoscan (Joyce-Loeb, Gateshead, Co. Durham) with a red filter.

Immunoelectrophoresis. Two-dimensional Laurell immunoelectrophoresis was performed on concentrated fractions by the method of Clarke & Freeman (1967) on 4·5 cm × 7·5 cm plates.

Immunodiffusion. Transferrin, haptoglobin, haemopexin and caeruloplasmin in fractions were estimated by single radial immunodiffusion (Mancini, Carbonara & Heremans, 1965).

Radioactive tocopherol studies

D-α-[5-Me-3H]Tocopherol (100 μCi) in 100 μl of benzene was evaporated under a stream of nitrogen, redissolved in 200 μl of ethanol, mixed with 0·23 mmol (100 mg) of DL-α-tocopherol as a carrier and then taken orally by a human volunteer and washed down with milk. Fractionation experiments were carried out on blood samples collected after 8 h.

Tests for radioactive purity. Because of the reported instability of labelled tocopherol (MacMahon & Neale, 1970) the radioactive purity of the preparation was checked by thin-layer chromatography. α-[3H]Tocopherol (1 μCi) was dissolved in 100 μl of benzene containing 100 μg of DL-α-tocopherol and applied to a 20 cm × 20 cm plate of silica gel-G previously activated by heating for 15 min at 100°C. The plates were developed in benzene for a distance of 10 cm. The α-tocopherol region was located by spraying with Emmerie-Engel reagent (0·3 g of FeCl3,6H2O and 0·6 g of bathophenanthroline in 100 ml of acetic acid). After development the plate was dried and divided into 5 cm × 2 cm strips. Areas of silica gel were scraped off into 1 ml of toluene and 0·1 ml of this solution was taken for counting. Of the radioactivity 96% was located in the zone corresponding to α-tocopherol; the remainder was located at the origin.

The nature of the radioactivity in serum was also checked by thin-layer chromatography. Serum (1 ml) was mixed with 2 ml of ethanol and extracted twice with 4 ml of heptane. After centrifugation the upper layers were removed, pooled and evaporated to dryness in vacuo at 25°C. The residue was redissolved in 100 μl of benzene and chromatographed on plates of
silica gel-G as described above. Of the radioactivity 89.5% was located in the zone corresponding to \( \alpha \)-tocopherol; the remainder was located at the origin.

Determinations of radioactivity. Serum (100 \( \mu l \)) or protein fraction in buffer was added to 2 ml of Soluene 100 contained in a counting vial. The sample dissolved within 2 h. The solution was acidified with 8 drops of acetic acid and 15 ml of scintillation fluid containing 0.3 g of POPOP and 5 g of PPO in 1 l of toluene was added. Radioactivity was measured in a Packard Tri-Carb scintillation counter. Counting efficiency was determined by the automatic external standard method. Efficiencies were between 17 and 20%. Results are expressed as d.p.m./ml.

Other measurements

\( \beta \)-Lipoprotein was precipitated from serum with dextran sulphate and CaCl\(_2\) (Burstein, Scholnick & Morfin, 1970). Dextran sulphate solution (5 g/100 ml), 0.275 ml, and 0.5 ml of 1 mol/l CaCl\(_2\) were added to 5 ml of serum. After standing for 1 h at 4°C the serum was centrifuged at 500 \( g \) for 20 min and the supernatant removed and dialysed against 0.15 mol/l NaCl for 24 h. Serum tocopherols were measured by the method of Hashim & Schuttringer (1966).

RESULTS

General characteristics of serum antioxidant activity

Preliminary experiments showed that serum antioxidant activity depended on proteins or protein-bound substances. Dialysis of serum against several changes of phosphate-saline buffer or against 0.05 mol/l Tris–HCl (pH 8.0) for up to 24 h at 4°C did not result in any loss of antioxidant activity. Protein-free ultrafiltrates obtained after pressure ultrafiltration of serum had no detectable antioxidant activity. Serum antioxidant activity was partially abolished by heating at 60°C for 15 min and virtually abolished by heating at 90°C (Table 1).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Antioxidant activity (% inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>57</td>
</tr>
<tr>
<td>Dialysed against:</td>
<td></td>
</tr>
<tr>
<td>Tris–HCl buffer, pH 8.0</td>
<td>56</td>
</tr>
<tr>
<td>NaCl–phosphate buffer, pH 7.4</td>
<td>56</td>
</tr>
<tr>
<td>Heated for 15 min at 60°C</td>
<td>31</td>
</tr>
<tr>
<td>Heated for 15 min at 90°C</td>
<td>6</td>
</tr>
<tr>
<td>Ultrafiltration</td>
<td>0</td>
</tr>
</tbody>
</table>
Fractionation of serum antioxidant activity

Fig. 1 shows the elution pattern of serum antioxidant activity after chromatography of serum on a DEAE-cellulose column. Two peaks of antioxidant activity were consistently

![Absorbance at 280 nm](image)

![Antioxidant activity](image)

![Transferrin and Caeruloplasmin](image)

**Fig. 1.** Separation of antioxidant components in serum after DEAE-cellulose chromatography in relation to tocopherol, transferrin and caeruloplasmin. Dialysed serum containing [5-Me-3H] tocopherol was applied to a 40 cm x 5.2 cm column of DEAE-cellulose and eluted with a concentration gradient of 0.05-0.5 mol/l Tris-HCl buffer, pH 8.0. Alternate fractions were pooled and antioxidant activity (■), radioactivity (○), transferrin (△), caeruloplasmin (□) and absorbance at 280 nm (——) measured as described in the text.

found in each of six experiments. Examination of proteins in pooled fractions from the first peak (A) by polyacrylamide electrophoresis and Laurell immunoelectrophoresis showed that the major protein component was transferrin (Fig. 2a). In addition there were two other minor
components. The transferrin concentration and antioxidant activity in individual fractions conformed closely to the same pattern (Fig. 1). To purify further the antioxidant activity of peak A concentrated pooled fractions corresponding to this peak were applied to a column

![Graph of Fig. 2](image)

**Fig. 2.** Densitometric tracing of polyacrylamide-gel electrophoresis of antioxidant-containing fractions isolated after DEAE-cellulose chromatography (peaks A and B, Fig. 1) and gel filtration. Electrophoresis was performed as described in the text. (a) First peak (A) eluted after DEAE-cellulose chromatography. (b) Fraction obtained after gel filtration of peak (A). (c) Second peak (B) obtained after DEAE-cellulose chromatography. (d) Fractions obtained after gel filtration of peak B. The absolute mobilities of the pure proteins studied were 2.53 cm for transferrin and 2.34 cm for caeruloplasmin (relative to albumin these were 0.56 and 0.51 respectively).

of G-150. The antioxidant activity was eluted in fractions corresponding to transferrin (Fig. 3a), which was homogeneous on examination by polyacrylamide electrophoresis (Fig. 2b) and by Laurell immunoelectrophoresis.

The second peak (B) of antioxidant activity shown in Fig. 2 contained albumin, caeruloplasmin and four other components (Fig. 2c). Peak B eluted on DEAE-cellulose after the albumin peak and had the same elution pattern as caeruloplasmin. However, gel filtration of pooled
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fractions of this peak did not completely identify antioxidant activity as the function of a single protein (mainly because of the relatively large quantity of albumin present) (Fig. 3b).

![Graph](image)

**Fig. 3.** Gel filtration of antioxidant fractions separated after DEAE-cellulose chromatography. Fractions from each of the two antioxidant-containing peaks (A and B in Fig. 1) obtained from DEAE-cellulose chromatography of serum were pooled, concentrated and applied to a 70 cm x 2.6 cm column of Sephadex G-150 and eluted at a flow rate of 30 ml/h. (a) Elution of antioxidant fractions from the first peak (A). (b) Elution of antioxidant fractions from the second peak (B). Antioxidant activity (●—●) and u.v. absorbance (——) were measured as described in the text.

**TABLE 2. Antioxidant activity and tocopherol in normal and β-lipoprotein-depleted serum**

Tocopherol and antioxidant activity were measured in serum before and after precipitation of lipoprotein with dextran sulphate. Experimental details are given in the text. Results are averages ± SD of four experiments.

<table>
<thead>
<tr>
<th></th>
<th>Tocopherol (µmol/100 ml)</th>
<th>Antioxidant activity (% inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal serum</td>
<td>2.87 ± 0.16</td>
<td>62 ± 6.0</td>
</tr>
<tr>
<td>β-Lipoprotein-depleted serum</td>
<td>0.39 ± 0.11</td>
<td>56 ± 5.4</td>
</tr>
</tbody>
</table>

**Relation of serum antioxidant activity to tocopherol**

Tocopherol in serum is bound almost exclusively to β-lipoprotein and can be removed by selective precipitation with dextran sulphate (Davis, Kelleher & Losowsky, 1969). Prepar-
ations depleted of \( \beta \)-lipoprotein containing less than 10\% of the original tocopherol showed no significant decline in antioxidant activity compared with the original serum (Table 2). The relation between tocopherol and antioxidant activity was investigated by chromatography of serum containing \( \alpha \)-\( \alpha \)-[5-Me-\( 3^H \)]tocopherol. The elution pattern of tocopherol did not

**TABLE 3. Antioxidant effect of \( \alpha \)-tocopherol**

\( \alpha \)-Tocopherol was prepared as an emulsion with Tween 20 and antioxidant activity measured as described in the text.

<table>
<thead>
<tr>
<th>Concentration (( \mu \text{mol/100 ml} ))</th>
<th>Antioxidant activity (% inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.3</td>
<td>0</td>
</tr>
<tr>
<td>11.6</td>
<td>2.3</td>
</tr>
<tr>
<td>23.2</td>
<td>9.8</td>
</tr>
<tr>
<td>69.6</td>
<td>29.4</td>
</tr>
<tr>
<td>116</td>
<td>63.3</td>
</tr>
</tbody>
</table>

**Fig. 4.** Loss of inhibitory activity of transferrin after addition of iron. Various amounts of Fe(NO\(_3\))\(_3\) were added to the preparation of iron-free human transferrin and the inhibition was measured as described in the text. Calculations of molar ratios were made on the basis of a molecular weight of 77 000 (Charlwood, 1971).

correspond to either of the two antioxidant peaks (Fig. 1). It eluted in fractions before the first antioxidant peak, corresponding to the reported elution pattern of \( \beta \)-lipoprotein (Peters & Chiauzzi, 1965). Of the radioactivity applied to the column 75\% was recovered.

Emulsions of pure \( \text{D}L\)-\( \alpha \)-tocopherol produced the same degree of inhibition as normal serum only at a concentration of 116 \( \mu \text{mol/100 ml} \) (Table 3), i.e. at approximately 50 times the
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**Inhibitory effect of transferrin and chelating agents**

Pure iron-free human transferrin had a marked inhibitory action on lipid peroxidation in the incubated brain homogenate. The inhibitory effect was progressively reversed by the addition of Fe³⁺ and complete reversal was obtained after the addition of 2.1 mol of Fe³⁺/mol of transferrin (Fig. 4).

<table>
<thead>
<tr>
<th>Chelating reagent</th>
<th>10⁻⁵ × Final concn. (mol/l)</th>
<th>MDA formation (nmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>8.4</td>
</tr>
<tr>
<td>8-Hydroxyquinoline</td>
<td>5</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>12.2</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>12.4</td>
</tr>
<tr>
<td>2,2'-Bipyridyl</td>
<td>5</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>11.0</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>11.6</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>12.8</td>
</tr>
</tbody>
</table>

The chelating agents hydroxyquinolone, EDTA and 2,2'-bipyridyl were as effective as tocopherol in inhibiting lipid autoxidation in the brain homogenate. Complete inhibition by each agent was obtained at a final concentration of 5 × 10⁻⁵ mol/l (Table 4).

**Relation of antioxidant activity to haemopexin and haptoglobin**

Both haem and haemoglobin are effective catalysts of lipid autoxidation of pure fatty acids (Tappel, 1954). Neither of the serum proteins with especial affinity for these, haemopexin and haptoglobin, was eluted in the antioxidant-containing fractions (peaks A and B) obtained after DEAE-cellulose chromatography of serum.

**DISCUSSION**

Protection against the non-enzymic oxidation (autoxidation) of polyunsaturated fatty acids is an essential function of all animal tissues (Dormandy, 1969), and the powerful antioxidant effect of plasma is well recognized (Barber, 1962; Vidlakova, Erazimova, Horki & Placer, 1972; Slater, 1972). The antioxidant property of vitamin E has led to the assumption that it may be the principal protective agent in biological systems, and this may be so in some animal
species. The experiments described in the present paper provide strong evidence, however, that its contribution to the antioxidant behaviour of normal adult human serum is negligible. When tested against brain homogenate whole serum is approximately 50 times more powerful as an autoxidation inhibitor than could be accounted for by its tocopherol content. Moreover, the differential precipitation of the tocopherol-carrying β-lipoprotein fraction has virtually no effect on its antioxidant potency. These findings do not, of course, exclude the possibility that when administered in pharmacological doses the vitamin can significantly enhance serum antioxidant activity (Abrams, Gutteridge, Stocks, Friedman & Dormandy, 1973; Barnes, Gutteridge, Stocks & Dormandy, 1973). There is also circumstantial but strong evidence that the role of the vitamin may be critical in the perinatal period (Oski & Barness, 1967).

Macromolecules and macromolecular complexes have an undoubted though ill-understood capacity to act as free-radical stabilizers (Dormandy, 1969, 1971; Matushita, Ibuki & Aoki, 1963), and we considered the possibility at the outset of the present study that the antioxidant effect of serum might be largely the non-specific function of the serum proteins. This, too, was clearly disproved by our results. When tested against the spontaneous autoxidation of brain homogenate the antioxidant effect of serum was shown to depend on two protein fractions together representing no more than 4% of the total serum proteins. The effect was unrelated to the total protein concentration.

The antioxidant potency of the two active fractions, provisionally termed peaks A and B, can be appreciated by comparing it with the potency of tocopherol. Assuming a molecular weight of 80 000–180 000 for the proteins, their antioxidant activity on a molar basis is 200–500 times greater. The component in peak A appears to be transferrin, and both direct and indirect evidence suggests that its antioxidant activity is largely a function of its iron-binding capacity. The component in peak B has not yet been as clearly defined. However, since caeruloplasmin is one of the main proteins in this function, an analogy with transferrin would suggest that the antioxidant activity of this fraction depends on copper binding. Copper has been shown to be an effective catalyst of the autoxidation of linoleic acid (Haase & Dunkly, 1969), though its role in lipid autoxidation in tissues has been less fully explored than that of iron.

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REFERENCES


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