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o-Quinones Formed in Plant Extracts

THEIR REACTION WITH BOVINE SERUM ALBUMIN

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1. The reactions between chlorogenoquinone, the *o*-quinone formed during the oxidation of chlorogenic acid, and bovine serum albumin depend on the ratio of reactants. 2. When the serum albumin is in excess, oxygen is not absorbed and the products are colourless. This reaction probably involves the thiol group of bovine serum albumin; it does not occur with bovine serum albumin which has been treated with *p*-chloromercuribenzoate, iodoacetamide or Ellman's reagent. 3. When bovine serum albumin reacts with excess of chlorogenoquinone, oxygen is absorbed and the products are red. The red colour is probably formed by reaction of the lysine ϵ-amino groups of bovine serum albumin, as it is prevented by treating the protein with formaldehyde, succinic anhydride or *O*-methylisourea. 4. Bovine serum albumin modified by a 1·5-fold (BSA-Q) and a fivefold (BSA-Q2) excess of chlorogenoquinone were separated by chromatography on DEAE-Sephadex A-50, and some of their properties observed. 5. Reaction of BSA-Q2 with fluorodinitrobenzene suggests that the terminal α-amino group, as well as lysine ϵ-amino groups, are combined with chlorogenoquinone.

The *o*-quinones produced during the oxidation of polyphenols in leaf extracts combine with and modify the properties of a wide range of leaf proteins. As there is little detailed information on the reactions involved, the study of the reactions of chlorogenoquinone with amino acids and peptides (Pierpoint, 1969) was extended to its reaction with BSA.* (The trivial names chlorogenoquinone and caffeoquinone are suggested for the *o*-quinones produced from chlorogenic acid and caffeic acid respectively.)

BSA is assumed to have the amino acid composition reported by Spahr & Edsall (1964) and a molecular weight of 66000. Its single peptide chain contains 62 lysine residues, one *N*-terminal asparate and, at most, one free thiol group. Mixed dithiols and dimers formed by reaction of the thiol group (Andersson, 1966) are likely to contribute to the heterogeneity of most preparations. A reaction between this protein and simple *o*-quinones, especially *o*-benzoquinone, has been known for many years (e.g. Cooper, 1913) but two more recent papers are most relevant. Mason (1955) briefly discussed the likely involvement of protein amino and thiol groups, and emphasized that the product of the reaction depends on the ratio of reactants; Haider, Frederick & Flaig (1965) provided evidence that *o*-benzoquinone reacts with both the lysine ϵ-amino groups and aspartate α-amino group of albumin.

* Abbreviation: BSA, bovine serum albumin; FDNB, fluorodinitrobenzene; TBNS, 2,4,6-trinitrobenzenesulphonic acid.

EXPERIMENTAL

Materials. *O*-Methylisourea hydrochloride was prepared from cyanamide and methanol as described in Rabjohn (1963), and recrystallized from methanol. Crystalline BSA (puriss; 99%; batch nos. 35342, 35343 and 38143) was obtained from Koch–Light Laboratories Ltd., and non-crystalline preparations (fatty acid-poor; B grade) from Calbiochem Ltd. Crystalline material was used for most of the experiments reported. Non-crystalline material was used in most exploratory experiments, and no significant differences were noted between the relevant properties of the two preparations. Both contained about 15% of polymers when analysed on Sephadex G-100 and about 0·5 thiol group/molecule.

The source of chlorogenic acid and caffeic acid, and the composition of buffers, is described in the preceding paper (Pierpoint, 1969).

Reaction of chlorogenoquinone and proteins. The production of chlorogenoquinone from chlorogenic acid by an excess of *o*-diphenol oxidase (EC 1.10.3.1), and its reaction with protein, were usually performed in manometric flasks. The conditions of the reaction, the measurement of oxygen absorption and spectrophotometric examination of the products were essentially as already described (Pierpoint, 1969).

Chromatography on DEAE-Sephadex. About 5g. of

DEAE-Sephadex A-50 (total capacity 3·5m-equiv./g.) was allowed to swell overnight in sodium phosphate–potassium phosphate buffer (0·05M; pH 5·5) containing KCl (0·1M), and, after washing with more of this solution, packed into a column approx. 2·3cm. × 25–28cm. It was washed further with large quantities of phosphate buffer–KCl until equilibrated. The sample to be analysed, containing usually 40–65mg. of protein, was diluted seven- to eight-fold in phosphate buffer–KCl, its pH adjusted to 5·5, and then applied to the top of the column. The chromatogram was usually developed with 100ml. of phosphate buffer–KCl, and then with a linear KCl gradient made from 100ml. of 0·1M-KCl and 100ml. of 1·0M-KCl, both buffered at pH 5·5 with 0·05M-phosphate buffer. The flow rate was usually 30–40ml./hr. Fractions of the eluate were examined for ultraviolet absorption at 280nm., and for potassium content. The latter depends on the phosphate buffer in the eluent as well as the KCl.

As the concentration of KCl in the eluting buffer increased, the column of DEAE-Sephadex shrank greatly. To regenerate it, it was washed well in 0·05M-phosphate buffer (pH 5·5)–1·0M-KCl, removed from the column and allowed to swell in a large volume of starting buffer before the column was repacked.

A few experiments were done with DEAE-cellulose (Whatman DE 32; microgranular), which does not change volume so much as DEAE-Sephadex. They were not pursued, however, as BSA behaved quite differently on the two materials. Thus a column of DEAE-cellulose of similar exchange capacity to the DEAE-Sephadex columns, and equilibrated with 0·1M-KCl buffered with phosphate (0·05M) at pH 5·5, did not appreciably adsorb BSA (cf. Fig. 4). BSA was absorbed from phosphate buffer (0·05M; pH 5·5) not containing KCl, but its subsequent elution with a KCl gradient showed greater heterogeneity than did its elution from DEAE-Sephadex.

Chromatography on Sephadex G-100. Sephadex G-100 was equilibrated with phosphate buffer (0·05M; pH 5·5) containing 0·1M-KCl, and packed in a column 40cm. × 3·4cm. The sample to be analysed was diluted with phosphate–KCl, and 10ml., containing about 50mg. of protein, was added to the top of the column. The column was washed with phosphate–KCl at a rate of 30–36ml./hr. and fractions of the eluate were examined for u.v. absorption at 280nm. Recovery of BSA from G-100 and DEAE-Sephadex columns was usually 75–96%.

Ultracentrifugation. This was done by Dr J. Carpenter in a Spinco model E analytical centrifuge operated at 59780rev./min. and 16·5°. Schlieren patterns were obtained with the bar angle at 55° and photographed at 16min. intervals.

Electrophoresis of proteins. This was done on cellulose acetate membranes (Schleicher & Schüll) by Mr D. Wilson with a Shandon Universal Electrophoresis (Kohn, mark II) apparatus. The electrolyte was 0·02M-phosphate buffer, pH 5·5, and the current and voltage across each membrane (2·5cm. × 15cm.) was 0·75–1mA and 300–350v. Each run lasted 1–2hr. at room temperature, and the protein was located with Nigrosin and Ponceau S stains.

Amino acid composition of proteins. BSA derivatives eluted from DEAE-Sephadex were dialysed against distilled water for at least 24hr. and evaporated to dryness at 35° under reduced pressure. Samples (10–14mg.) were mixed with 6–7ml. of constant-boiling HCl in Pyrex tubes, sealed *in vacuo* (Boulter, 1966) and kept at 110° for 24hr. The hydrolysates were evaporated to dryness in a rotary evaporator, freed from excess of HCl and dissolved in 10ml. of 0·01M-HCl containing norleucine (0·1mM) as standard.

The amino acids were analysed by Miss M. Byers with a Technicon single-column automatic analyser and a standard elution gradient. When hydrolysates of guanidinated BSA were analysed, the gradient was extended with additional pH 5 buffer (citrate–HCl containing 3·5%, w/v, NaCl) to recover the homoarginine. The identity of this acid was confirmed by comparing its elution with that of an authentic specimen. Its concentration was calculated by using a norleucine equivalent of 1·07, which was also derived from this specimen.

Determination of protein amino groups. Free amino groups in BSA and quinone-treated BSA were identified and estimated with FDNB as described by Fraenkel-Conrat, Harris & Levy (1955). The proteins were caused to react with FDNB in bicarbonate solution, hydrolysed under N_2 in constant-boiling HCl, for 16hr. at 105°, and the products extracted and separated by paper chromatography. The extracts prepared from quinone-treated protein contained some dark material that did not move on the chromatograms. The only DNP-amino acid identified in the ether extract was DNP-aspartate, and ϵ-DNP-lysine was, except for a trace of *o*-DNP-tyrosine, the only one in the aqueous extract. Estimates of DNP-aspartate and ϵ-DNP-lysine were not corrected for losses occurring during hydrolysis or chromatography.

Determination of thiol groups. Thiol groups were estimated as described by Ellman (1959). To ensure maximum colour development with BSA it was necessary to increase fourfold the concentration of 5,5′-dithiobis-(2-nitrobenzoic acid), and to incubate the mixture at 30° for 30min. before measuring the extinction. *N*-Acetylcysteine was used as a standard instead of cysteine; it is more stable in alkaline solution, gives the same amount of colour, and the colour produced is stable for much longer.

Potassium. This was determined with an EEL flame photometer, with solutions of KCl (0·025–0·26mM) as standards.

Chemical modification of BSA. Formaldehyde-treated BSA was prepared by incubating a 15% solution of BSA (fatty acid-poor) with 7% formaldehyde in phosphate buffer (pH 7; 0·1M) at room temperature for 18hr. The mixture was diluted with 2vol. of water, dialysed for 30hr. in the cold and the protein concentration estimated spectrophotometrically.

Succinylated BSA was prepared by allowing 50ml. of a solution of BSA (2%, fatty acid-poor) to react with 50mg. of succinic anhydride in dioxan as described by Habeeb (1967). After dialysis, the product was concentrated under reduced pressure. From the results of Habeeb it seems likely that this procedure succinylated about 20 of the amino groups of the BSA.

TBNS was caused to react with BSA (fatty acid-poor) by incubating a 10% protein solution (1·5mM) with 2–20 times its concentration of TBNS in tris buffer (pH 7; 0·1M) at room temperature for 4hr. The pH was adjusted to 7 with NaOH from time to time, and then excess of TBNS removed by dialysis against distilled water overnight. The preparation used in the experiment described in Fig. 3 had been made with 3mM-TBNS.

BSA was guanidinated with *O*-methylisourea hydrochloride in conditions similar to those described by Hughes, Saroff & Carney (1949). The extent of the reaction was controlled by adjusting the concentration of starting materials and pH. The two preparations described in Table 4, in which approximately 38 and 85% of the lysine groups had reacted, were made from the two reaction mixtures: (1) BSA (fatty acid-poor), 20%; *O*-methylisourea hydrochloride, 0·34 M, pH 9·2; (2) BSA, 14·4%; *O*-methylisourea hydrochloride, 0·29 M, pH 10·2. The mixtures were kept at 0° for 1 and 4 days respectively with occasional adjustments of the pH to the starting values. They were then dialysed against distilled water for 24 hr., neutralized and concentrated. Their protein contents were estimated spectrophotometrically at 280 nm.

The extent to which lysine groups had reacted with *O*-methylisourea was estimated approximately by measuring the disappearance of non-protein N from samples of the reaction mixtures. The samples (0·1 ml.) were deproteinized with trichloroacetic acid (2 ml. of a 10%, w/v, solution) and the N content of the supernatant fluid was determined by a standard micro-Kjeldahl procedure. These measurements suggested that enough *O*-methylisourea had disappeared to account for the guanidination of approx. 38 and 70% of the lysine residues in the two preparations; amino acid analysis on a hydrolysed sample of the second preparation showed that 84–88% of the lysine residues had been converted into homoarginine.

RESULTS

Effect of BSA on the oxidation of chlorogenic acid. BSA has only a small effect on the rate at which *o*-diphenol oxidase oxidizes chlorogenic acid; a concentration of 134 mg./ml. slows the rate by about a third, but keeps it constant over a longer period of time, probably by preventing some of the enzyme inactivation that accompanies the oxidation.

Its effect on the complete oxidation of chlorogenic acid by an excess of enzyme depends on the molecular ratio of BSA to chlorogenic acid. As this ratio increases to 1, progressively less oxygen is absorbed, and the reaction mixture becomes less brown and more red. These red colours have a broad absorption maximum between 480 and 540 nm., and are fairly stable, fading over 24 hr. With a two- or three-fold excess of BSA, only 1 atom of oxygen is absorbed/molecule of chlorogenic acid and the products are no longer red, but almost colourless. These changes are illustrated in Fig. 1 for a sample of crystalline BSA, and were observed with BSA of different degrees of purity from several commercial sources. They are unlikely to be caused by low-molecular-weight contaminants, as they are not altered by exhaustive dialysis and do not occur with heat-denatured BSA.

The oxidation of caffeic acid is affected by BSA in the same way: red products are formed only when the molecular ratio of protein to caffeic acid is near to, or less than, 1.

Reaction between chlorogenoquinone and an excess of BSA. The effect of an excess of BSA on the oxidation of the *o*-diphenols resembled that of amino acids containing thiol groups (Pierpoint, 1969). BSA usually contains some free thiol groups (Ellman, 1959), and the sample used in the experiment of Fig. 1 contained 0·54 thiol group/molecule of protein. Attempts were therefore made to see if removing the free thiol groups would modify the reaction of protein with quinones. That it does so

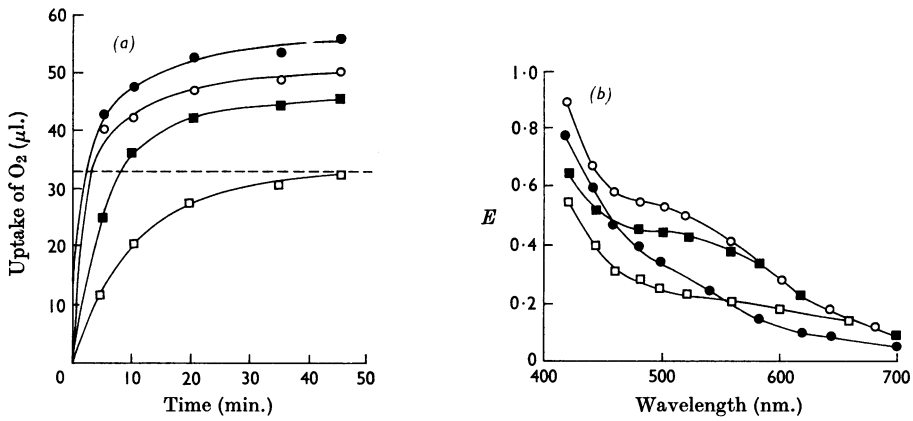

Fig. 1. Effect of BSA on the oxidation of chlorogenic acid. Chlorogenic acid (3 μmoles) was oxidized by an excess of enzyme (0·12 ml.) alone (●) and in the presence of 1·5 (○), 3·0 (■) and 7·0 (□) μmoles of BSA. The oxygen absorbed in the reaction was measured (the broken line on the graph corresponds to 1 atom absorbed/molecule of chlorogenic acid), and the spectra of the reaction mixtures were measured after these had been diluted with an equal volume of water.

Table 1. *Effect of BSA treated with* p*-chloromercuribenzoic acid on the oxidation of chlorogenic acid*

BSA (1g. in 5ml. of 0·1M-tris buffer, pH7) was incubated at room temperature for 30min. with 21mg. of *p*-chloromercuribenzoic acid, briefly centrifuged and then dialysed overnight against tris buffer (0·1M; pH7). A similar sample was put through the same process without *p*-chloromercuribenzoic acid. The two protein solutions were adjusted to the same concentration, and their effect on the oxygen absorbed and colours produced during the oxidation of chlorogenic acid (3μmoles) was measured.

Protein added	Amount added (μmoles)	Oxygen absorbed (atoms/mol. of chlorogenic acid)	Colour of final reaction medium	E_{500} of diluted final reaction medium
None	—	1·5	Brown	0·31
BSA	1·36	1·5	Pink–brown	0·45
	2·72	1·4	Red	0·47
	6·80	1·0	Pale brown	0·26
BSA treated with *p*-chloromercuribenzoic acid	1·36	1·5	Pink–brown	0·48
	2·72	1·5	Red	0·72
	4·08	1·57	Red	0·72
	6·80	1·57	Red	0·81

is shown in Table 1. BSA treated with *p*-chloromercuribenzoic acid no longer decreased the amount of oxygen absorbed, nor, when it was in excess, prevented the formation of coloured products; large amounts of it gave redder reaction mixtures, as was shown by their extinction at 500nm. Exactly the same effect was obtained with BSA treated with iodoacetamide or Ellman's reagent.

As the product of the reaction of chlorogenoquinone with excess of cysteine is an addition compound (Pierpoint, 1966), it seems likely that the reaction with an excess of BSA gives a protein–*S*-phenolic compound (BSA-S-QH_2). Another possible reaction, however, is the oxidation of protein monomers with free thiol groups to S·S-linked dimers; this occurs when cytochrome *c* is treated with some oxidizing agents (Little & O'Brien, 1967). It is unlikely to occur to a great extent in the reaction between BSA and chlorogenoquinone as it could not account for the decreased oxygen absorption. Moreover, ultracentrifugation (Fig. 2) and chromatography on Sephadex G-100 failed to detect a significant increase in the amount of dimer initially present in BSA after treatment with half its equivalent of quinone. These experiments in fact indicated (e.g. Fig. 2) that the molecular weight of the protein was not materially affected by reaction with quinone.

It was of interest to see if chlorogenoquinone reacted with ovalbumin, which has four unreactive thiol groups per molecule. However, a two- to three-fold excess of ovalbumin neither decreased the oxygen absorbed during the oxidation of chlorogenic acid nor prevented the solution browning. These thiol groups are therefore as inaccessible to the quinone as they are to Ellman's reagent or to indole-5,6-quinone (Mason & Peterson, 1965). They were not made more accessible to chlorogenoquinone by treatment with 10M-urea for 2hr. although, after this, at least one thiol group per molecule of partly soluble protein reacted with Ellman's reagent.

Reaction between BSA and an excess of chlorogenoquinone. The reaction of BSA with an excess of chlorogenoquinone does not, as judged by chromatography on Sephadex G-100, greatly alter the molecular weight of the protein. The red colour of the products, however, indicates that the reaction has involved other groups on the protein as well as the thiols. These are likely to be the free ϵ-amino groups of lysine and the α-amino group of the terminal asparate. Evidence for this was obtained by studying the way the quinone reacted with BSA in which these groups had been chemically modified.

Reaction between chlorogenoquinone and BSA with modified amino groups. Treatment of BSA with formaldehyde or succinic anhydride completely prevented it forming red products with an excess of quinone. The final colour of the reaction mixture was the brown of oxidized chlorogenic acid (e.g. Table 2). It had no trace of an absorption band near 500nm., but a slightly stronger absorption between 450 and 700nm. probably because of light scattered by the faintly opalescent solutions. However, the reaction that occurred between the thiol groups of the protein and chlorogenoquinone was not affected. Oxygen absorption was restricted by an excess of protein to the one atom involved in the formation of quinone, and pale-coloured products were formed (Table 2).

Formaldehyde does not react specifically with protein amino groups. Even in mild conditions it reacts with the imidazole and phenolic residues of serum albumin (Fraenkel-Conrat & Olcott, 1948). Succinic anhydride is probably more specific. Under the conditions used it has little or no effect

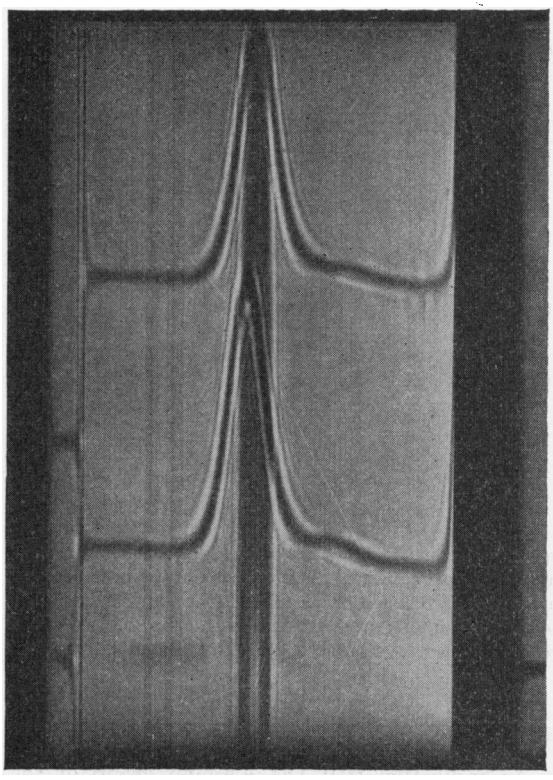

Fig. 2. Ultracentrifugation of quinone-treated albumin. Chlorogenic acid (0·75 μmole) was oxidized by *o*-diphenol oxidase (0·06 ml.) in conditions similar to those of Fig. 1, with crystalline albumin (1·5 μmoles) added either before the oxidation (lower trace) or after the oxidation was complete. Both solutions were diluted (× 8·5) to a protein concentration of 13 mg./ml., ultracentrifuged, and the above schlieren pattern was photographed after 96 min. Sedimentation was from left to right, and the sedimentation coefficient for the small (dimer) peak was 1·4 times that of the large (monomer) one.

on tyrosine groups in BSA (Habeeb, Cassidy & Singer, 1958), but it causes conformational changes in the protein that may affect the reactivity of various groups (Habeeb, 1967). BSA was therefore treated with two other reagents, TBNS and *O*-methylisourea, thought to be specific for amino groups. Although TBNS has recently been shown to react also with the thiol groups of proteins (Freedman & Radda, 1968), the urea derivative is apparently specific for the ϵ-amino groups of lysine (Chervenka & Wilcox, 1956) and was expected to indicate whether the reaction of these groups with chlorogenoquinone is chromogenic.

No red product was formed from chlorogenoquinone and BSA that had been treated with TBNS. However, the orange–yellow protein

Table 2. *Effect of succinylated BSA on the oxidation of chlorogenic acid*

BSA was caused to react with succinic anhydride so as to succinylate about a third of its amino groups. Its effect on the oxidation of 2·4 μmoles of chlorogenic acid was compared with that of equivalent amounts of untreated protein.

Protein added	Amount added (μmoles)	Oxygen absorbed (atoms/mol. of chlorogenic acid)	Final colour of reaction medium
None	—	1·72	Brown
BSA	1·25	1·38	Red
	2·5	1·38	Red
	5·2	1·00	Pale yellow
Succinylated BSA	1·25	1·75	Brown
	2·5	1·28	Pale brown
	5·2	0·95	Pale yellow

derivative clearly combines with the quinone, for it becomes noticeably paler, prevents the browning of the solution, and decreases the oxygen absorbed in the reaction (Fig. 3). The reaction resembles that of thiol groups with chlorogenoquinone. The most probable explanation is that the bound TBNS, or the -NH- linking it to the protein, combines with the quinone to give a stable addition compound that is not further oxidized. Evidence for this was obtained from the reaction of the TBNS derivative of triglycylglycine ethyl ester with caffeoquinone (Table 3). The large oxygen uptake that characterizes the reaction of the peptide (Pierpoint, 1969) is prevented when the terminal amino group is blocked by TBNS, but the brown products of the oxidation of caffeic acid do not appear and oxygen uptake is restricted to that required to convert caffeic acid into quinone. Thus TBNS derivatives of amino acids still react with quinones but in quite a different way from the amino acids. This new reaction makes it difficult to state with certainty that red products are not formed from TBNS bound to BSA because the TBNS has blocked the chromogenic groups.

Three preparations of guanidinated BSA, in which different proportions of the lysine residues had been specifically converted into homoarginine, were caused to react with chlorogenoquinone, with results summarized in Table 4. Guanidinating about a third of the lysine groups did not perceptibly alter the reactions of the protein: red products, with absorption spectra similar to those formed from untreated BSA, were formed when there was an excess of chlorogenoquinone, and when the protein was in excess the reaction seemed to involve only the protein thiol group and give paler products.

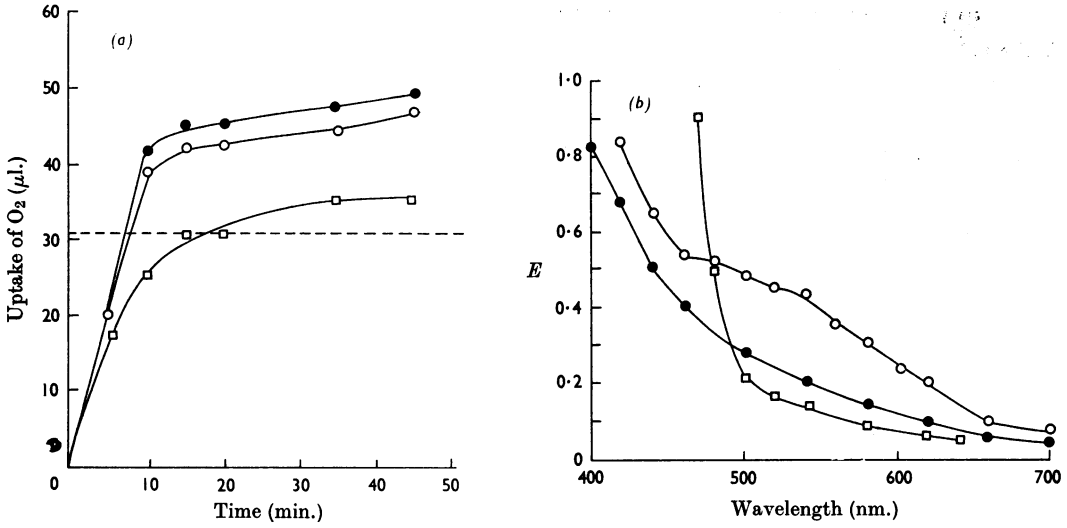

Fig. 3. Effect of BSA treated with TBNS on the oxidation of chlorogenic acid. Chlorogenic acid (3 μmoles) was oxidized by an excess of *o*-diphenol oxidase (0·08 ml.) in tris buffer (pH 7; 66 mM) alone (●) and in the presence of either 1·95 μmoles of BSA (○) or 1·95 μmoles of TBNS-treated BSA (□) prepared as described in the Experimental section. Oxygen absorbed in the reaction was measured (the broken line corresponds to 1 atom absorbed/molecule of chlorogenic acid), and the spectra of the reaction mixtures were measured after these had been diluted with an equal volume of water.

Table 3. *Effect of ethyl triglycylglycine and its TBNS derivative on the oxidation of caffeic acid*

Caffeic acid (4·5 μmoles) was oxidized by *o*-diphenol oxidase (0·1 ml.) in phosphate buffer (pH 7; 0·12 M) with the additions listed below, and oxygen absorption was measured over 2 hr. Where TBNS and ethyl triglycylglycine were present together they were preincubated for 30 min. at 30°.

Additions	Concn. (mM)	Oxygen absorbed (atoms/mol. of caffeic acid)
None	—	1·46
TBNS	16·7	1·4
Ethyl triglycylglycine	16·7	3·6
TBNS + ethyl triglycylglycine	16·7	1·1
Benzenesulphinic acid	33	1·06

More extensive guanidination modified both reactions. No red products were apparent (Table 4), which suggested that the chromogenic reaction involved the lysine ϵ-amino groups. The reaction of the protein thiol group, normally seen when excess of protein was present, was also markedly decreased. There is no evidence that thiol groups react with *O*-methylisourea; the decrease in reactivity is therefore possibly a consequence of a change in protein conformation brought about by guanidination.

An indication that the α-amino group of the terminal aspartate residue in BSA is not involved in the chromogenic reaction with chlorogenoquinone, was obtained with copper-treated BSA. This terminal group binds a single Cu^+ ion with such affinity that its reactivity is decreased (Peters, 1960; Bradshaw, Sheaver & Gurd, 1968). When BSA was treated with copper sulphate (1·3 mol./mol. of protein), and care taken to dialyse away unbound copper sulphate that otherwise affects the oxidation of chlorogenic acid, the treated protein reacted with excess of chlorogenoquinone to give red products with spectra similar to those given by untreated protein. This does not, of course, suggest that the aspartate amino group does not react with the quinone, but only that its reaction is not responsible for a significant amount of the colour produced.

Chromatographic separation of quinone-treated BSA. A quinone-modified BSA could be partially separated from the red reaction mixtures by chromatography on the anion-exchanger DEAE-Sephadex at pH 5·5, near the isoelectric point of BSA. Under these conditions BSA is not strongly absorbed, whereas the quinone derivative, which contains slightly fewer amino groups and some introduced carboxyl groups, is more strongly held. Non-ionic attractive forces between the bound phenolic groups and the dextran base of Sephadex

Table 4. *Effect of guanidinated BSA on the oxidation of chlorogenic acid*

Two samples of BSA (fatty acid-poor), in which approximately 38 and 86% of the lysine groups had been guanidinated with *O*-methylisourea, were tested for their effect on the oxidation of chlorogenic acid (3 μmoles) under the standard conditions. The colours of the final reaction mixtures were measured, after dilution with an equal volume of water, as E_{500}.

Expt. no.	Protein added	Amount (μmoles)	Oxygen absorbed (μatoms/mol. of chlorogenic acid)	Final colour of reaction medium	E_{500} of diluted final reaction medium
1	None	—	1·36	Brown	0·4
	Albumin	1·23	1·25	Red	0·56
		2·45	1·27	Red	0·56
		5·6	0·9	Pale brown	0·36
	38% guanidinated albumin	1·14	1·29	Red	0·56
		2·3	1·31	Red	0·59
		5·2	0·98	Pale red–brown	0·45
2	None	—	1·5	Brown	0·3
	Albumin	0·57	1·38	Red	0·4
		1·36	1·38	Red	0·45
		2·5	1·38	Red	0·36
		6·1	0·9	Pale brown	—
	86% guanidinated albumin	0·57	1·38	Brown	0·32
		1·36	1·38	Brown	0·32
		2·5	1·42	Brown	0·32
		6·1	1·4	Brown	—

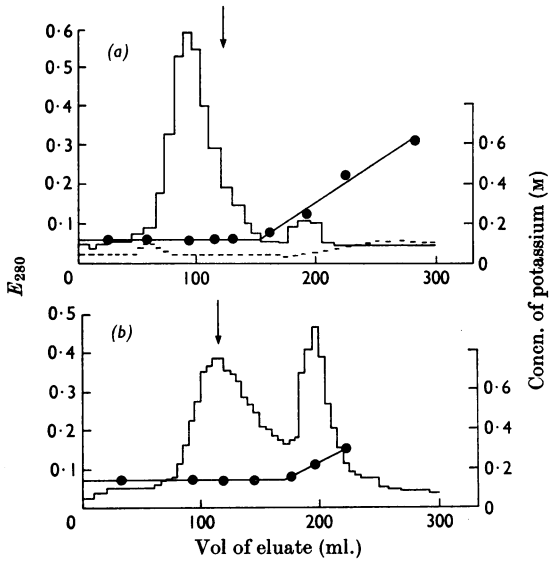

Fig. 4. Chromatographic separation of quinone-treated BSA. The BSA (2·1 μmoles) was dissolved in 3 ml. of tris buffer (66 mM; pH 7·0) containing *o*-diphenol oxidase (0·1 ml.) and, after chlorogenic acid (3 μmoles) had been added, was incubated for 45 min. at 30°. A sample (1 ml.) was diluted eightfold with phosphate buffer (0·05 M; pH 5·5) containing KCl (0·1 M), and its pH adjusted to 5·5. It was chromatographed on DEAE-Sephadex A-50. The distribution of u.v.-absorption and potassium (●) among fractions of the eluate is shown in the lower Figure, where the arrow marks the beginning of the KCl gradient. The upper figure shows the results when chlorogenic acid (——) or BSA (– – –) was omitted from the reaction mixtures.

(Woof & Pierce, 1967) may also contribute to the stronger absorption of the quinone-treated BSA.

Fig. 4 shows this separation. When untreated BSA is chromatographed on DEAE-Sephadex in 0·05 M-phosphate buffer containing 0·1 M-potassium chloride, followed by a potassium chloride gradient, the bulk of it is eluted before the gradient is applied, and only a small proportion (5–6%, occasionally 10%) appears when the concentration of potassium chloride increases. Both fractions have typical protein spectra. By contrast, the red–pink reaction mixture obtained by treating BSA (2·1 μmoles) with chlorogenoquinone (3 μmoles) contains a larger proportion (25–40%) of u.v.-absorbing material that is more strongly absorbed and eluted as a second peak. This material, referred to as BSA-Q, probably corresponds to the red–pink compounds, although much of its colour fades during the chromatography. Its spectrum has a shoulder at 320 nm. (Fig. 5), which suggested the presence of phenolic material. The spectrum of the material of the first peak is also higher at 320 nm. than is that of untreated BSA (Fig. 5), which suggested that it, too, has been modified, to a smaller extent, by the quinone. It may therefore contain some BSA-S-QH_2 as well as unmodified BSA. The 320 nm. absorption in both peaks is unlikely to be from adventitious polymerized quinone; this material is strongly absorbed by DEAE-Sephadex and only eluted in significant amounts by more concentrated potassium chloride (Fig. 4).

The BSA-Q material in the experiment shown

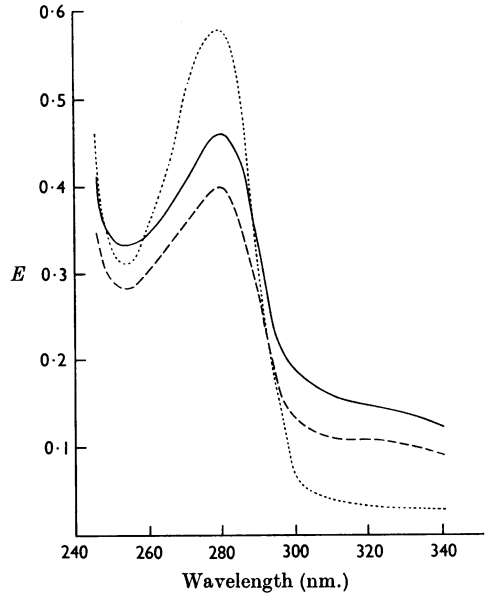

Fig. 5. Spectra of chlorogenoquinone-treated BSA. Spectra are those of three eluate fractions obtained from the experiment described in Fig. 4, taken from the first (---) and second (——) peaks of quinone-modified BSA, and the first peak of unmodified BSA (·····).

in Fig. 4 may contain only one or two groups per protein molecule modified by reaction with chlorogenoquinone. However, if all the quinone generated (3 μmoles) had reacted with the protein that appears in the BSA-Q peak (about 0·7 μmole), then each protein molecule might contain four to five modified groups, or even more if each quinone molecule reacts with more than one amino group. As quinone–amino acid bonds are more resistant than peptide bonds to hydrolysis (Ladd & Butler, 1966; Haider *et al.* 1965), evidence on the number of modified groups was sought by comparing the amino acids present in hydrolysates of the two chromatographically separated components of quinone-treated BSA. The results (Table 5) show that the amino acid compositions of the two hydrolysates resemble each other closely, and are moreover similar to those derived from purified BSA by Spahr & Edsall (1964). The largest differences occur in the contents of threonine, serine and methionine, all of which are difficult to estimate either because of instability in the conditions of the hydrolysis or because they are present in small amounts. BSA-Q hydrolysates contain slightly less of the three amino acids that are expected to react with chlorogenoquinone, but the percentage differences for lysine (2·2) and aspartate (2·8) are within the errors of the estimations. That for cystine is

Table 5. *Amino acids in hydrolysates of the two components of quinone-modified BSA*

A red solution of quinone-treated BSA was resolved chromatographically into two components similar to those shown in Fig. 4. The pooled fractions containing each component were dialysed and evaporated to dryness and, after hydrolysis, amino acids determined. The analytical results were converted into moles/mole of protein, by assuming that 1 molecule of protein contained 62 molecules of leucine (Spahr & Edsall, 1964) and that this amino acid was recovered completely from both protein components.

	Amino acid (mol./mol. of protein)		Difference	
	First peak	Second peak (BSA-Q)	Difference (mol./mol. of protein)	Difference (%)
Asp	53·3	51·8	−1	−2·8
Thr	33·6	31·5	−2	−6·3
Ser	26·2	25	−1	−4·6
Glu	82·3	80·6	−1	−2·1
Pro	27·8	27·7	0	−0·4
Gly	16·5	16·9	0	+2·4
Ala	45·9	45·6	0	−0·6
Val	35·0	35·1	0	−0·3
CyS	33·8	31·3	−3	−7·4
Met	3·6	4·1	0	+13·9
Ile	13·8	14·1	0	+2·2
Leu	62·0	62·0	0	0
Tyr	19·0	18·4	−1	−3·1
Phe	27·0	27·0	0	0
Lys	59·0	57·7	−1	−2·2
His	16·9	16·9	0	0
Arg	23·1	22·4	−1	−3

probably more significant, but the existence of disulphide-bound cysteine in BSA (Andersson, 1966), and its possible presence in the first chromatographic fraction, makes this difference difficult to interpret. These analyses therefore suggest that only a few of the amino groups of BSA-Q have reacted with chlorogenoquinone.

Separation and characterization of a BSA fraction (BSA-Q2) more extensively modified by chlorogenoquinone. A derivative of BSA (BSA-Q2) containing more bound quinone was prepared by exposing BSA to a five- or six-fold excess of chlorogenoquinone generated successively in four equal amounts. If the quinone were generated all at once, much of it polymerized rather than reacted with the protein. BSA-Q2 was also purified chromatographically and some of its properties were observed.

A typical preparation of BSA-Q2 and its separation are shown in Fig. 6. After the four treatments with chlorogenic acid, the preparation is a dark red–brown. Much of the brown polymeric material is strongly absorbed on to the top layers of the DEAE-Sephadex column, whereas the red protein

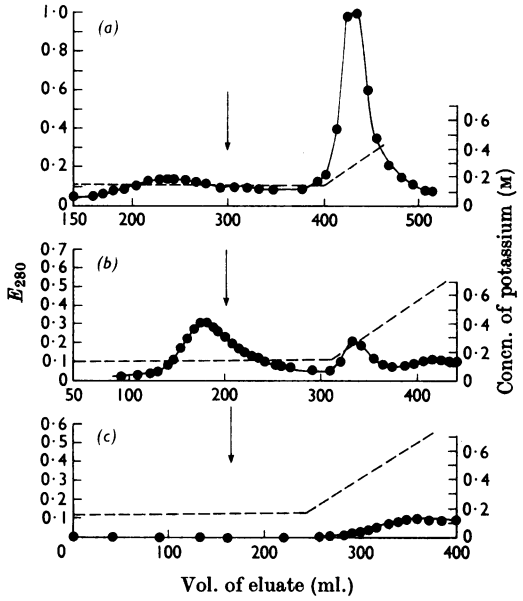

Fig. 6. Chromatographic separation of chlorogenoquinone-treated BSA (BSA-Q2). Crystalline BSA (2·3 μmoles) was dissolved in 3·1 ml. of 33 mM-tris (pH 7) containing o-diphenol oxidase (0·3 ml.), chlorogenic acid was added (3 μmoles in 0·1 ml.) and the mixture incubated for 30 min. at 30°. Three further lots of chlorogenic acid were added, each followed by an incubation. The pH of the mixture was adjusted to 5·5, and 1 ml., diluted sevenfold with 0·05 M-phosphate buffer (pH 5·5) containing KCl (0·1 M), was chromatographed on DEAE-Sephadex A-50 with more of this buffered KCl. A KCl gradient was applied to the column at the time indicated by an arrow (upper trace). For comparison, the same reaction mixture from which BSA had been omitted was chromatographed (lower trace), as well as the reaction mixture to which the BSA had been added after the final incubation (middle trace).

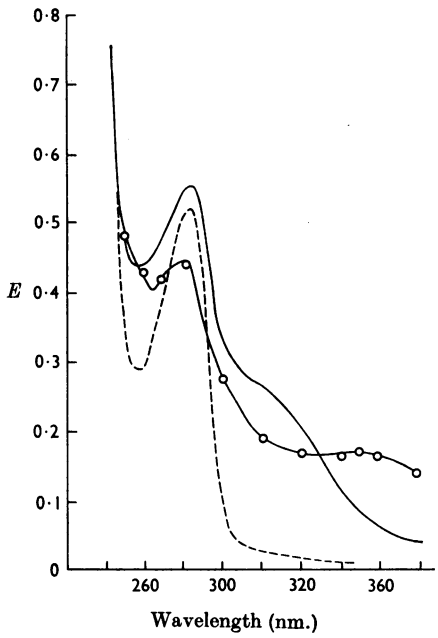

Fig. 7. Spectrum of chlorogenoquinone-treated BSA (BSA-Q2). Chromatographic fractions containing the BSA-Q2 peak were pooled and dialysed before the spectrum was measured (——). A similar preparation in phosphate buffer was made alkaline (pH > 10) with NaOH (○). The broken line shows the spectrum of untreated serum albumin (0·5 mg./ml.).

derivative moves very slowly down the column, losing much of its colour as it moves. Most (60–80%) of it is eluted as a sharp peak (BSA-Q2) when the potassium concentration is 0·2 M; only a minor fraction is eluted in the position of the major BSA component before the gradient is applied to the column. Fig. 6 also shows, for comparison, that polymerized quinone is slowly eluted from the DEAE-Sephadex, whereas BSA added to previously oxidized chlorogenic acid mainly emerges before the gradient is applied, and only a small proportion (10–20%), contaminated with quinone polymer, emerges in a second peak.

The first minor peak of protein derived from the BSA-Q2 preparation is not all unchanged BSA; its absorption spectrum has a hump near 320 nm. indicating some phenolic component. However, attention has been given to the second major peak of material, in recognition that it may be contaminated with a small proportion of the minor BSA component or with polymerized quinone, and that it is unlikely to be a single molecular species. Any of these considerations may account for the spectral differences between separate fractions of this peak; the absorption at 320 nm. increased from 44% of the absorption at 280 nm. in the first fractions to 55% in the later ones. Fig. 7 shows a typical spectrum of the pooled BSA-Q2 peak, and shows that the shoulder at 320 nm. is, like the absorption of other chlorogenic acid derivatives, shifted to longer wavelengths by alkali.

On ultracentrifugation, BSA-Q2 has the same sedimentation constant as untreated BSA, although the main component is rather more broadly spread. Like BSA it contains a small proportion of a second component that sediments about 1·4 times as fast, which suggests that it is a dimer or its derivative. However, BSA-Q2 can be distinguished from BSA by electrophoresis on cellulose acetate membranes. At pH 5·5 BSA moves slowly towards the anode whereas BSA-Q2 travels, as a single band, 50–60%

Table 6. *Free amino groups in BSA and quinone-treated BSA (BSA-Q2)*

Crystalline BSA (1·5 μmoles) was dissolved in 2 ml. of tris buffer (pH 7; 34 mM) containing *o*-diphenol oxidase (0·2 ml.). Chlorogenic acid (8 μmoles) was added in four equal lots and the mixture incubated at 30° for 30 min. after each addition. The mixture was diluted with 20 ml. of phosphate buffer (0·05 M; pH 5·5) containing 0·1 M-KCl, divided into two lots and each was chromatographed on a DEAE-Sephadex A-50 column as described in Fig. 6. The well-defined BSA-Q2 peaks from each column were pooled, dialysed overnight and evaporated to dryness under reduced pressure. The resulting quinone-treated protein (88 mg.) was treated with FDNB, as was a control sample (50 mg.) of untreated BSA, and after hydrolysis the DNP-aspartate and ε-DNP-lysine were estimated. No corrections were made for losses during hydrolysis or chromatography.

Protein	Amount treated with FDNB (μmoles)	DNP-aspartate recovered		ε-DNP-lysine recovered	
		(μmole)	(μmole/μmole of protein)	(μmoles)	(μmoles/μmole of protein)
BSA	0·75	0·31	0·42	27	36
Chlorogenoquinone-treated BSA	1·3	0·14	0·11	14·2	11

faster in the same direction. The attachment of residues containing carboxyl groups to some of the amino groups of BSA would be expected to have this effect.

The free amino groups of BSA-Q2 and of untreated BSA were estimated by causing their reaction with FDNB and measuring the DNP-aspartate and ε-DNP-lysine that could be recovered after hydrolysis (Fraenkel-Conrat *et al.* 1955). The results (Table 6) must be interpreted cautiously because of the large losses that occur during hydrolysis and chromatographic separation of DNP-amino acids, and which are assumed to be the same for BSA and BSA-Q2. They show that only a quarter as much DNP-aspartate and a third as much ε-DNP-lysine can be recovered from BSA-Q2 as from BSA. This suggests that chlorogenoquinone reacts with both the α- and ε-amino groups of albumin, approximately to the same extent, making them unavailable to FDNB. Haider *et al.* (1965) suggested that the α-amino group of BSA is more reactive to *o*-benzoquinone and *p*-benzoquinone than are the ε-amino groups.

The recovery of ε-DNP-lysine from BSA-Q2 is poor (Table 6 of Haider *et al.* 1965); apparently 25 lysine residues per molecule will not, because of quinone treatment, react with FDNB. However, if all the chlorogenoquinone (8 μmoles) involved in the preparation of BSA-Q2 had reacted with that amount of protein (about 0·75 μmole) chromatographically isolated as BSA-Q2, only about 11 of its lysine residues might be expected to have reacted with quinone. A similar effect is seen in the data of Haider *et al.* (1965; Table 7); treating BSA with a ten-fold molar excess of *o*-benzoquinone decreases the amount of ε-amino group that reacts with FDNB, not by the expected one-sixth, but by more than three times this amount. Possible explanations for this apparent unavailability of ε-amino groups to FDNB are that each quinone molecule reacts with more than one amino group, or that it is a consequence of conformational changes induced by reaction with the quinone.

DISCUSSION

The reaction between chlorogenoquinone and an excess of albumin involves the thiol groups of the protein, does not result in oxygen absorption and gives colourless products. It resembles the reaction of chlorogenoquinone with other thiol compounds and can therefore be written:

$$\text{BSA-SH} + \text{Q} \rightarrow \text{BSA-S-QH}_2 \qquad (1)$$

This reaction may also occur when there is an excess of quinone, but here it is accompanied by the reaction of some of the amino groups of the protein:

$$\text{BSA-NH}_2 + \text{Q} \rightarrow \text{BSA-NH-QH}_2 \qquad (2)$$

Both these phenol–protein compounds may be further oxidized to coloured quinone derivatives by an excess of chlorogenoquinone (Mason, 1955);

$$\text{BSA-S-QH}_2 + \text{Q} \rightarrow \text{BSA-S-Q} + \text{QH}_2 \qquad (3)$$

$$\text{BSA-NH-QH}_2 + \text{Q} \rightarrow \text{BSA-NH-Q} + \text{QH}_2 \qquad (4)$$

where, for convenience, the reactions are written as if the modified thiol and amino groups are on different protein molecules. If these reactions went to completion, 2 atoms of oxygen would be absorbed for every molecule of chlorogenic acid oxidized in the presence of albumin. However, albumin did not increase the oxygen absorbed in the oxidation. It seems reasonable to conclude therefore that reactions (3) and (4) do not go to completion, and that the red protein derivatives contain phenolic in addition to quinone residues. These may well account for the chlorogenic acid-like spectral shift

that occurs when the BSA-Q2 preparation is made alkaline (Fig. 7).

It is possible that most of the phenolic groups in the protein are BSA-S-QH_2 and that reaction (3), in contrast with reaction (4), does not occur to an appreciable extent. This is suggested by the reaction of chlorogenoquinone with succinic anhydride-treated protein. Although only the thiol group of the modified albumin appears to react with the quinone (Table 2), the colours formed when quinone is in excess are not distinguishable from those formed from chlorogenoquinone alone. But even if this conclusion is wrong, and BSA-S-QH_2 is oxidized by excess of quinone, it seems certain that this oxidation is not the cause of red colours of quinone-treated BSA.

The most likely origin of the red colours, suggested by the experiments with guanidinated and copper-treated BSA, is the oxidation of lysine-bound QH_2 groups. However, no stable red colours were observed during the reaction between chlorogenoquinone and either lysine or α-*N*-acetyl-lysine (Pierpoint, 1969). Other lysine derivatives were therefore caused to react with chlorogenoquinone but with similar results: although, for instance, red colours are initially formed from a sample of poly-L-lysine whose molecular weight was of the same order as that of BSA, they faded to brown within 2 or 3min. This does not necessarily throw doubt on the relevance of lysine-bound quinone to the colour of the quinone-treated BSA: it more likely indicates the stability of lysine–quinone in BSA compared with the same structure in smaller compounds and in poly-L-lysine. Presumably the occurrence of secondary reactions, such as poly-substitution of a quinone nucleus by adjacent amino groups or condensations of adjacent quinone nuclei, are restricted by the spatial organization of the protein molecule.

However, some secondary reactions in the modified protein are likely to occur. In the preparation of BSA-Q2 they may have occurred during the successive additions of chlorogenoquinone, and almost certainly during chromatography when the colour fades from red to pink–brown. It is tempting to use the small recovery of ϵ-DNP-lysine from FDNB-treated BSA-Q2 as evidence that more than one lysine ϵ-amino group has been substituted into each quinone nucleus. However, as was pointed out, the values in this experiment must be used cautiously, and they are also open to an alternative explanation; conformational changes in the BSA, caused by reaction of the quinone, may have made the ϵ-amino groups less accessible to FDNB.

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