

THE EFFECT OF ULTRASONIC IRRADIATION ON PROTEINS

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Abstract

Myoglobin, apomyoglobin, *S*-carboxymethyl bovine serum albumin, soluble *S*-carboxymethyl high-sulphur (SCMKB) and low-sulphur (SCMKA) proteins from wool, and also insoluble wool fibres have been subjected to ultrasonic irradiation in formic acid and the modified proteins studied by gel-permeation chromatography and other methods. The amount of breakdown decreases with decrease of the intensity of transient cavitation, but still occurs to a small extent under conditions of stable cavitation. The breakdown in 6M guanidine hydrochloride is greater than in formic acid. Results vary greatly from one protein to another, although all are free of disulphide bonds and likely to be random coils in formic acid. Thus, myoglobin aggregates whilst apomyoglobin is split into half. In contrast to results of other workers with different macromolecules, there is no evidence for the occurrence of a limiting molecular weight below which degradation does not occur. However, where degradation does occur there is clear evidence of non-random fission near the centre of the molecule, as observed by others. Some modification of insoluble keratin results from ultrasonic irradiation in formic acid.

I. INTRODUCTION

The ability of ultrasonic irradiation to cause non-random degradation of macromolecules in solution has been known for many years, but it is not yet possible to describe with any detail the mechanism of the process or to predict the outcome of irradiation of any particular macromolecule (Peacocke and Pritchard 1968). However, it is clear that irradiation of a particular intensity causes reduction in molecular weight down to a lower limit below which degradation does not occur (Sato *et al.* 1951; Mostafa 1958; Nishihara and Doty 1958; Gooberman and Lamb 1960; Peacocke and Pritchard 1968), and that there is a preference for breakage near the centre of the molecule (Sato *et al.* 1951; Nishihara and Doty 1958; Ovenall *et al.* 1958; Freifelder *et al.* 1962; Smith and Temple 1968). These principles which have been established for synthetic polymers, proteins, and nucleic acids in a variety of solvents indicate that the primary breakdown is the result of a mechanical rather than a chemical process, but chemical processes associated with cavitation (Weissler 1959; El'piner 1964) may produce secondary effects.

We have been concerned with the disruption of keratin fibres by ultrasonic irradiation in formic acid and the separation and analysis of the various histological

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components (Bradbury and Chapman 1964; Bradbury *et al.* 1965, 1970; Peters and Bradbury 1972). Checks were made to show that the amino acid analysis of the insoluble keratin is unchanged by ultrasonic irradiation in formic acid (Bradbury *et al.* 1965), but there is still the possibility of bond fission which would affect any studies on soluble keratin proteins produced from histological components (Bradbury and O'Shea 1972). We have therefore studied by gel-filtration chromatography the effect of ultrasonic irradiation in formic acid on a range of *S*-carboxymethylated (SCM) proteins. A novel feature of this study, compared with others on ultrasonic irradiation of proteins, is that here the proteins are unfolded (largely random coils) in the solvents used.

II. EXPERIMENTAL

Sperm whale myoglobin and bovine serum albumin were obtained from Mann Laboratories and pepsin from Worthington Biochemicals. Apomyoglobin was prepared by a published procedure (Harrison and Blout 1965). SCM-proteins were prepared by the method of Hirs (1967).

Soluble *S*-carboxymethyl low-sulphur (SCMKA) and *S*-carboxymethyl high-sulphur (SCMKB) proteins from wool were prepared as described previously (Bradbury and O'Shea 1972). Formic acid (reagent grade) was redistilled before use to remove solid residues. All other materials used were of reagent grade purity.

(a) Ultrasonic Irradiation of Solutions

A Mullard-MSE 500 W ultrasonic disintegrator was used. The ultrasonic irradiations of frequency 20 kHz were transmitted from the titanium probe (ratio of end areas is 9 : 1) into the solution (50 ml) which was contained in a small round-bottomed beaker (Bradbury and Chapman 1964). The probe was positioned about 2 cm below the surface of the liquid and 1.5 cm above the bottom of the vessel. The vessel was cooled by immersion in a stirred ice-salt bath. The instrument was tuned to transient cavitation (Peacocke and Pritchard 1968) which coincided with maximum amperage at any particular power setting.

The point at which transient cavitation commenced on increase of the power (the threshold of cavitation) was determined by measuring the amount of iodine liberated from a solution of potassium iodide in formic acid after irradiation for 30 min. The absorbance at 480 nm of the treated solution (Liu 1965) was compared with that of a control (since some air oxidation of iodide occurs in the acid media). It was found that the threshold of cavitation corresponded to a power reading of 2 on the meter of the instrument and that above this value there was a linear relation between absorbance at 480 nm and the power setting up to the maximum value of 8.

In irradiation of protein solutions 0.08–0.10 g of protein was dissolved in 50 ml of liquid (formic acid which was outgassed if necessary by boiling and cooling under an atmosphere of nitrogen, or 6M guanidine hydrochloride) and the ultrasonic irradiation carried out as already described, with passage of nitrogen gas over the surface of the liquid, if necessary. After treatment at the requisite power setting for a definite time (usually 2 hr) the solution was centrifuged at 4000 *g* at 4°C for 1 hr to remove titanium chips from the probe. Solutions of formic acid were lyophilized and those containing 6M guanidine hydrochloride were concentrated to small volume by passage of solvent through a microporous membrane (No. UM05) in a Diaflo cell.

(b) Gel Filtration in 8M Urea

About 60 mg of protein was chromatographed on a column of Agarose (Biogel A) at 4°C and pH 8.0 in 8M urea under the conditions described in detail elsewhere (Bradbury and O'Shea 1972). The column was calibrated for determination of the molecular weight of proteins free of disulphide bonds and in an unfolded state by determination of the elution volumes of SCM-insulin, SCM-ribonuclease, myoglobin, SCM-pepsin, and SCM-bovine serum albumin. A linear relationship between elution volume and log(molecular weight) was found.

(c) Viscosity Measurements

The intrinsic viscosity of proteins was determined in 6M guanidine hydrochloride, by use of an Ubbelohde dilution viscometer and standard procedures (Bradbury 1970).

III. RESULTS

The results of the gel-filtration chromatograms of myoglobin, apomyoglobin, bovine serum albumin, and the two major wool protein fractions SCMKA and SCMKB are given in Figures 1–3. The rather surprising production of a substance of increased molecular weight (presumably an aggregate) of myoglobin shown in Figure 1(b), accompanied by a colour change of the solution from red to brown, is confirmed by a small increase in the intrinsic viscosity (η) measured in 6M guanidine

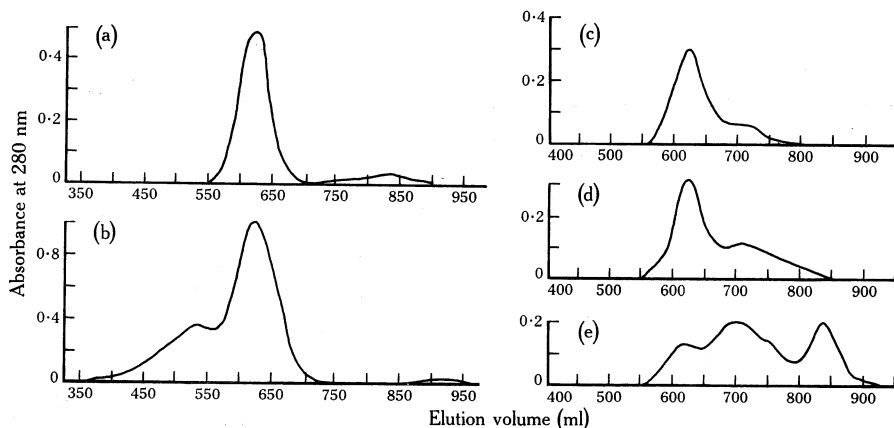


Fig. 1.—Gel-filtration chromatograms of (a) myoglobin, (b) myoglobin after ultrasonic irradiation for 2 hr in formic acid in an atmosphere of nitrogen at a power setting of 8, (c) unmodified apomyoglobin, (d), (e) apomyoglobin after irradiation for 2 hr in formic acid (d) and 6M guanidine hydrochloride (e), both at a power setting of 8.

hydrochloride from 0.161 to 0.168 dl/g, after 4 hr of ultrasonic irradiation under the stated conditions. The breakdown of apomyoglobin by ultrasonic irradiation in formic acid, shown in Figure 1(d) by the increase in the peak at 710 ml, was too small to be observed by the less sensitive viscosity measurements; η was unchanged. However, the large amount of degradation in 6M guanidine hydrochloride [Fig. 1(e)] was matched by a decrease in η from 0.157 to 0.140.

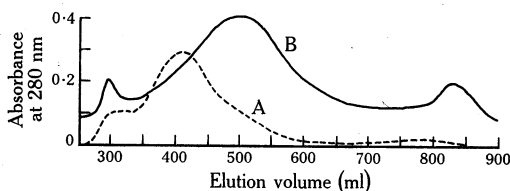


Fig. 2.—Gel-filtration pattern of SCM-bovine serum albumin before treatment (curve A) and after irradiation in formic acid for 2 hr at a power setting of 4 (curve B).

As shown in Figure 3(b) the change in the gel-filtration chromatogram of SCMKB due to ultrasonic treatment appears quite small, although there are decreases in the amount of material at elution volumes 300–460 ml and 650–750 ml. Ion-exchange chromatography using a long column of DEAE-cellulose and the procedure of

Darskus *et al.* (1969) showed that the treatment produced only small changes in the pattern. The *N*-terminal amino acids of both samples were kindly determined by Dr. L. S. Swart by the dinitrophenyl method of Sanger using a Technicon DNP-autoanalyser (Kesner *et al.* 1963). The untreated sample of SCMKB contained six *N*-terminal residues totalling 10 μ moles per gram and the ultrasonically treated sample 11 *N*-terminal residues [the seven which normally occur in keratin samples

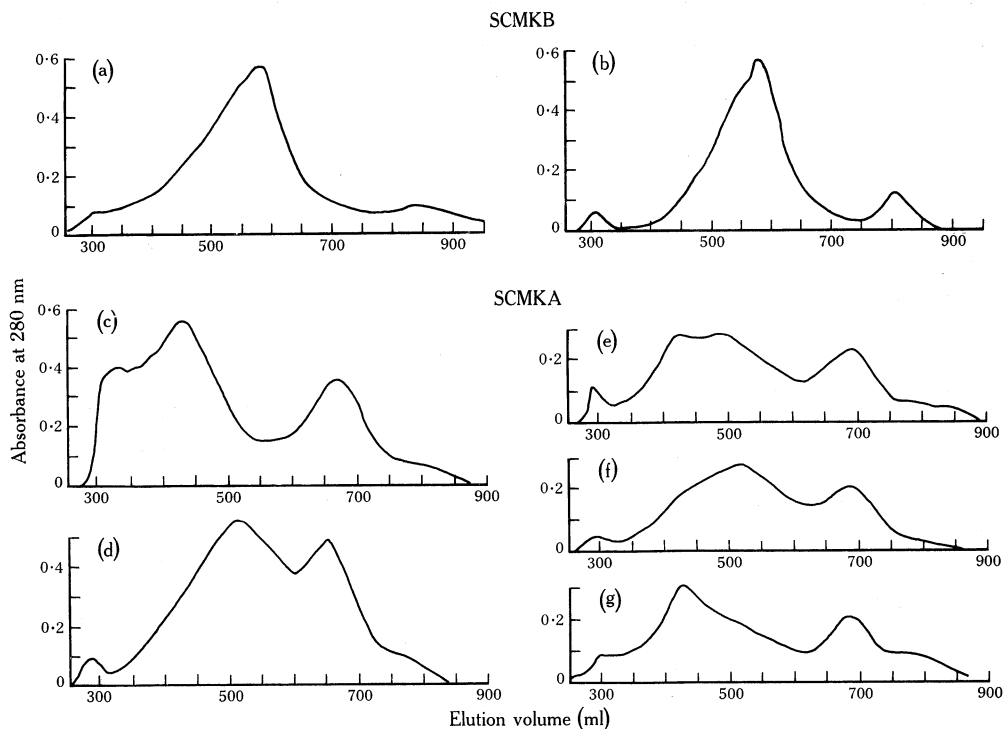


Fig. 3.—Gel-filtration chromatograms of *S*-carboxymethylated (*a*, *b*) high-sulphur (SCMKB) and (*c*–*g*) low-sulphur (SCMKA) proteins from wool. (*a*, *c*) Before treatment; (*b*, *d*) after irradiation for 2 hr in formic acid at a power setting of 8; (*e*, *f*, *g*) after irradiation in formic acid for 2, 7, and 7 hr at power settings of 4, 2.3, and 1.9 respectively.

(Crewther *et al.* 1965) and cysteic acid, histidine, leucine, and phenylalanine] which amount in total to 20 μ moles per gram. Although this represents a considerable increase, yet these levels are common for keratin fibres and for soluble proteins therefrom (Crewther *et al.* 1965; Kulkarni *et al.* 1971) and still amounts to a total level of end groups which is small compared with that expected for a mixture of proteins of predominant molecular weights between 15,000 and 30,000. This is because of the presence of *N*-acetyl end groups (O'Donnell *et al.* 1962; Haylett *et al.* 1971), which may have been hydrolysed to some extent by the ultrasonic treatment with the production of new *N*-terminal end groups, without any appreciable amount of peptide-bond hydrolysis.

The gel-filtration chromatograms of SCMKA in Figures 3(c)–3(g) show evidence of very considerable reduction in molecular weight which lessens as the intensity of the treatment is reduced. However, even the mildest treatment [Fig. 3(g)] produces an increase in the *N*-terminal amino acids from a total of 7 μ moles per gram in untreated SCMKA (sum of cysteic acid, serine, and threonine) to a total of 16 μ moles per gram (due to the 11 amino acids described above) for the treated sample. Once again this increase can be attributed very largely to hydrolysis of *N*-acetyl groups, since Figures 3(c) and 3(g) are similar except for the removal of a large amount of aggregated material (Thompson and O'Donnell 1965; Bradbury and O'Shea 1972) (elution volume 300–400 ml, hence molecular weight > 70,000) from the latter, and the occurrence of a very small peak due to degraded material at 520 ml.

An attempt was made to determine the effect of ultrasonic irradiation in formic acid on insoluble keratin. A 1 g sample of chopped wool (Bradbury and Chapman 1964) was subjected to ultrasonic irradiation in 50 ml formic acid for 30 min at a power setting of 8 and the low- and high-sulphur proteins prepared (Bradbury and O'Shea 1972). There was 20% of high-sulphur protein (SCMKB) obtained from the control and only 14% from the treated sample and an increase in the amount of low-sulphur protein (SCMKA) from the latter. On gel filtration, the SCMKA from the treated sample showed much more aggregated material (molecular weight > 70,000) than that from virgin wool. Chromatography on DEAE-cellulose of the SCMKB from ultrasonically irradiated wool showed a similar chromatogram to that obtained from untreated wool.

IV. DISCUSSION

(a) *Intensity and Time of Irradiation*

The effect of variation of ultrasonic intensity and time of treatment is shown in Figures 3(c)–3(g) with respect to the complex protein mixture SCMKA. The changes which are noted on irradiation at high power [Fig. 3(d)] are a decrease in the region of elution volumes < 400 ml which is attributed to aggregated material (Thompson and O'Donnell 1965; Bradbury and O'Shea 1972), removal of the major peak at 440 ml (molecular weight \simeq 50,000), and its replacement by a major peak at 530 ml (molecular weight \simeq 28,000), and enhancement of the peak at 690 ml (molecular weight \simeq 10,000). As the intensity of irradiation is decreased so the amount of change is progressively decreased but even at a power setting of 1.9, which is below the threshold of transient cavitation, there is still a considerable reduction in the amount of aggregated material and a very small peak evident at 520 ml. This condition apparently corresponds with stable cavitation (Peacocke and Pritchard 1968) and degradation of DNA can still occur under these conditions (Pritchard *et al.* 1966).

(b) *Effect of Different Liquids*

The intensity of cavitation in a liquid decreased with decrease in its surface tension and is a complex function of the vapour pressure and viscosity of the liquid (Noltingk and Neppiras 1950, 1951; Hueter and Bolt 1955).

The breakdown of cellular keratin has been studied extensively in various liquids (Bradbury and Chapman 1964), but the present study has been deliberately confined to formic acid, because of its importance in the keratin field (Bradbury and Chapman 1964; Bradbury *et al.* 1965, 1970; Bradbury and O'Shea 1972; Peters and Bradbury 1972), with only one comparative study in aqueous 6M guanidine hydrochloride [Fig. 1(e)]. Since the protein molecule (apomyoglobin) is probably a random coil in both solvent systems (Bradbury and King 1969; Tanford 1970), the much greater breakdown in 6M guanidine hydrochloride is presumably due to the greater intensity of transient cavitation in that solvent.

(c) *Effect on Different Soluble Proteins*

The soluble proteins are all free of disulphide bonds and are most likely to be random coils in formic acid (Bradbury and King 1969), with the possible exception of SCMKA which may retain about one-quarter of its helical content (Harrap 1963). In spite of this apparent similarity of structure of the proteins in formic acid, yet ultrasonic irradiation produces very different effects from one to another. Thus, irradiation of myoglobin in an atmosphere of nitrogen apparently causes only aggregation of material from an original molecular weight of $\approx 17,000$ to about twice the size [$\approx 29,000$, Fig. 1(b)]. Aggregation of macromolecules during irradiation in an atmosphere of hydrogen gas has been observed (Peacocke and Pritchard 1968), but not before in an atmosphere of nitrogen. In previous studies, the less-sensitive viscosity method has been used to detect changes for proteins. On the other hand, irradiation of apomyoglobin in formic acid caused no aggregation but rather a small amount of degradation from molecular weight $\approx 17,000$ to ≈ 9000 [i.e. approximate halving of the molecule, Fig. 1(d)], which was greatly accentuated in aqueous guanidine hydrochloride. SCMKA is both disaggregated and degraded by irradiation [Figs. 3(d)–3(g)].

It is particularly interesting that aggregation occurs in the presence of the haem group in myoglobin and a small amount of degradation occurs in its absence (apomyoglobin). Apparently the haem group stabilizes the myoglobin even in the presence of denaturant, which would be expected to cause dissociation of the haem group (Acampora and Hermans 1967), but may not completely remove all structure from the protein (globin) chain (Hermans *et al.* 1969). However, the fact of this stabilization of the myoglobin structure (which actually facilitates aggregation during ultrasonic irradiation in formic acid) can only be explained on the basis of some residual interaction between the haem group and the globin chain in formic acid.

In seeking valid generalizations which would be applicable to all these proteins, we consider whether there is a lower molecular weight limit below which degradation does not occur at a particular intensity (say setting 8), as has been observed in other cases (Sato *et al.* 1951; Mostafa 1958; Nishihara and Doty 1958; Goberman and Lamb 1960; Peacocke and Pritchard 1968).

This is clearly not the case since apomyoglobin breaks down slightly to material of molecular weight ≈ 9000 in formic acid (and molecular weight ≈ 2600 as well in guanidine hydrochloride), SCM-bovine serum albumin breaks down to a major

peak at elution volume 500 ml of molecular weight $\approx 37,000$ and a considerable range of material of much lower molecular weight (Fig. 2), whilst SCMKB shows little change [Figs. 3(a), 3(b)]. A possible reason for our inability to obtain this behaviour is that although we are dealing with proteins which are largely random coils, there are still some residual non-covalent interactions and possibilities of ordered areas, e.g. SCMKA (Harrap 1963), remaining in formic acid, which precludes the obtaining of consistent behaviour. Also the variability of sequence of the various proteins [as compared with the invariance of say a polystyrene chain (Smith and Temple 1968)] would allow for great variability in the secondary effects due to irradiation, in particular chemical attack. Since 6M guanidine hydrochloride may be a better denaturant for proteins than formic acid (Bradbury and King 1969; Tanford 1970) it would be useful to repeat these studies in the former solvent, to see whether a molecular weight limit would be obtained.

A second generalization is that the degradative process is non-random and causes splitting near the centre of the macromolecule (Sato *et al.* 1951; Nishihara and Doty 1958; Ovenall *et al.* 1958; Freifelder and Davison 1962; Smith and Temple 1968).

In those cases in which degradation occurs this conclusion is, in general, correct. Thus apomyoglobin breaks down slowly from molecular weight $\approx 17,000$ to ≈ 9000 in formic acid (but in guanidine hydrochloride another major peak is observed at molecular weight ≈ 2600), SCM-bovine serum albumin breaks down from molecular weight $\approx 65,000$ to $\approx 37,000$ and some smaller units, and SCMKA from molecular weight $\approx 50,000$ to a major peak at $\approx 28,000$.

(d) *Effect on Insoluble Keratins*

It is difficult to assess the effect of ultrasonic irradiation in formic acid on insoluble cellular material such as keratin fibres and small histological components therefrom. The results obtained in this work indicate that irradiation produces a definite effect on the precipitation procedure used for the separation of SCMKA and SCMKB, since there is 6% less of the latter and more of the former produced from irradiated than from virgin fibres. However, the fractions themselves seem to be superficially similar to those from untreated wool, although more work is needed to confirm this point and also to investigate the reason for the differences.

The work on soluble SCMKA and SCMKB shows that they are degraded strongly and very slightly respectively by irradiation in formic acid at power setting 8 and therefore it is likely that some degradation will also occur to these proteins, when they are built into an insoluble heavily cross-linked structure (insoluble keratin). However, the extent of the protection afforded by such a structure is unknown and presents an interesting and unsolved problem.

V. ACKNOWLEDGMENTS

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