Biochem. J. (1973) **133**, 821–826
Printed in Great Britain

Loss of Tryptophan Associated with Photo-Polymerization and Yellowing of Proteins Exposed to Light over 300nm

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(*Received* 6 *March* 1973)

S-Carboxymethyl-lysozyme and *S*-carboxymethyl-ribonuclease A were irradiated with light of wavelength greater than 300nm. Photo-oxidative loss of tryptophan from the *S*-carboxymethyl-lysozyme was accompanied by yellowing of the protein and the formation of covalently cross-linked polymers. *S*-Carboxymethyl-ribonuclease, which contains no tryptophan, showed little yellowing and no polymer formation. The irradiated *S*-carboxymethyl-lysozyme was similar to the proteins of the brown cataractous human lens nucleus and to bovine lens proteins exposed to sunlight *in vitro* in that it (1) was insoluble in non-denaturing solvents, (2) contained a new fluorescence, (3) was brown in colour, and (4) contained covalent cross-links that are not disulphide bonds.

Pirie (1968) suggested that the dark-brown proteins of the cataractous human lens contain covalent cross-links other than disulphide bonds. This has been confirmed by Buckingham (1972). These cross-linked brown proteins are not present in the normal human lens or in many types of cataract. The insoluble brown protein of the lens nucleus has a lower tryptophan and histidine content than protein from normal post-mortem lenses; the fluorescence (emission 350nm) of tryptophan is diminished and a new fluorescence (emission 440–460nm) develops (Pirie, 1972). Buckingham & Pirie (1972) found that exposure of lens proteins to sunlight *in vitro* caused both yellowing and cross-linking.

To study the relation, if any, between photo-oxidative changes in a protein and the alterations to the proteins of the brown cataractous human lens, proteins of known structure were exposed to light over 300nm of a spectral composition similar to daylight. The proteins were first converted into their *S*-carboxymethyl derivatives to eliminate cross-link formation by disulphide interchange or from photo-oxidation products of the disulphide bond and/or thiol groups. This precaution was taken because some of the covalent cross-links of the brown cataractous proteins are not disulphides. The experiments were performed in the absence of a photosensitizer and with the proteins fully denatured to eliminate interactions resulting from the tertiary structure of the protein and consequent precipitation during irradiation. Protein unfolding may be an early change in nucleo-cortical cataract (Harding, 1972). Two proteins were chosen for investigation. Lysozyme was selected for its high tryptophan content and ribonuclease was selected because it contains no tryptophan.

Materials and Methods

Materials

Lysozyme (3×crystallized, dialysed and freeze-dried, grade 1) and ribonuclease A (5×crystallized, type 1-A) from the Sigma (London) Chemical Co., London S.W.6, U.K., were reduced and *S*-carboxymethylated by the method of Crestfield *et al.* (1963). The extent of modification was found by the determination of *S*-carboxymethylcysteine on the amino acid analyser after acid hydrolysis under vacuum. Both the modified proteins ran as single bands on polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate.

Methods

Irradiation. The *S*-carboxymethylated proteins were irradiated at a concentration of 10mg/ml in 6M-guanidinium chloride (specially purified for biochemical work, BDH Chemicals Ltd., Poole, Dorset, U.K.). The measured pH (glass electrode) of the protein solutions fell within the range 4.6–4.8 and remained constant throughout the period of irradiation. *S*-Carboxymethyl-lysozyme was also irradiated as a solid (100mg) and at a concentration of 5mg/ml in a sodium acetate–acetic acid buffer, pH5.0 (McKenzie, 1969), containing 1% sodium dodecyl sulphate. In all cases appropriate controls were stored in the dark at room temperature during the period of irradiation. Protein solutions were irradiated in a water-cooled (<20°C) magnetically stirred reaction cell exposed to the air with an MLU 300W sunlamp (Phillips, Eindhoven, The Netherlands), which emitted energy over a similar wavelength

range to sunlight (manufacturer's literature). Samples of the proteins (1 mg) were removed for analysis at various times during the irradiation.

After irradiation the proteins were recovered from solution either by precipitation with ice-cold ethanol (2×9 vol.), washing with diethyl ether and drying in air at 20°C, or by gel filtration on a column (2cm×130cm) of Sephadex G-25F equilibrated with 50% acetic acid, followed by freeze-drying.

Spectroscopy. Ultraviolet spectra were determined on a Pye Unicam SP. 8000 recording u.v. spectrophotometer with 1 cm-path-length silica cells.

Amino acid analysis. This was performed after hydrolysis of the proteins with 6M-HCl containing 1% (v/v) 0.3M-thioglycollic acid *in vacuo* for 24h at 110°C or by the method of Liu & Chang (1971), utilizing hydrolysis with toluene 4-sulphonic acid in the presence of tryptamine, which enabled all the amino acids, including tryptophan, to be determined in a single step. Hydrolysates were analysed on a Locarte amino acid analyser by using a single column for neutral, acidic and basic amino acids.

Gel chromatography. The untreated and irradiated proteins were examined by chromatography as dodecyl sulphate complexes on 6% (w/v) agarose (Bio-Gel A-5M, 200–400 mesh; Bio-Rad Laboratories Ltd., St. Albans, Herts., U.K.), as described by Reynolds & Tanford (1970), with Blue Dextran [Pharmacia (G.B.) Ltd., London W.5, U.K.] and Dnp-β-alanine to determine the column parameters. To form the complexes of some samples of the irradiated *S*-carboxymethyl-lysozyme in buffers that contained sodium dodecyl sulphate, it proved necessary to maintain the solution at 100°C for 2min, after which any insoluble material (always <5% by wt.) was removed by centrifugation and discarded. Protein in the column effluent was determined by the method of Lowry *et al.* (1951) with crystallized bovine plasma albumin (Armour Pharmaceutical Co., Eastbourne, U.K.) as standard.

Gel electrophoresis. The untreated and the irradiated proteins were examined by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate on 10% gels, as described by Weber & Osborn (1969). To determine the molecular weights of species present in the irradiated proteins a calibration curve was plotted by using lysozyme cross-linked with diethyl pyrocarbonate by the method of Wolf *et al.* (1970).

Electrophoresis and chromatography. High-voltage electrophoresis of acid hydrolysates was done at 50–70 V/cm in a cooled-plate apparatus (Camag, Muttenz, Switzerland) on Whatman 3MM chromatography paper at pH 1.9 with the volatile buffer system described by Sargent (1969). Separation in the second dimension was achieved by descending paper chromatography in BAWP solvent [butan-1-ol–acetic acid–water–pyridine (15:3:12:10, by vol.); Waley & Watson, 1953]. Amino acids were detected with ninhydrin–cadmium acetate reagent (Heilman *et al.*, 1957); tryptophan and other indole-containing compounds were detected by their fluorescence when examined under a Hanovia lamp no. 16, maximum emission 365nm, and/or their reaction with Ehrlich reagent (Smith, 1969).

Determination of N-terminal amino acids. The *N*-terminal amino acids of the untreated and irradiated proteins were determined by the dansyl chloride method of Gray (1967) and the Dns-amino acids were identified on polyamide layers by using the system of Woods & Wang (1967) with the modification described by Croft (1972).

Results

Effect of light on S-carboxymethyl-lysozyme

Spectral changes. On irradiation, the solutions of *S*-carboxymethyl-lysozyme changed from colourless to a golden brown, but the protein remained in solution. The absorption maximum of the untreated protein at 282nm due to tryptophan was replaced by a species absorbing at over 300nm and near 260nm (Fig. 1). This indicated that tryptophan was photo-oxidized during irradiation. The rate of yellowing, arbitrarily defined as the rate of change of the ratio E_{310}/E_{282}, was constant for the first 49h of exposure, after which little change was detected (Fig. 2). The irradiated solutions of *S*-carboxymethyl-lysozyme fluoresced more strongly than the control solution kept in the dark.

Changes in amino acid composition. Amino acid

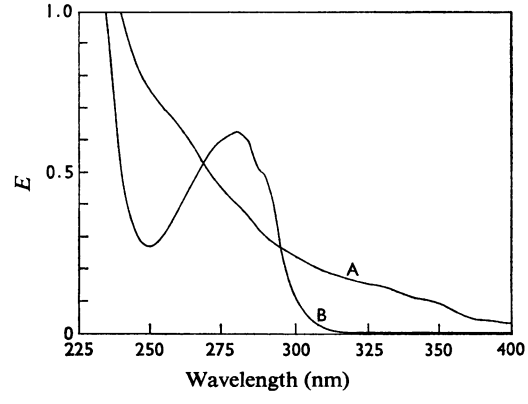

Fig. 1. *Change in the absorption spectrum of S-carboxymethyl-lysozyme after irradiation*

Absorption spectrum of 0.033% protein solutions in 6M-guanidinium chloride measured in 1 cm silica cells. Curve A, *S*-carboxymethyl-lysozyme irradiated for 72h; curve B, untreated *S*-carboxymethyl-lysozyme.

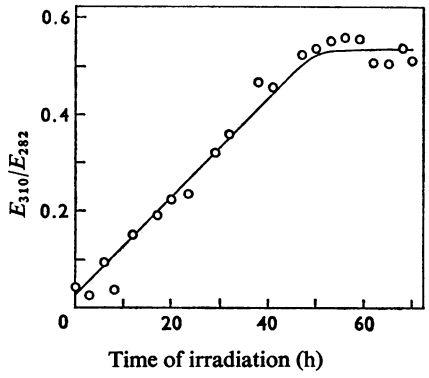

Fig. 2. *Rate of yellowing of S-carboxymethyl-lysozyme*

The experimental conditions used for the irradiation of the protein are described in detail in the text. Samples (1 mg) were taken from the reaction cell at the times indicated, diluted with 6M-guanidinium chloride to give 0.033% solutions, and the absorption spectra recorded. The ratio E_{310}/E_{282} was then calculated for each sample.

analysis of samples of protein taken after different times of irradiation and hydrolysed with toluene 4-sulphonic acid provided the following information (Table 1). (1) Tryptophan was destroyed most rapidly during the initial stages of the irradiation and after 72h exposure no tryptophan could be detected in the hydrolysed protein (Fig. 3). An additional peak appeared on the amino acid analyser trace, which eluted between phenylalanine and tryptophan in the same position as an authentic sample of kynurenine. Its identity was confirmed by the separation of an acid hydrolysate on paper in two dimensions by electrophoresis and chromatography. A spot that fluoresced blue was found, giving an orange colour with Ehrlich reagent and running in the position of kynurenine. At no stage in the irradiation was kynurenine detected in more than trace amounts. No other ninhydrin-positive or Ehrlich-positive breakdown products of tryptophan were identified. (2) *S*-Carboxymethylcysteine was progressively destroyed during irradiation (Fig. 3) and its disappearance was accompanied by the detection of cysteic acid on the amino acid analyser (Table 1). (3) Methionine, tyro-

Table 1. *Amino acid compositions of untreated and irradiated proteins*

Results are expressed as the number of residues/molecule; residues were calculated by assuming that *S*-carboxymethyl-lysozyme contains 8 leucine residues/molecule and that *S*-carboxymethyl-ribonuclease contains 2 leucine residues/molecule. Irradiation was carried out for the stated time by using experimental conditions described in the text. *S*-Carboxymethyl-lysozyme was hydrolysed for 48h with toluene 4-sulphonic acid in the presence of tryptamine by the method of Liu & Chang (1971), and *S*-carboxymethyl-ribonuclease was hydrolysed for 24h with 6M-HCl (for details see the text). No corrections were made for destruction of amino acids during hydrolysis. Abbreviations: Cys(O_3H), cysteic acid; CmCys, *S*-carboxymethylcysteine; Kyn, kynurenine.

Protein ...	*S*-Carboxymethyl-lysozyme			*S*-Carboxymethyl-ribonuclease		
Time of irradiation (h) ...	0	35	72	0	35	72
Amino acid						
Cys(O_3H)	0	Trace	0.3	0	0.6	0.9
CmCys	7.9	3.5	0	7.6	1.7	0
Asp	20.9	21.3	22.1	15.3	15.4	15.2
Thr	6.8	6.9	6.8	9.3	9.5	9.2
Ser	9.2	8.8	8.9	13.8	13.6	13.2
Glu	5.3	5.2	5.5	12.1	12.0	12.2
Pro	2.1	2.3	2.0	4.1	4.2	4.2
Gly	11.9	12.1	11.8	3.0	3.1	3.0
Ala	12.2	13.2	13.9	11.9	12.3	12.1
Val	5.8	6.0	5.4	8.6	8.7	8.9
Met	1.9	2.0	1.8	3.7	3.7	3.8
Ile	5.9	5.8	5.8	2.3	2.2	2.0
Leu	8.0	8.0	8.0	2.0	2.0	2.0
Tyr	3.0	2.9	2.8	5.8	3.5	1.8
Phe	3.0	3.1	2.9	2.9	3.0	2.0
Kyn	0	Trace	Trace	0	0	0
Trp	5.8	0.7	Trace	0	0	0
Lys	6.2	5.8	6.1	10.0	10.2	10.3
His	1.0	0.9	0.8	3.8	3.9	3.8
Arg	10.9	11.1	11.2	4.1	4.0	4.2

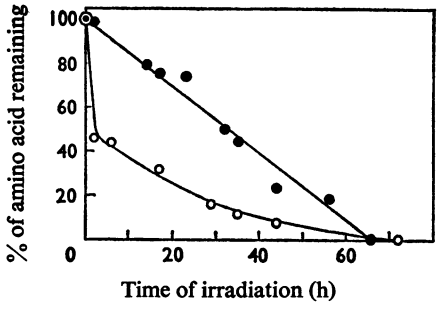

Fig. 3. *Destruction of S-carboxymethylcysteine and of tryptophan during irradiation of S-carboxymethyl-lysozyme*

The percentages of *S*-carboxymethylcysteine (●) and tryptophan (○) remaining in the irradiated *S*-carboxymethyl-lysozyme were determined by amino acid analysis of samples (1 mg) taken at the times indicated and hydrolysed with toluene-4-sulphonic acid by the method of Liu & Chang (1971).

sine and histidine were not photo-oxidized during 72h of irradiation under the acidic conditions used (Table 1). (4) The alanine content of the irradiated *S*-carboxymethyl-lysozyme was slightly greater than that of the untreated protein (Table 1).

Polymer formation during irradiation. Gel chromatography (Fig. 4) of sodium dodecyl sulphate complexes of the irradiated and untreated proteins on 6% agarose showed that high-molecular-weight species were present in the irradiated *S*-carboxymethyl-lysozyme. This material, arbitrarily defined as having a partition coefficient (K_d) of less than 0.46, comprised 51% of protein eluted from the column. Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate confirmed that irradiated protein contained molecules joined by cross-links that were stable in a highly dissociating medium. A series of bands were detected (Fig. 5) that had lower electrophoretic mobilities than the untreated *S*-carboxymethyl-lysozyme and it was assumed that these bands were covalently cross-linked polymers of *S*-carboxymethyl-lysozyme.

The molecular weights of the polymeric bands were determined from a calibration curve (Fig. 6) plotted by using polymers of lysozyme cross-linked with diethyl pyrocarbonate as protein standards (Wolf *et al.*, 1970). This procedure was necessary because of the considerable effect of covalent cross-links on the electrophoretic mobility of proteins in sodium dodecyl sulphate solution in polyacrylamide gels (Griffith, 1972). These results indicated that the polymers were integral multiples of monomeric *S*-carboxymethyl-lysozyme. The polymeric bands developed during the course of irradiation of *S*-

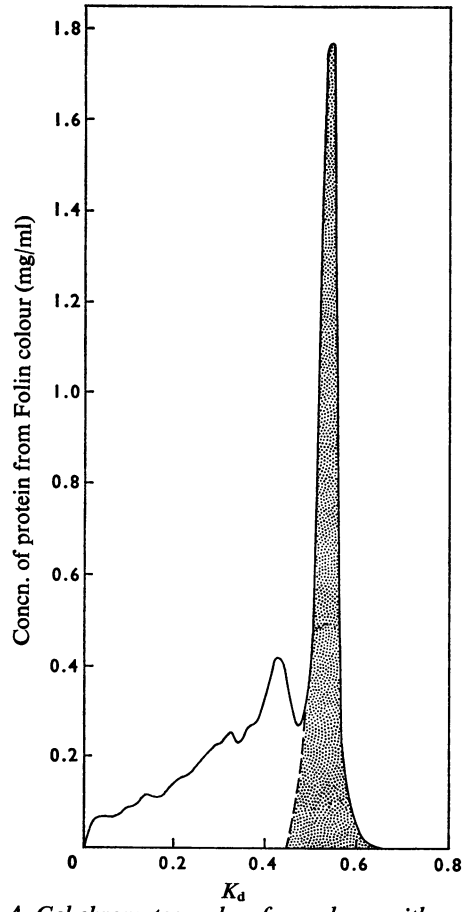

Fig. 4. *Gel chromatography of complexes with sodium dodecyl sulphate of S-carboxymethyl-lysozyme*

Protein–sodium dodecyl sulphate complexes were prepared as described in the text. Samples containing 6–10mg of protein were applied to a column (1.4cm×60cm) of 6% agarose and eluted with 0.1% sodium dodecyl sulphate, 5mM-NaH_2PO_4, 14.4mM-Na_2-PO_4, pH7.2, at 3.6ml/h. Blue Dextran ($K_d = 0$, determined at 630nm) and Dnp-β-alanine ($K_d = 1$, determined at 360nm) were used as markers to determine the K_d of the eluted protein. —— is the elution profile of *S*-carboxymethyl-lysozyme irradiated for 72h and ▩ shows the elution profile of untreated *S*-carboxymethyl-lysozyme.

carboxymethyl-lysozyme. The band corresponding to the dimer appeared first, followed by the trimer and so on. The amount of material present in each band decreased with increasing molecular weight. Different preparations of irradiated *S*-carboxymethyl-lysozyme gave similar patterns on polyacrylamide-gel electrophoresis.

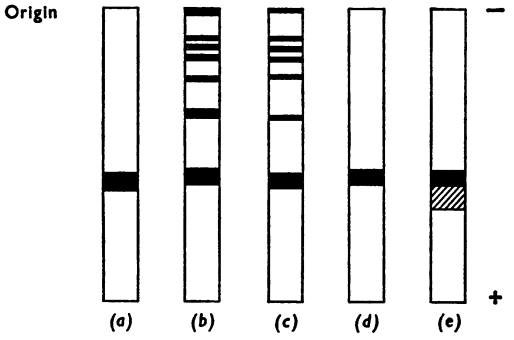

Fig. 5. *Polyacrylamide-gel electrophoresis of untreated and irradiated proteins on* 10% *gels in the presence of* 0.1% *sodium dodecyl sulphate as described by Weber & Osborn* (1969)

(*a*) Untreated *S*-carboxymethyl-lysozyme; (*b*) *S*-carboxymethyl-lysozyme irradiated for 72h; (*c*) lysozyme cross-linked with diethyl pyrocarbonate by the method of Wolf *et al.* (1970); (*d*) untreated *S*-carboxymethyl-ribonuclease; (*e*) *S*-carboxymethyl-ribonuclease irradiated for 72h.

No components of lower molecular weight than that of the untreated protein were detected after irradiation by either of the above methods and no new *N*-terminal amino acid residues were detected. Therefore the amount of chain splitting in the irradiated protein must have been small.

When polymeric irradiated *S*-carboxymethyl-lysozyme was reduced and *S*-carboxymethylated for a second time and examined by gel chromatography and polyacrylamide-gel electrophoresis, no decrease in the proportion of high-molecular-weight material in the irradiated protein was observed.

Samples of *S*-carboxymethyl-lysozyme that had been irradiated as a solid or in a solution of sodium dodecyl sulphate, sodium acetate and acetic acid, pH5.0, showed similar colour changes and formation of covalently cross-linked polymers.

Effect of light on ribonuclease

After irradiation under the same conditions as lysozyme for 72h, *S*-carboxymethyl-ribonuclease changed from colourless to a very pale yellow. Amino acid analysis (Table 1) showed that *S*-carboxymethyl-cysteine and tyrosine (see the Discussion section) were the only amino acids to be photo-oxidized.

Gel electrophoresis of the irradiated protein (Fig. 5) showed no components of higher molecular weight than the control protein, but a diffuse band of greater electrophoretic mobility indicated that chain cleavage of the protein had occurred during irradiation.

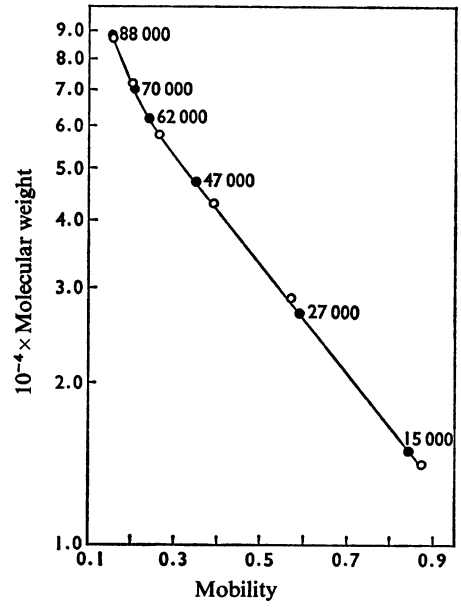

Fig. 6. *Determination of the molecular weights of species in irradiated S-carboxymethyl-lysozyme by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate on* 10% *gels as described by Weber & Osborn* (1969)

The mobilities (○; relative to Bromophenol Blue) of the polymers of lysozyme cross-linked with diethyl pyrocarbonate prepared by the method of Wolf *et al.* (1970) are plotted as a function of the log of their molecular weights. From this curve, the molecular weights of polymers (shown on the figure) from irradiated *S*-carboxymethyl-lysozyme (●) were deduced.

Discussion

The results shown in Table 1 indicate that photo-oxidation of tryptophan was the main modification in *S*-carboxymethyl-lysozyme during the early stages of irradiation. Specificity was achieved by working at pH4.6 (tyrosine and histidine are not photo-oxidized below pH6; Galiazzo *et al.*, 1972), by reduction and *S*-carboxymethylation of the proteins and the use of a powerful denaturing agent as solvent to minimize differences between residues in their susceptibility to photo-oxidation.

Traces of kynurenine and a small amount of alanine were the only possible breakdown products of tryptophan to be positively identified. Several fluorescent compounds were detected but could not be characterized. The absorption spectrum and amino acid analysis cannot show whether the initial breakdown

product of tryptophan in *S*-carboxymethyl-lysozyme was kynurenine or *N'*-formylkynurenine, which was detected by Pirie (1971) as a breakdown product of the tryptophan in lysozyme and other proteins exposed to sunlight at pH7.

Only *S*-carboxymethylcysteine and tyrosine were photo-oxidized on irradiation of *S*-carboxymethyl-ribonuclease and the modification of the tyrosine is surprising as no change was detected in the tyrosine content of the irradiated *S*-carboxymethyl-lysozyme.

The marked difference in the colour between the *S*-carboxymethylated lysozyme and ribonuclease supports the observations of Lennox & Rowlands (1969) that photo-oxidative breakdown products of tryptophan are the major cause of photo-oxidative yellowing.

No polymers were detected in irradiated *S*-carboxymethyl-ribonuclease; it therefore seems probable that photo-oxidative breakdown products of tryptophan form the covalent cross-links in irradiated *S*-carboxymethyl-lysozyme. The polymerization is probably initiated by a free-radical mechanism. The free-radical scavenger glutathione is capable of retarding the yellowing and polymerization (K. J. Dilley, unpublished results).

Since the photo-polymerization of *S*-carboxymethyl-lysozyme is not reversed by reduction, it differed from that observed by Marciani & Tolbert (1972) on γ-irradiation of unprotected lysozyme.

The action of light on *S*-carboxymethyl-lysozyme gives a product that resembles the cross-linked proteins of the brown cataractous human lens; both are insoluble in non-denaturing solvents, are dark-brown, contain a 'new' fluorescence, contain covalent cross-links that are not disulphides, and both contain traces of kynurenine, which has been detected in acid hydrolysates of brown cataractous human lens protein by Pirie (1972) and K. J. Dilley (unpublished results).

Further work is required to establish whether these similarities arise from the action of light on lens proteins *in vivo*.

I am extremely grateful to Dr. A. Pirie for helpful advice and discussion, to Dr. J. J. Harding and my other colleagues in this Laboratory for suggestions and encouragement and to Mr. A. G. Skuce and Mr. H. F. Wilcox for their technical assistance. The support of the Royal National Institute for the Blind is gratefully acknowledged.

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