Chemistry of Ultrasound. Part IV.[1] Effects of Ultrasound on Some Amino-acids

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Nine amino-acids (glycine, alanine, glutamic acid, glutamine, phenylalanine, histidine, methionine, cysteine, and cystine), together with imidazole as a control, were irradiated at 800 kHz in dilute aqueous solution. The nature of the products has been established and mechanisms accounting for their formation are suggested.

THERE is evidence that enzymes undergo extraordinary transformations when subjected to ultrasonic irradiation.[2] Reports of biological activation and deactivation of proteins and enzymes by ultrasound[3,4] prompted us to study the chemical effects of ultrasound on amino-acids. Nine amino-acids were irradiated as aqueous 0.006M-solutions at 800 kHz in an argon atmosphere for 6 h; the products were subjected to amino-acid analysis and the results compared with those from sample blanks. All the amino-acids were irradiated at their isoelectronic points, and histidine was also irradiated at pH 2 and 10. Imidazole was also irradiated, as a control, at pH 2, 7, and 10. Results, expressed as percentages of starting material, are given in Table 1.

TABLE 1

Solution products from the 6 h 800 kHz irradiation of amino acids at 21 °C in aqueous 0.006M-solution; acoustical power $k = 11.6 \times 10^{-6}$ mol l^{-1} min^{-1} of H_2O_2 at 21 °C

Amino-acid	Recovered starting material (%)	Ammonia (%)	Other amino-acids (%)	RNH_2 [a] (%)	HCHO (%)	MeOH (%)	Other products [b]	Material lost as gas or volatile [c] (%)
Gly	92	7		1	2		Glycolic acid	2
Ala	91	5		4	2	2	Lactic acid Acetaldehyde	8
Glu	89	5	0.5 [d]	5	3			10
Gln	85	5	0.5 [e]	9	2			12
Phe	58	5	1 [f] 1 [g]	35	3		Phenylacetaldehyde Phenolic compounds *p*-Benzoquinones	24
His, pH 2	63	16	3 [d]	18	(+) [h]		Polymers	
His, pH 7	51	16	1 [d]	32	(+) [h]		Polymers	6
His, pH 10	47	13	0.5 [d]	39	(+) [h]		Polymers	
Met	67	7	4 [i] 2 [k]	20	4	1		15
Cys	39	4	46 [l] 2 [m] 1 [n]	8	2			10
Cys-Cys	83	12	6 [m]		4			11

[a] The quantities expressed are the maximum values necessary to maintain nitrogen balances. [b] Spot tests were conducted for amides, nitro-compounds, nitriles, oximes, urea, and nitrous and nitric acids. All were negative. For procedures, see F. Feigl, 'Spot Tests in Organic Analysis,' Elsevier, New York, 1960. [c] Determined by lyophilization of irradiated samples. [d] Aspartic acid. [e] Glutamic acid. [f] Tyrosine. [g] Unknown. [h] Tollens test was positive. Quantitative analysis was not possible, colourimetrically, owing to the tendency of histidine samples to become coloured in acidic solution. [i] Met *S*-oxide. [k] Two unknowns, 1% each. [l] Cys-Cys. [m] Cysteic acid. [n] Serine.

Gas evolution from irradiated samples was monitored by employing a gas burette, and gaseous products (hydrogen, carbon monoxide, and, in some cases, methane) were analysed by g.c. (Table 2).

Water saturated with argon yielded an average of 19 ml of gas over a 6 h irradiation period. This was exclusively hydrogen. Thus any hydrogen evolved from an organic compound in 0.006M-solution would be insignificant in comparison with that produced from the water, and the volume of hydrogen evolved from irradiated samples could be used as an internal standard. By use of this standard, the amounts of gas evolved during irradiation of amino-acids could be compared. As seen from Table 2, the method does not hold for samples which evolve large amounts of gas; presumably these compounds are themselves evolving significant amounts of hydrogen. (It should be noted that the breakage of a C–H or N–H bond enjoys a considerable energy advantage over the breakage of an O–H bond.)

After amino-acid analysis, the irradiated solutions were examined for methanol and formaldehyde by colourimetric methods. Spot tests were performed for formic acid, organic nitro-compounds, nitriles, amides, nitrous and nitric acids, and, where appropriate, acetaldehyde, phenylacetaldehyde, phenolic compounds, and *p*-benzoquinones. Irradiated glycine and alanine solutions, after concentration, were subjected to t.l.c. and the results compared with those from glycolic acid–glyoxylic acid and lactic acid–pyruvic acid samples, respectively. Spots corresponding to hydroxy-acid were detected in each case.

[1] Part III, R. G. Fayter and L. A. Spurlock, *J. Acoust. Soc. America*, 1974, **56**, 1461.

[2] I. E. El'piner, 'Ultrasound: Physical, Chemical and Biological Effects,' Consultants Bureau, New York, 1964.

[3] L. Chambers and E. J. Florsdorff, *J. Biol. Chem.*, 1936, **114**, 75.

[4] I. E. El'piner and L. I. Stekol-nikov, (*a*) *Biokhimiya*, 1963, **28**, 501; (*b*) *Doklady Akad Nauk S.S.S.R.*, 1962, **146**, 700; (*c*) *ibid.*, p. 929.

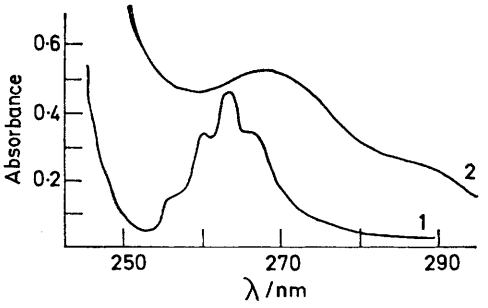
Glycine and alanine were the most stable to ultrasound, giving rise to ammonia, carbon monoxide, and formaldehyde. The appearance of glycolic acid and lactic acid in irradiated solutions of glycine and alanine, respectively, leads us to include them as products of deamination. This has precedent in the irradiation of methylamine to give methanol,[5] and of glycyl-DL-leucylglycine to give glycolic acid.[6] The occurrence of acetaldehyde in irradiated alanine samples and of phenylacetaldehyde in phenylalanine samples suggested the general formation of similar aldehydes from other amino-acids *via* deamination and decarboxylation. The detection of phenylacetaldehyde from phenylalanine was reinforced by a previous report.[7] The principal products of deamination and decarboxylation observed in our study are summarized in Scheme 1. Since nitrogen balances were poor in the reactions of compounds which evolved large amounts of carbon monoxide, and since no oxidative products of nitrogen were found, it is most likely that the missing nitrogen was present as the primary amine resulting from decarboxylation. Analytical difficulties rendered identification of these amines impossible in the presence of starting material, ammonia, and other products.

TABLE 2

Analysis of gas evolution accompanying 6 h 800 kHz irradiations of amino-acids; acoustical power $k = 11.6 \times 10^{-6}$ mol l^{-1} min^{-1} of H_2O_2

Amino-acid	Volume evolved (ml)	Relative proportions [a]			Volume ratio (relative to hydrogen as unity)	
		H_2	CO	CH_4	CO	CH_4
Gly	20.5	95	5		0.05	
Ala	22	85	15		0.18	
Glu	23	84	16		0.20	
Gln	24.5	79	21		0.27	
Phe	28+ (4 h)	67	32	1	0.48	0.015
His, pH 2	21	90	10		0.11	
His, pH 7	21	86	14		0.16	
His, pH 10	19	98	2		0.02	
Imidazole	21	83	15	3	0.18	0.04
Met	29	66	33	2	0.50	0.03
Cys	20.5	95	5		0.05	
Cys-Cys	23.5	68	30	1	0.44	0.015
H_2O	19	100				

[a] Average values, based on a minimum of four runs.

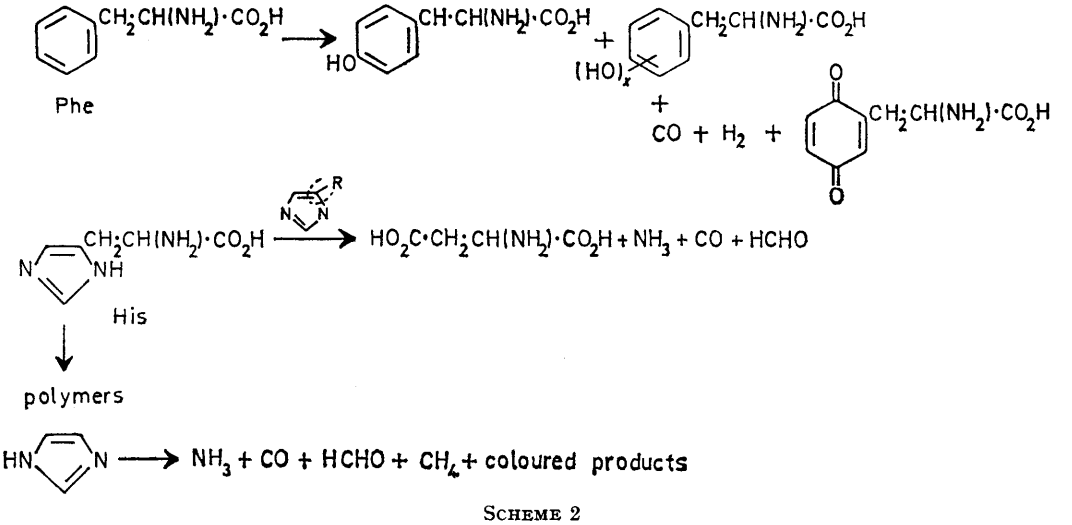


The u.v. spectra of irradiated histidine, imidazole, and phenylalanine solutions were examined after 6 h in order to assess the effects on the aromatic rings. It was necessary, first, to reduce the hydrogen peroxide present with platinum black in order to eliminate the strong end absorption in the regions of interest.

Phenylalanine samples, diluted 2 : 1, revealed an increased, broadened absorption maximum centred at 265 nm with a shoulder at *ca.* 280 nm (Figure). Treatment of the phenylalanine sample with 5% palladium–carbon and hydrogen did not materially alter this spectrum. Treatment of the same sample with platinum, hydrogen, and a drop of perchloric acid, however,

$$RCH(NH_2)\cdot CO_2H \longrightarrow H_2 + CO + NH_3 + HCHO + RCH(OH)\cdot CO_2H + RCHO + RCH_2NH_2 + ROH$$

SCHEME 1



SCHEME 2

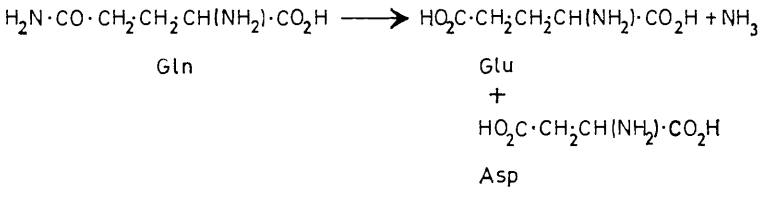
[5] R. G. Fayter, Ph.D. Thesis, Brown University, 1973.

[6] I. E. El'piner and A. V. Sokol'skaya, *Doklady Akad. Nauk S.S.S.R.*, 1962, **147**, 1220.

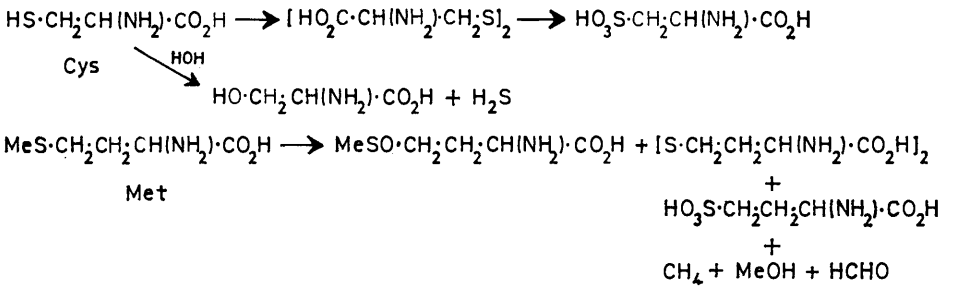
[7] B. Robert, R. O. Prudhomme, and P. Grabar, *Bull. Soc. Chim. Biol.*, 1955, **37**, 897.

eliminated all absorption. These data coupled with appropriate spot tests and amino-acid analysis indicated the presence of phenolic compounds and *p*-benzoquinone (Scheme 2).

Histidine and imidazole samples (pH 7), diluted 50 : 1, gave u.v. absorptions which were reduced by 46 and 54% respectively in comparison with unirradiated blanks. Further, histidine samples gave rise to yellow solutions upon irradiation. The colour darkened upon exposure to air, and pH 2 and pH 7 samples deposited a black polymeric precipitate. The pH 10 samples did not do this, but adjustment to pH 2 caused a similar precipitation.



SCHEME 3



SCHEME 4

A precise description of the polymer was not possible, but it was apparent that it was acidic, and from u.v. monitoring of the reaction it was clear that it was formed at the expense of the imidazole ring. Oddly, the polymer appeared to be formed by a combination of the action of ultrasound and the subsequent exposure of the solution to oxygen, since the degree of colouration of irradiated solutions, and subsequent precipitation of black amorphous solid, could be controlled by exclusion of air. Light had no effect on the process. The influence of pH was noteworthy: samples at low pH showed a great tendency to darken, with colour appearing early in the irradiation. Medium pH samples by contrast became yellow towards the end of the irradiations, and basic samples darkened only after lengthy exposure to air subsequent to irradiation. In spite of this, the recovery of histidine was poorest from basic solution, and considerably better in acidic solution. This seems to indicate that stabilization of the imidazole ring to ultrasound is imparted by protonation. The generalization is not strictly true for all products, however, as aspartic acid, clearly formed at the expense of the imidazole ring, occurs in a quantity six times greater at pH 2 than at pH 10. Apparently several modes of destruction of the imidazole ring are possible and the probability of any one mode occurring depends upon the pH of the medium. In this regard, it is noteworthy that imidazole, when irradiated, also showed this tendency to become coloured, with similar pH dependence. No polymer precipitation was observed, however. The proportion of ammonia produced from imidazole at pH 7 amounted to 82% of that produced by histidine under identical conditions. This verified that much of the ammonia observed from irradiated histidine samples is due to destruction of the heterocyclic ring (Scheme 2).

Glutamine evolved more gas than glutamic acid, consistent with the observation that dicarboxylic acids are generally less reactive and evolve less gas than simple carboxylic acids.[5] Interestingly, the amide unit of glutamine appears to be stable to ultrasound; only small amounts of deamination to glutamic acid were discerned. Likewise, decarboxylation of the distal carboxy-group of glutamic acid, giving aspartic acid, appears to be sluggish (Scheme 3).

The sulphur-containing amino-acids (Scheme 4) undergo many of the reactions to be expected from their exposure to conditions favouring oxidation. Cysteine is converted into cystine, and cysteic acid is formed from both cysteine and cystine. The amount of cysteic acid formed in irradiated cysteine solutions is less than that from irradiations of cystine. This is consistent with the usual sequence of oxidation of thiols to sulphonic acids. Cysteine also gave some hydrogen sulphide, detectable by odour, but present in insufficient amount to be quantitatively analysed. The product from this removal of the thiol group is the hydroxy-amino-acid serine, detected in trace amounts by amino-acid analysis.

Surprisingly, methionine showed a degree of instability in an ultrasonic field only slightly less than those of histidine and phenylalanine. Both decarboxylation and deamination contribute significantly to its reactivity. Oxidation to methionine *S*-oxide, though not the *SS*-dioxide, is also significant. (This latter mode of

reactivity was anticipated in light of the earlier studies of di-n-butyl sulphide.[8]) Formaldehyde production is also greater from methionine than from most other amino-acids. Formaldehyde may, alternatively, be formed *via* hydroxylation and removal of the terminal *S*-methyl group. Similarly the methane and methanol formed are believed to be derived from that source.

Upon conclusion of irradiation, a small amount of precipitate was noted from methionine samples. Amino-acid analysis indicated this to be homocystine.

In a final experiment (Table 1), irradiated samples were lyophilized to determine the quantity of material lost *via* gas evolution. Material balances of solution products, obtained by freeze-drying irradiated samples, generally coincide with our analyses for the amounts of material lost through gas evolution. Imidazole provided the one exception, but in this instance it is likely that many of the products formed upon irradiation were low molecular weight volatile compounds which would be lost during freeze-drying. The material obtained from freeze-drying the imidazole samples appeared to be polymeric.

In summary, the stability of a variety of naturally occurring amino-acids to ultrasonic irradiation has been determined. We shall next turn our attention to di- and tri-peptides to determine the stability of the peptide linkage. The amide linkage of glutamide was remarkable stable under irradiative conditions. If the amide linkage is stable to ultrasound, all reactions involving the α carbon site (*e.g.* deamination and decarboxylation) will effectively be eliminated. This will greatly simplify our task.

EXPERIMENTAL

Irradiations were conducted with a Macrosonics Multisons 180 VF high frequency ultrasonic generator. The sound source was a cobalt barium titanate transducer, model HSF, operation with maximum efficiency at 800 kHz with a rated power output of 85 W (or power comparable with hydrogen peroxide formation from water under argon of $k = 11.6 \times 10^{-6}$ mol l^{-1} min^{-1} at 21 °C). The water volume was 300 ml and the height of the water column 17 cm. On the irradiation vessel (int. diam. 4.8 cm) were affixed, at the lower end, a disc of 500 D Mylar film serving as the sound-transparent bottom, and, at the upper end, gas inlet and outlet fixtures for control of the internal atmosphere. The vessels were fitted to an external cooling jacket with a reproducible distance (*ca.* 1 cm) between the surface of the transducer and the bottom of the vessel. Sound coupling between source and vessel occurred through the water circulating through the cooling jacket.

Spectrophotometric measurements were performed with a Bausch and Lomb Spectronic 20 instrument. Amino-acid analyses were conducted with a Beckmann 121 Automated Amino Acid Analyser. The g.l.c. analyses were carried out with a Perkin-Elmer F-11 hot wire chromatograph and an F & M 500 gas chromatograph. Glycine, alanine, phenylalanine, and methionine *S*-oxide and *SS*-dioxide were purchased from the Nutritional Biochemicals Company. Glutamic acid, glutamine, cysteine, cystine, methionine, and histidine were obtained from Aldrich Chemical Company. All materials were checked for purity by amino-acid analysis.

Standardization of the Irradiation Apparatus.—The irradiation apparatus was initially standardized by relating its acoustical power output, under specified conditions, to the occurrence of a known, ultrasonically induced chemical reaction: the production of hydrogen peroxide in water.[9] Pure water was obtained for irradiation by distillation from a concentrated phosphoric acid solution. Prior to distillation, the reaction vessel was purged with the appropriate gas. The distillation apparatus was purged with steam and filled with the appropriate gas. The water was then distilled directly into the reaction vessel to a column height of 17 cm (300 ml). By this method, water was obtained at pH 7.

The vessel was then placed in a cooling jacket mounted over a transducer. The temperature was maintained at 21 °C while the solution was irradiated at 800 kHz and an intensity corresponding to 800 V_{pp} on an oscilloscope. Samples were removed at regular intervals for hydrogen peroxide analysis. The rate of hydrogen peroxide formation was followed colourimetrically by the tri-iodide anion method.[9]

Irradiation of Aqueous Solutions of Amino-acids.—Water for irradiation was purified as described above. Water saturated with argon and under an argon atmosphere (300 ml) was distilled directly into a reaction vessel containing in each case sufficient amino-acid for a 0.006M-solution. Solutions were thus obtained at a pH equivalent to the isoelectric point of the amino-acid. In cases where an amino-acid was only available as its hydrochloride, an appropriate amount of solid potassium hydroxide was added to the final solution. Samples irradiated at pH 2 and pH 10 were brought to the specified pH with concentrated hydrochloric acid or solid potassium hydroxide as required. Irradiations were conducted at 800 kHz, acoustical power $k = 11.6 \times 10^{-6}$ mol l^{-1} min^{-1} of hydrogen peroxide, and a gross solution temperature of 21 °C. The samples were irradiated as a column 17 cm high.

Amino-acid Analysis.—The irradiated amino-acid solutions were subjected to automated amino-acid analysis with sample blank comparison, by using well defined protein hydrolysate separation techniques. It was necessary to dilute samples 10 : 1 for analysis with sodium citrate buffer of doubly distilled water. Quantitative assessment of the amino-acids and ammonia present was accomplished by correcting for individual sensitivity to the ninhydrin reaction employed in their detection using a commercially prepared standard amino-acid solution mixture. The reported proportions of unknown amino-acids are, of necessity, not corrected for sensitivity to the ninhydrin reaction.

Irradiated amino-acid solutions were also subjected to two-dimensional ascending paper chromatographic analysis (solvents pyridine–acetone–ammonium hydroxide–water, 45 : 30 : 5 : 20 ml; then propan-2-ol–formic acid–water, 75 : 12.5 : 12.5). After drying in air, the chromatogram was sprayed with ninhydrin reagent and heated at 110—115 °C for *ca.* 1 min.[10]

Analyses of Formaldehyde and Methanol.—The reagent was prepared by dissolving veratrole (1 ml) in concentrated sulphuric acid (10 ml). Samples of irradiated amino-

[8] L. A. Spurlock and S. Reifsneider, *J. Amer. Chem. Soc.*, 1970, **92**, 6112.

[9] A. O. Allen, *J. Phys. Chem.*, 1952, **56**, 575.

[10] C. J. Spinella, *Clinical Chem.*, 1969, **15**, 1011.

acid solutions (1.0—5.0 ml, depending upon the formaldehyde concentration) were mixed with reagent (1 ml) and concentrated sulphuric acid (10 ml) in a 25 ml volumetric flask. The solution was cooled to room temperature, then distilled water was added to bring the volume to 25 ml at room temperature. Optical densities were determined at 540 nm (1 cm cell). The concentration of formaldehyde in the original sample (in mmol l^{-1}) is given by $(D_s - D_b) \times 0.671 \times$ dilution, where D_s is the optical density of the sample and D_b that of the reagent (diluted with sulphuric acid and distilled water), and the dilution is the ratio of the volume of the final solution to the volume of the original sample.

Analysis of methanol was accomplished by treating the sample with two drops of aqueous 5% potassium permanganate for 1 min at room temperature. The excess of permanganate was then destroyed with aqueous 0.5% sodium hydrogen sulphite, and the treated sample was analysed colourimetrically, as previously described. The quantity of methanol (mmol l^{-1}) was determined by subtracting the amount of formaldehyde present in the unoxidized sample from that present in the treated sample.

Analyses of Gaseous Products.—The volumes of gas evolved from 0.006M-solutions of amino-acids irradiated as previously specified were monitored by employing a gas burette attached to the reaction vessel. The composition of the gases evolved was then determined by g.c. ($\frac{1}{4}$ in $\times$ 16 ft molecular sieve column and $\frac{1}{4}$ in $\times$ 16 ft charcoal column to determine hydrogen, carbon monoxide, and methane proportions). Both argon and helium were employed as carrier gases.

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