The Specific Cleavage of Tyrosyl–Peptide Bonds by Electrolytic Oxidation*

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ABSTRACT: Tyrosyl–peptide bonds are cleaved specifically by electrolytic oxidation in aqueous media under mild conditions. A second component is formed by oxidation of the phenolic ring which permits additional peptide bond cleavage to occur upon boiling the solution. Electrolytic cleavage of the peptide model, phloretylglycine, has been examined under a variety of conditions to attain maximum cleavage yields, which reach 45% prior to and 65% following boiling. The specificity of the method is demonstrated by oxidative cleavage at the tyrosine residues of angiotensin and insulin.

The selective cleavage of tyrosyl–peptide bonds by a coupling of chemical oxidation (with *N*-bromosuccinimide or bromine) and nucleophilic participation of the peptide linkage has been described in previous reports from this laboratory (Wilson and Cohen, 1963, and earlier papers cited therein). However, such a cleavage method may not be termed specific, since peptide bonds following tryptophan are cleaved more rapidly than those following tyrosine (Schmir and Cohen, 1961; Witkop, 1961) and those adjacent to histidine are cleaved either simultaneously or subsequently (Shaltiel and Patchornik, 1963). In the course of our studies on chemical methods for mapping the surface of proteins in solution,[1] we have observed that mild electrolytic oxidation effects the specific cleavage of tyrosyl–peptide bonds with little or no attack upon those following tryptophan or histidine.[2]

At a platinum anode and at high voltages, a polypeptide will follow the Hofer–Moest modification of the Kolbe electrolysis (Weedon, 1952, 1960), wherein the carboxyl terminal is lost and is replaced by the solvent anion. The decarboxylation thus achieved has been utilized to effect stepwise degradation of polypeptides from the carboxyl terminal (Boissonas, 1953). In a subsequent study of the method, it was observed that the aromatic rings of phenylalanine and tyrosine are destroyed in the course of electrolysis (Thompson, 1954). At lower voltages, these events do not occur and oxidation can be limited largely to the phenolic ring of tyrosine.

At the platinum anode, phloretic acid (I) is oxidized to the dienone spirolactone, II, in 20% yield (Scott *et al.*, 1963). Similarly, phloretylglycine (III) is cleaved to II and glycine in yields of 25–45%. Subsequent to the oxidation step, the course of cleavage, *via* an iminolactone intermediate, is the same as in the *N*-bromosuccinimide method (Scheme I) and is applicable to a polypeptide of any chain length. Peptides not containing tyrosine, such as glycylleucine (Davies *et al.*, 1964) and alanylleucine (Iwasaki *et al.*, 1963), are not cleaved by electrolysis. In peptides containing tryptophan, both the peptide bond and the indole nucleus remain intact (Iwasaki *et al.*, 1963; Iwasaki and Witkop, 1964). Although other functional groups of proteins, *e.g.*, imidazole, thioether, disulfide, and amine, are subject to electrolytic oxidation, the rates of oxidation are considerably lower than that of tyrosine. In this first report on the electrolytic cleavage of tyrosyl peptides, the effect of reaction variables on yield and on the nature of the products is described.

Protection of Amino Groups. Amino terminals originally present in the polypeptide and those subsequently released by electrolytic cleavage are subject to further destruction at the anode,[3,4] as shown by a time-dependent decrease in ninhydrin values (Figure 1, curve A). Adequate protection of amino groups may be achieved by conducting the electrolysis at the lowest practical voltage and in a moderately acidic medium. In principle, oxidation of phenolic systems can be achieved at a potential difference of slightly over 1 v (Fieser, 1930); however, for the quantity of substrate normally used (0.05–0.5 mmole), the application of 6–8 v was found necessary to complete a run within a 3–4-hr period.

The effect of increasing protonation on the survival of

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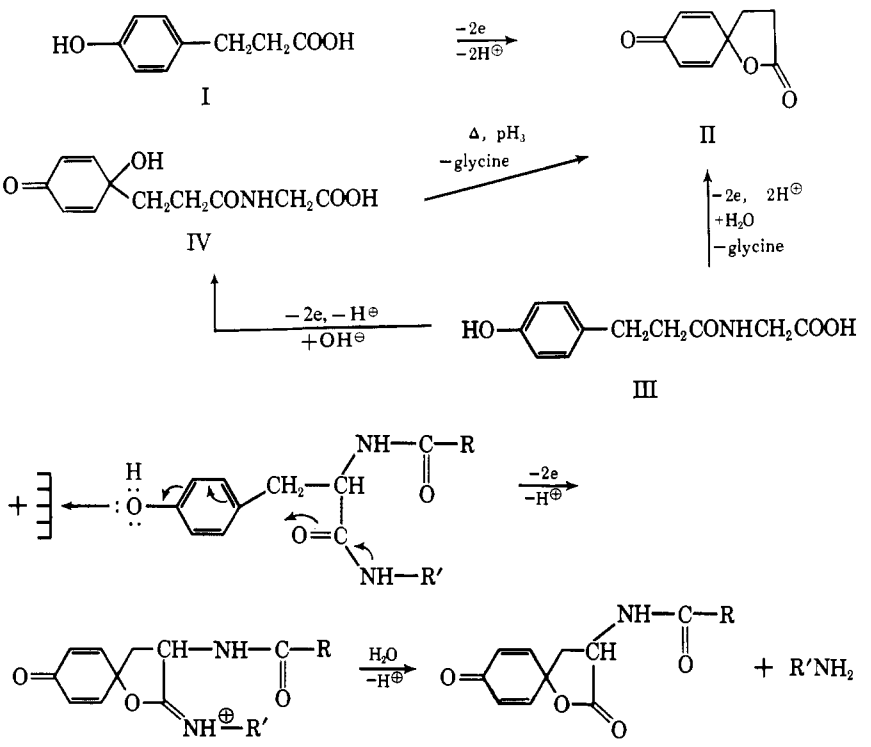
[1] To be published.

[2] A similar specificity in the cleavage of nitrotyrosyl–peptide bonds has already been reported (Iwasaki *et al.*, 1963; Iwasaki and Witkop, 1964).

[3] All other basic nitrogen functions in the polypeptide are probably also subject to slow oxidative attack.

[4] The fate of the amino group is known only to the extent that it becomes nonreactive in the ninhydrin assay; see, however, Takayama (1933).

SCHEME I



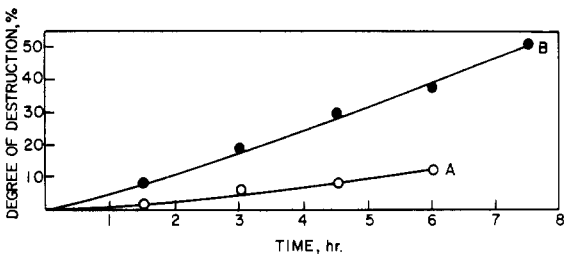


FIGURE 1: Effect of electrolytic oxidation on glycine (A) (7 v, 50 ma, pH 3) and on dienone spirolactone (II) (B) (7 v, 50 ma, 6 hr), as determined by ninhydrin assay and by the decrease in absorption at 230 mμ, respectively.

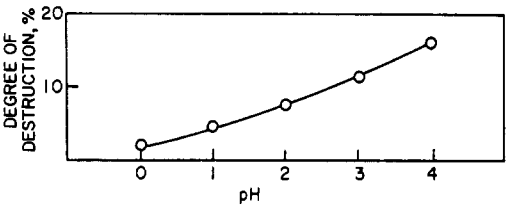


FIGURE 2: Effect of pH on stability of glycine during electrolytic oxidation (7 v, 50 ma, 6 hr), as determined by ninhydrin assay.

amino groups is shown in Figure 2. The use of media more acidic than pH 1 offers no particular advantage, since destruction of amino groups becomes negligible below this value and since polypeptides containing hydroxyamino acids or aspartic acid may undergo some hydrolytic cleavage (Hill, 1965). The protection achieved by protonation may be due either to the decreased availability of electrons on nitrogen (thus raising its oxidation potential) or to electrostatic repulsion of the positive pole by the anode, or both. For reasons which will be developed subsequently, a pH range of 2–3 was used routinely in the present series of experiments.

If the original substrate is devoid of basic nitrogen functions, *e.g.*, the cyclic polypeptide rufomycin A, the basic product formed by electrolytic cleavage may be removed from solution and protected to some extent by addition of an acidic resin such as Dowex 50 to the anode cell (Iwasaki *et al.*, 1963; Iwasaki and Witkop, 1964).

Stability of the Dienone Spirolactone System. The species, II, resulting from oxidation of the phenolic moiety of phloretylglycine, is reasonably stable in the pH range 3–6 (Figure 3). The dienone disappears from solution according to (pseudo) first-order kinetics, the rate being followed by a decrease in optical density at 230 mμ. Beyond the first 20–30% reaction, the ultraviolet spectrum of the dienone chromophore becomes obscured by that of its decomposition products. In the presence of aqueous halogen acid, reduction to the original phenolic system may also occur (Schmir *et al.*, 1959). Even in the region of maximum pH stability, II suffers further destruction of undetermined nature at the oxidizing electrode (Figure 1, curve B). The rate of destruction (3–6%/hr) is essentially independent of applied voltage in the region 2–12 v.

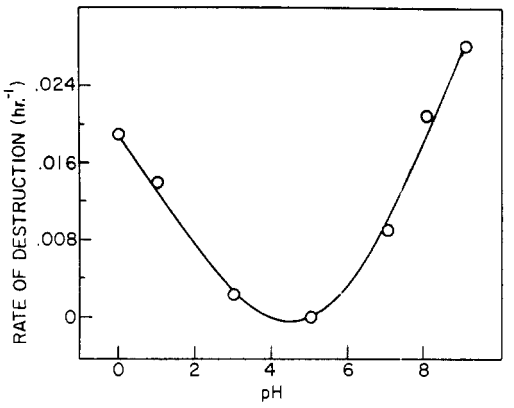


FIGURE 3: Effect of pH on stability of dienone spirolactone (II), as determined by the decrease in absorption at 230 mμ.

Experimental Conditions. The rate and progress of a cleavage experiment with phloretylglycine may be followed initially by the disappearance of the phenolic peak at 280 mμ and by ninhydrin assay for glycine released (Figure 4). Disappearance of the phenolic chromophore cannot be followed to completion since the peak becomes submerged in the much more intense absorption of the dienone chromophore.[5] Even after the ninhydrin assay has reached a constant value, absorption at 280 mμ continues to decrease due to electrolytic destruction of the dienone chromophore. A typical time study of glycine release is shown in Figure 5; for low concentrations of substrate (10^{-3}–10^{-4} M), there is little advantage in conducting an electrolysis for more than 5 hr. Indeed, it can be shown that phloretylglycine has almost disappeared from the anolyte at this point. Cleavage yields (based on ninhydrin assay),[6] under a variety of experimental conditions, are summarized in Table I, column 5. The yield is essentially independent of temperature in the range 0–80° and is not significantly improved by conducting the electrolysis at pH values below 3. Cleaner reactions and higher yields are often realized by adding a solution of the peptide to the anolyte gradually (Table I). Although maximal yields will be obtained by use of lower voltages, the time necessary to complete a run may be impractical, and a reasonable balance of the two factors must be sought. The electrolysis of preparative quantities of substrate (10–100 mmoles) usually requires several days for completion; the reaction time can be shortened

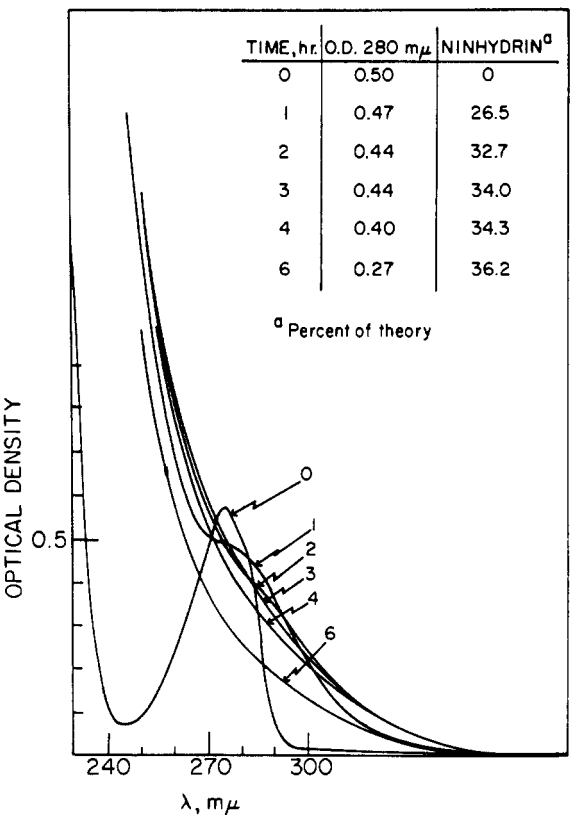


FIGURE 4: The changes in ultraviolet absorption and cleavage yields of phloretylglycine following electrolysis.

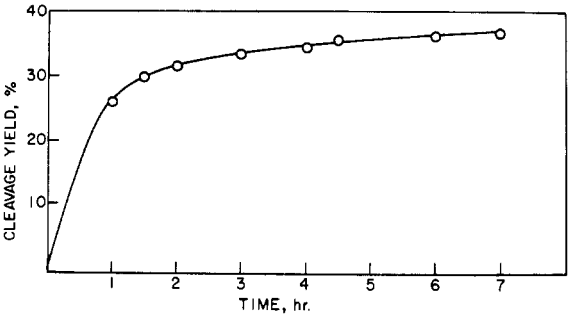


FIGURE 5: Time dependence in the electrolytic release of glycine from phloretylglycine (7 v, 50 ma, pH 3) (ninhydrin assay).

severalfold by a considerable increase in electrode surface and/or the use of higher voltages. In the latter case, it may be advisable to maintain pH values below 3 and to cool both the electrolysis cell and the agar bridge (see Experimental Section).

Other Products of Electrolytic Oxidation. In the course of efforts to improve the cleavage yield of phloretylglycine beyond 45%, it was discovered that the ninhydrin color value could be raised to 65–70% by boiling

[5] Electrolysis of the phenolic substrate is accompanied by the generation, in solution, of a yellow color which is discharged almost simultaneously with the complete loss of the phenolic chromophore at 280 mμ. Although the species responsible for the color is unknown, the phenomenon has been utilized in practice as an indication of the end point of the oxidation process.

[6] It is recognized that the ninhydrin values may also include ammonia, resulting from further degradation of glycine; however, assay of random samples has indicated the amount of ammonia to be very small.

TABLE I: Electrolytic Oxidation of Phloretylglycine.

Voltage (v)	Current (ma)	Concn[a] (mM)	Time (hr)	Yield[b] Unboiled	Yield[b] Boiled[c]	Special Conditions[d]
7	50	0.37	4	35	59	...
7	50	0.37	4	36	58	50°
7	50	0.37	4	33	60	80°
9	50	0.37	4	43	66	...[e]
7	40	4.1	25	29	43	...
7	40	4.1	32	28	50	...
12	150	4.1	21	21	37	...
30	250	4.1	10	22	41	...
58	800	4.1	4.5	27	42	Cooling[f]
20	250	4.1	12	33	48	pH 0
76	1000	12	18	20	28	Cooling
76	1000	12	22	14	20	Cooling

[a] In each run, approximately 100 ml of a solution of the substrate, at the specific concentration in 0.1 M potassium sulfate, was used. [b] Expressed as per cent of theory as determined by ninhydrin assay. [c] All electrolysis mixtures were boiled for 3 hr at pH 3. [d] Unless specified otherwise, the reaction mixture was maintained at 25° and pH 3 throughout the run. [e] Gradual addition of substrate (see Experimental Section). [f] Cooling of the electrolysis cells and agar bridge were necessary to maintain a temperature of *ca.* 25°.

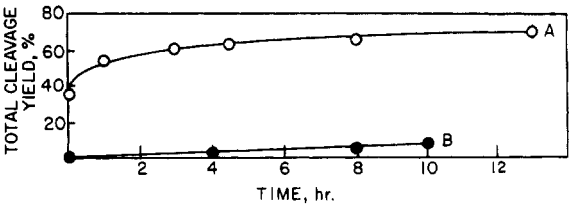


FIGURE 6: Effect of boiling time on release of glycine from the hydroxydienone (IV) (A) and phloretylglycine (III) (B) at pH 3.

the reaction mixture at pH 3 for 3 hr (Figure 6A). In Table I, column 6, the increase in yield following the boiling of the various electrolysis mixtures is summarized. The additional release is not due to phloretylglycine, which hydrolyzes to an extent of less than 2% under the same reaction conditions (Figure 6B). At pH values less than 3, the increase in ninhydrin value occurs more rapidly but phloretylglycine itself hydrolyzes to a considerably greater extent. Although the parent peptide is completely stable to boiling at pH 5, the species which releases glycine at pH 3 fails to do so at the higher pH value.

The labile material was isolated from large-scale (100–300 mg) electrolyses by extraction with methyl or ethyl acetate and purification on preparative thin layer plates. The compound was obtained as a colorless oil to which the hydroxydienone structure, IV, is assigned on the basis of the following data:[7] (a) ultraviolet absorption at 225 mμ (ε 10,500, 95% ethanol), suggesting a dienone chromophore; (b) an A_2B_2 pattern in the aromatic region of the nuclear magnetic resonance spectrum; (c) release of glycine only after heating aqueous solutions of the purified material; (d) sodium borohydride reduction with regeneration of the phenolic spectrum and identification of the product as phloretylglycine; and (e) rearrangement of the compound in concentrated H_2SO_4 to a phenolic compound (λ_{max} 286 mμ) (Schmir *et al.*, 1959; Scott *et al.*, 1963).

Clearly, the hydroxydienone, IV, is formed by solvolytic participation of water in competition with intramolecular participation of the peptide bond.[8] Conceivably, the latter process may be rendered difficult if particular peptide bonds within a protein molecule are bound by conformational factors into a geometry unfavorable for participation; however, such is unlikely with a simple dipeptide where geometry is not a consideration.

The effect of pH on the rate of cleavage of IV is in accord with previous observations on the intramolecularly catalyzed hydrolysis of γ-hydroxybutyramide (Bruice and Marquardt, 1962), which shows maximum stability in the neutral pH range. Whether a particular electrolysis mixture warrants the subsequent boiling procedure depends, of course, on the desire for qualitative or quantitative determination of the new end groups and for preparative isolation of a fragment.

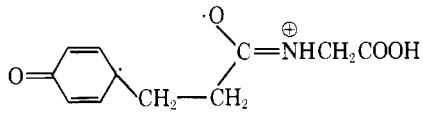
Mechanistic Considerations. Although electrochemical oxidation has been classically formulated as a means of effecting one-electron transfer, the rapid discharge of a

[7] Although the compound appeared homogeneous by thin layer chromatography, trace impurities rendered the analytical values somewhat beyond the range of acceptability.

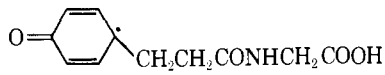
[8] Studies on electrolysis in aprotic solvents are in progress.

second electron to generate a positively charged species must also be considered. The suggestion has been made that the formation of alcohols and ethers in the course of a Kolbe electrolysis (the Hofer–Moest side reaction) is due to the reaction of solvent with carbonium ions generated at the electrode (Walling, 1957). Furthermore, it has been shown that the electrolytic oxidation of structurally appropriate carboxylic acids leads to the same pattern of rearrangement and solvolysis products which results from the chemical generation of the corresponding carbonium ion (Corey *et al.*, 1960).

At the anode, an initially formed phenoxyl radical has the choice of dimerizing with a neighbor or of surrendering a second electron. In a highly polar medium, the potentials necessary to remove one or two electrons from a phenol are sufficiently close not only to permit, but to favor, the latter process.[1,9] In view of the general resistance of amides to electron loss, we do not consider a species such as V to be a plausible participant in the over-all cleavage process (Scheme I). Since resonance-stabilized radicals show little tendency to decompose water (Dürckheimer and Cohen, 1962), it is equally doubtful that the hydroxydienone (IV) is formed directly from the radical species VI.



V



VI

The observed order of reactivities toward bromine or *N*-bromosuccinimide, tryptophan > tyrosine ≧ histidine probably represents the ease of formation of a bromonium ion intermediate (Witkop, 1961). The observed order in anodic oxidation, tyrosine ≫ tryptophan or histidine, is a function of relative oxidation potentials and is, perhaps, the more predictable of the two series. In view of the mechanistic differences between the two modes of oxidation, such a reversal in reactivity need not be surprising.

Electrolysis of Polypeptides. The electrolysis of *N*-acetyltyrosylglycylglycine (7 v, pH 3, 4 hr) resulted in a cleavage yield of 40% before boiling and 66% after boiling. Glycylglycine was identified as its dinitrophenyl (DNP) derivative by thin layer chromatography. DNP-glycine occurred in trace amounts only.

Angiotensin amide (VII) is cleaved exclusively at the tyrosyl–valyl bond. In addition to DNP-aspartic acid

Asp^{N}-Arg-Val-Tyr-Val-His-Pro-Phe

VII

[9] Although phloretylglycine is slowly oxidized by tri-*t*-butylphenoxyl, peptide bond cleavage is not a result of the radical oxidation.

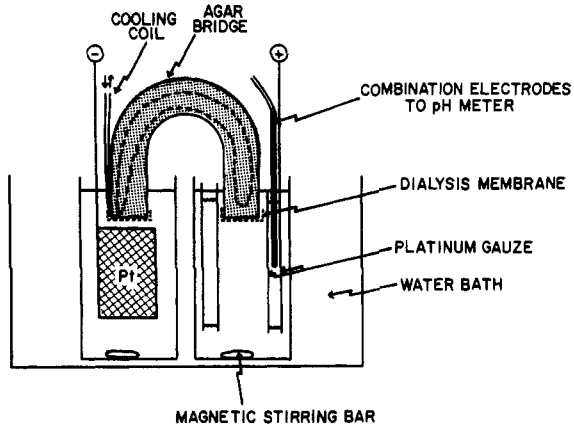


FIGURE 7: The apparatus used for electrolytic peptide cleavage.

(from the original amino terminal), DNP-valine was the only new derivative found by qualitative identification, either before or after boiling. The absence of *O*-DNP-tyrosine indicated oxidation to be complete; the absence of a proline terminal after boiling indicated that the imidazole ring of histidine had not been oxidized in the manner observed with *N*-bromosuccinimide (Shaltiel and Patchornik, 1963), although degradation to aspartic acid did occur to a small extent. These conclusions were supported by total amino acid analysis of the electrolysis mixture (see Experimental Section).

The electrolysis of crystalline zinc insulin was performed in a manner similar to that for the simpler polypeptides and the release of amino terminals determined qualitatively. In addition to the original glycine and phenylalanine, both threonine and leucine were identified as their DNP derivatives in the ether phase, mono-DNP-cystine, ε-DNP-lysine, and *O*-DNP-tyrosine in the aqueous phase. Significantly, DNP-glutamic acid was not observed in any experiment, but *O*-DNP-tyrosine was found, indicating not only incomplete but selective cleavage. Following cleavage of three tyrosyl–peptide bonds, there remains a large insulin fragment containing 70% of the original residues. It is entirely conceivable that sufficient tertiary structure is present in this fragment to render the fourth bond, Tyr-Glu^{N}, inaccessible to the electrode. Exploratory studies with ribonuclease have already demonstrated that the platinum electrode can exercise a high degree of selectivity in attacking the surface of the molecule.[1]

Experimental Section

Apparatus (*Figure 7*). Two glass jars (5 × 10 cm, 125-ml capacity) were immersed in a large water bath and equipped for magnetic stirring. The cathode consisted of a platinum sheet (7.5 × 3 cm), external connection being made with a soldered platinum wire or alligator clip. The anode consisted of two concentric cylinders of fine platinum gauze (6.5 × 3.5 cm and

6.5 × 2.5 cm, total area 120 cm^2), joined together with platinum wire.

The bridge was formed of Pyrex tubing (17-mm i.d.), of sufficient length to permit 1–2 cm to be immersed in a cell containing 100 ml of solution. Acrylamide gel (Cyanogum 41) was used in early experiments (Iwasaki *et al.*, 1963), particularly since it tended to resist softening and cracking at high current densities and temperatures. It was found, however, that the gel (prepared with ammonium persulfate) released ammonia into the anolyte gradually; accordingly, it could not be used with the ninhydrin assay method. Because it tends to crack or soften, agar was found unsuitable at high temperatures (generated by high current densities). The difficulty was overcome by cooling the bridge with flowing water, either by imbedding fine polyethylene tubing within the bridge before the gel solidified or by wrapping a coil of such tubing on the outer surface of the bridge. Both ends of the U-tube were covered with dialysis membrane, held in place by platinum wire or rubber bands. This precaution was found helpful in preventing the agar from being displaced by the applied potential difference.

The gel should be made up with the same electrolyte as used in the cells, preferably at a higher concentration. The agar mixture was prepared by addition of 1.5 g of agar to a warm solution of 4.4 g of potassium sulfate in 50 ml of water. The mixture was heated and stirred until it began to boil, and was then poured into a U-tube immersed in an ice–water bath. The gel hardened in about 15 min.

The electrolyte used in the present series of experiments was 0.1 M potassium sulfate. In other instances, 10% acetic acid (pH 2.4), 0.1 M triethylammonium acetate (pH 5.2), and 0.4 M triethylammonium acetate–trifluoroacetate (pH 2.2) (Iwasaki *et al.*, 1963) have also been used. Since the electrolysis of water, concurrent with that of the substrate, effects a pH change at the separate electrodes, alkali must be added to the anolyte to maintain the desired pH. A thin combination electrode was immersed in the anolyte and the pH monitored continuously with an external pH meter. Potassium hydroxide (4 N) was added from a buret, as needed, to maintain a pH value of *ca.* 3.

Electrolytic Cleavage of Phloretylglycine. The anolyte consisted of 110 ml of 0.1 M potassium sulfate and contained 9.6 mg (0.043 mmole) of phloretylglycine (Schmir *et al.*, 1959). Of this solution, 10 ml was retained as a control. The catholyte consisted of 100 ml of 0.1 M potassium sulfate. The contents of both cells were stirred magnetically and maintained at *ca.* 25° by an external water bath during electrolysis. The conditions of each experiment are summarized in Table I, columns 1–4 and 7. Aliquots were withdrawn at 1-hr intervals and assayed both by ultraviolet spectroscopy and by the ninhydrin method (Spies, 1957), diluting the samples as necessary. The results of both procedures in a typical run are shown in Figure 4. Following treatment of an aliquot with fluorodinitrobenzene, DNP-glycine was identified on thin layer plates by comparison with an authentic sample (Brenner *et al.*, 1965). Silica gel G.F. was used as the adsorbent and development was effected with chloroform–methanol–acetic acid (95:5:1).

In the modified technique for adding the substrate gradually to the electrolyzing solution, 50 ml of 0.1 M potassium sulfate (pH 3) was placed in the anode compartment. The sample, dissolved in an additional 50 ml of 0.1 M potassium sulfate, was added dropwise over a 4-hr period and electrolysis continued an additional 10 min. The remainder of the procedure was similar to that described above.

Isolation of the Hydroxydienone (IV). Several electrolysis runs were carried out using 100–300 mg of phloretylglycine per experiment. The resulting mixtures were pooled, saturated with potassium sulfate, and extracted 20–30 times with 20-ml volumes of ethyl or, preferably, methyl acetate. Due to the thermal instability of the compound, continuous liquid–liquid extraction could not be used. The combined extracts were washed with 0.1 M sodium acetate and dried over sodium sulfate. Following filtration and removal of the solvent *in vacuo* at 25°, a brown oil was obtained. A portion of the material was redissolved in a small volume of methyl acetate, the solution was applied to preparative thin layer plates coated with silica gel G.F. (1-mm thickness), and the plates were developed with benzene–methanol–acetic acid (45:8:4). On plates of 0.25-mm thickness, the compound shows an R_F value of 0.25; on plates of 0.5-mm thickness, 0.29–0.30, and on 1-mm plates, 0.32–0.38. The dienone, II, runs considerably faster, R_F values varying from 0.66–0.71, according to plate thickness. Phloretylglycine (III) appears at the same position as the leading edge of the spot corresponding to IV; however, its presence in purified samples of the hydroxydienone (IV) may be readily excluded by the total absence of phenolic absorption at 270–280 mμ. The desired portion was removed from the thin layer plates, the silica gel extracted several times with methyl acetate, and the extract evaporated to dryness. A yellow oil remained which showed only one spot when rechromatographed in the same system.[7]

The compound shows an ultraviolet absorption maximum at 225 mμ (ε 10,500, 95% EtOH); the nuclear magnetic resonance spectrum shows, in part, an A_2B_2 quartet at 6.07, 6.24, 6.87, and 7.04 ppm (deuteriomethanol). The material, when freshly purified on thin layer plates, is negative in the ninhydrin test; following storage at room temperature for several days, the test is positive. When a solution of IV in 0.1 M potassium sulfate (pH 3) is boiled for 3 hr, the ninhydrin test is strongly positive; following dinitrophenylation of the hydrolysate, DNP-glycine was identified by thin layer chromatography. An aqueous solution of IV was stirred for 4 hr with an excess of sodium borohydride; the solution following acidification gave a peak at 275–280 mμ in the ultraviolet region. Thin layer chromatography on silica gel, using the solvent system described above, showed the reduction product to be identical with phloretylglycine, R_F 0.38. A sample of IV, dissolved in 1 ml of concentrated H_2SO_4, was stored at room temperature overnight. After the mixture had

been diluted with 0.1 M potassium sulfate, its ultraviolet spectrum showed a peak at 286 mμ.

Electrolysis of N-Acetyltyrosylglycylglycine. Using the apparatus and procedure described above for the electrolysis of phloretylglycine, a solution of 9.5 mg (0.56 mmole) of *N*-acetyltyrosylglycylglycine in 50 ml of 0.1 M potassium sulfate (pH 3) was added dropwise over 4 hr to an anode cell containing 50 ml of the same salt solution. At a potential difference of 7 v, the current was 50 ma. The final solution showed no phenolic absorption in its ultraviolet spectrum. Ninhydrin color yields (based on authentic glycylglycine) were 40% before boiling and 66% after boiling. The cleavage product was identified by thin layer chromatography of its DNP derivative on silica gel G.F. (0.5-mm thickness), using the solvent system previously described: DNP-glycylglycine, R_F 0.13; DNP-glycine, R_F 0.22.

Electrolysis of Angiotensin Amide. A sample of 5.4 mg of angiotensin amide[10] in 25 ml of 0.1 M potassium sulfate (pH 3) was added dropwise to an electrolyzing anolyte (8 v, 50 ma), consisting of 50 ml of 0.1 M potassium sulfate (pH 3). Addition was complete in 3 hr and the current was maintained an additional 10 min.

The reaction mixture was poured onto a column of Dowex 50W-X4 (H^+ form, 50–100 mesh) and the resin was washed with distilled water until the eluate was neutral (pH). The peptide material was removed from the column with 1 M ammonium hydroxide and the eluate evaporated to dryness *in vacuo* in two equal portions. One portion was treated with fluorodinitrobenzene in bicarbonate solution (Fraenkel-Conrat *et al.*, 1955). The resulting mixture of DNP-peptides was hydrolyzed by refluxing its solution in distilled, constant-boiling HCl for 18 hr under nitrogen. The hydrolysate was diluted with three volumes of water and extracted with ether. Both the aqueous and ethereal solutions were evaporated to dryness *in vacuo*.

The residue obtained from the ether layer was analyzed by thin layer chromatography with chloroform–methanol–acetic acid (95:5:1) as the developing solvent (Brenner *et al.*, 1965). Silica gel G.F. was used as the adsorbent. In addition to DNP-aspartic acid, arising from the original amino terminal, DNP-valine was the only other component observed. The residue obtained from the aqueous layer was analyzed by thin layer chromatography, using 1-propanol–concentrated ammonium hydroxide (7:3). The compound, *O*-DNP-tyrosine, was not present.

The second portion of the original electrolysis mixture was hydrolyzed by refluxing its solution in constant boiling HCl for 18 hr under nitrogen. The solution was taken to dryness and analyzed for total amino acid content on an automatic amino acid analyzer.[11] The results (Table II) indicate the total absence of tyrosine, a partial degradation of histidine to aspartic acid *via* an intermediate α-keto acid, and a slight loss of phenylalanine. The latter decrease may be the result of C-terminal decarboxylation *via* the Kolbe mechanism.

TABLE II: Amino Acid Composition of Angiotensin Amide.[a]

Amino Acid	Original	Electrolyzed
Aspartic acid	1.06	1.33
Arginine	1.00	0.89
Valine	2.02	2.07
Tyrosine	0.89	...
Histidine	0.94	0.70
Proline	1.12	1.14
Phenylalanine	0.97	0.90

[a] Expressed as moles per mole of peptide.

Electrolysis of Insulin. A sample of 7.6 mg of zinc insulin[12] was electrolyzed using conditions similar to those for angiotensin amide. The cleavage mixture was analyzed qualitatively by the fluorodinitrobenzene method, resolution and identification being performed as previously described, on silica gel thin layer plates. The results are summarized in Table III. Since DNP-cysteic acid fails to separate from mono-DNP-cystine in this chromatographic system, the presence of the former is not rigorously excluded.

TABLE III: Amino Terminals Released by Electrolytic Cleavage of Insulin.

DNP Derivatives	Original	Electrolyzed
Ether Fraction:		
DNP-glycine	+	+
DNP-phenylalanine	+	+
DNP-threonine	−	+
DNP-leucine	−	+
DNP-glutamic acid	−	−
Water fraction:		
ε-DNP-lysine	+	+
O-DNP-tyrosine	+	+
Mono-DNP-$(Cys)_2$	−	+

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[12] Supplied by the Pharmaceutical Division, Farbwerke Hoechst, A. G., Frankfurt-Hoechst, Germany.

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Ultraviolet Light Irradiated Collagen Macromolecules*

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ABSTRACT: Collagen macromolecules undergo photopolymerization when irradiated with ultraviolet light of 2537 A. The absorption spectrum of irradiated collagen shows an increase in extinction at the longer wavelengths (290–400 mμ). The decrease of ultraviolet fluorescences due to phenylalanine and tyrosine is accompanied by the concomitant appearance of new blue fluorescences excited at 350 mμ. Although the ultraviolet fluorescences of tyrosine and phenylalanine decrease uniformly with an increase in temperature, the intensity of the blue fluorescences shows an anomalous rise in the temperature range from 30 to 40°. This temperature range corresponds to that of a helix to random-coil transition as shown in the change of viscosity, optical rotation, and far-ultraviolet absorption. This transconformation temperature is lower in irradiated collagen than native collagen. The specific optical levorotation of irradiated collagen is greater than that of native collagen.

Irradiated collagen is still able to form segment-long-spacing (SLS)-like aggregates, even though the ability to form native fibers is lost. These studies show that the blue fluorescences result from the photoproducts of phenylalanine and tyrosine. These photoproducts are probably integral parts of the new linkages. The blue fluorescences are dependent on the structural conformation of a helical rigid body which is not seriously damaged by radiation.

It is known that ultraviolet light induces some proteins to aggregate and others to decompose (McLaren, 1949; McLaren and Shugar, 1964). However, very little is known about their molecular interaction or the change of molecular dimension in these processes. Most of the proteins studied so far are globular which contain disulfide, tryptophan, tyrosine, and phenylalanine. These processes effected by ultraviolet light at 2537 A involve, as the primary chemical reaction, photolysis of disulfide and aromatic residues (McLaren and Luse, 1961; McLaren and Shugar, 1964).

The aggregation of the structural proteins, fibrinogen (Slayter and Hall, 1964) and myosin A (Kaldor *et al.*, 1964), by ultraviolet light has recently been demonstrated. These proteins contain the above-mentioned four amino acid residues. Collagen, a stiff rodlike protein with the dimension of 2800 × 15 A does not contain disulfide bonds and tryptophan, but phenyl-

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