Chem. 247, 3176.
Gurd, F. R. N., Lawson, P. J., Cochran, D. W., and Wenkert, E. (1971), *J. Biol. Chem. 246*, 3725.
Horsley, W., Sternlicht, H., and Cohen, J. S. (1970), *J. Amer. Chem. Soc. 92*, 680.
Irwin, R., and Churchich, J. E. (1971), *J. Biol. Chem. 246*, 5329.
Kuhlmann, K. F., and Grant, D. M. (1968), *J. Amer. Chem. Soc. 90*, 7355.
Kuhlmann, K. F., Grant, D. M., and Harris, R. K. (1970), *J. Chem. Phys. 52*, 3439.
Levy, G. C., and Nelson, G. L. (1972), Carbon-13 Nuclear Magnetic Resonance for Organic Chemists, New York, N. Y., Wiley-Interscience.
Moon, R. B., and Richards, J. H. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 2193.
Parker, R. G., and Roberts, J. D. (1970), *J. Org. Chem. 35*, 996.
Rawitch, A. B. (1972), *Arch. Biochem. Biophys. 151*, 22.
Roberts, G. C. K., and Jardetzky, O. (1970), *Advan. Protein Chem. 24*, 448.
Solomon, I. (1955), *Phys. Rev. 99*, 559.
Wenkert, E., Clouse, A. O., Cochran, D. W., and Doddrell, D. (1969), *J. Amer. Chem. Soc. 91*, 6879.
Wien, R. W., Morrisett, J. D., and McConnell, H. M. (1972), *Biochemistry 11*, 3707.

Oxidative Modification of Proteins in the Presence of Ferrous Ion and Air. Effect of Ionic Constituents of the Reaction Medium on the Nature of the Oxidation Products†

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With the Technical Assistance of Kathrin McCollum

ABSTRACT: Proteins—represented by chymotrypsinogen, cytochrome *c*, myoglobin, phosvitin, and ribonuclease—undergo a structural modification upon brief exposure to ferrous ion, in air. The proteins become reducible by sodium borohydride, indicating that the modification is an oxidative one. The reaction does not occur when oxygen or ferrous ion is excluded, or when another cation (Fe^{3+}, Mg^{2+}, or Cu^{2+}) is substituted for Fe^{2+}. The ion exchange chromatographic profiles of acid hydrolyzates of the several oxidized proteins, labeled in their oxidatively modified side chains with [^{3}H]sodium borohydride, show a far-reaching similarity of composition in terms of labeled products. The nature of the reaction products is strikingly dependent on the composition of the reaction medium: different products are obtained after reaction in phosphate or in tris(hydroxymethyl)aminomethane buffer. The principal product obtained in sodium phosphate buffer has properties expected of a derivative of threonine. The principal product in tris(hydroxymethyl)aminomethane hydrochloride buffer is probably a derivative of lysine. This lysine derivative and some additional, minor products appear to be formed not only in proteins but also in poly-L-lysine. The reaction shares significant features with the spontaneous generation of collagen cross-links and may provide a model for the reaction catalyzed by lysine oxidase. Cytochrome *c*, compared with the other proteins tested, shows a particularly enhanced susceptibility to the oxidative modification but only in phosphate buffer.

Certain aspects of an earlier study of the aerobic reduction of ferricytochrome *c* by ferrous ion implied that the reaction might involve not only the heme group but also the protein moiety of the cytochrome molecule (Taborsky, 1972). In this article, we give evidence that the protein undergoes an oxidative modification in the course of the reaction. It is likely that this oxidation is a general feature of the aerobic interaction between proteins and ferrous ion, but cytochrome *c* appears to be particularly susceptible. This study also revealed that the oxidative modification of proteins is markedly affected by the ionic constitution of the reaction medium.

Materials and Experimental Procedure

Cytochrome *c* (horse heart; type III, Sigma), ribonuclease A (bovine pancreas; type IIA, Sigma), chymotrypsinogen A (bovine pancreas; 3× crystallized, Worthington), myoglobin (horse heart; 2× crystallized, Mann), and the synthetic polypeptides, poly-L-glutamic acid (mol wt 58,300; Miles) and poly-L-lysine (mol wt 11,100; Miles), were used as supplied. Phosvitin (hen egg volk) was prepared according to Joubert and Cook (1958) and was rendered metal-free as described earlier (Taborsky, 1963).

Ion exchange resins (Beckman and Bio-Rad), [^{14}C]amino acids (Schwarz-Mann), [^{3}H]sodium borohydride (New England Nuclear), [^{2}H]sodium borohydride (Alfa Inorganics), and other chemicals of reagent grade quality were used as obtained.

The routine experimental procedure consisted of four stages: (1) reaction with ferrous ion, in the presence of air, resulting in the oxidative modification of the protein (or poly-

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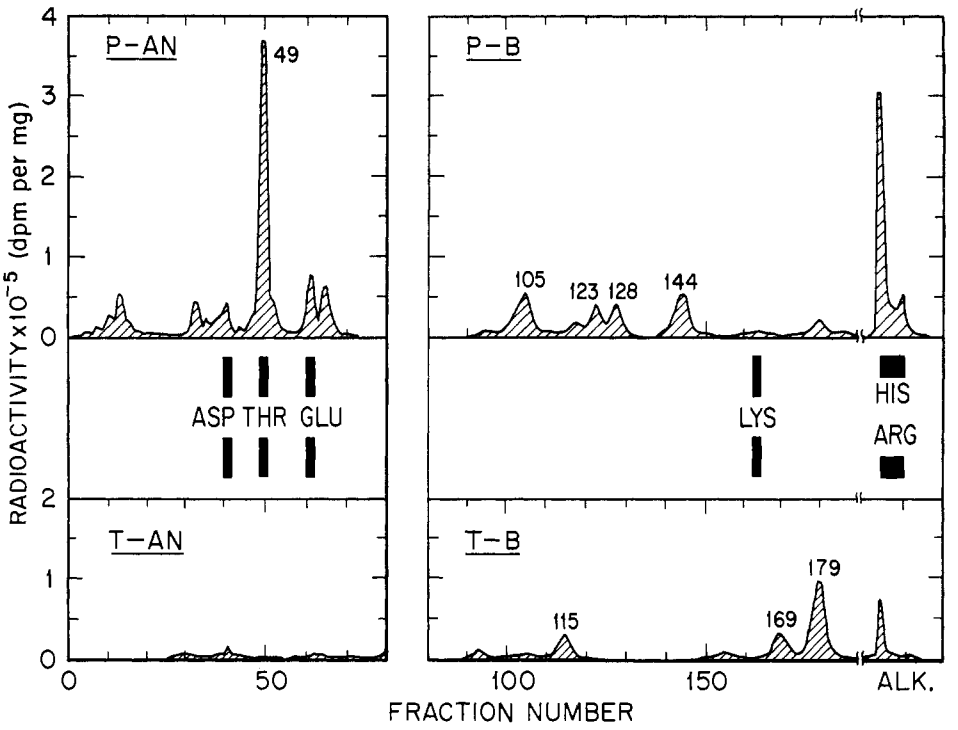


FIGURE 1: Chromatographic fractionation of the isotopically labeled products derived from ferricytochrome *c*, after reaction with Fe^{2+}, in air, and subsequent reduction with [^{3}H]sodium borohydride. Experimental details are given in the text. The four panels of the figure represent patterns of radioactive components of the hydrolyzates of phosphate-buffered (P) or Tris-buffered (T) reaction products, eluted from "acidic–neutral" (AN) or "basic" (B) columns of the amino acid analyzer. Positions of amino acid standards are shown in the center strip of the figure. AN patterns are presented only up to the position of glycine. Radioactive components beyond this point do not appear at an appreciable level. (For the same reason, the first 25 fractions of effluent were not analyzed in detail and are not shown in panel T-AN.) B patterns are shown up to the position of histidine. Any radioactive material still on the columns at this point will remain on the column even if the elution is continued for a total of about 400 fractions (past the position of arginine), unless the eluting buffer is replaced by alkali (ALK). Routinely, elution with alkali was started at about fraction 200. Components to which reference is made in the text are identified by numbers denoting the effluent fraction about which they are centered.

peptide); (2) treatment of this reaction mixture with [^{3}H]sodium borohydride, resulting in the reductive isotopic labeling of susceptible, oxidatively modified functional groups; (3) hydrolysis of the reductively labeled product; (4) analysis by ion exchange chromatography and radioactivity measurement. Details will be described in the following text. (Any nonroutine procedures will be noted under Results.)

Ferrous sulfate, freshly dissolved in water, was added to a buffered protein solution. A typical mixture contained about 0.7 mg/ml of protein (or polypeptide) and 0.8 mM $FeSO_4$, in 50 mM buffer (sodium phosphate or Tris-HCl) at pH 7.5. The solution was stirred in air, at about 21°, for 30 min. When it was desired to exclude air, the reaction mixture was prepared and the reaction was carried out in a glove box, flushed continuously with nitrogen gas.

Oxidized sites, generated during the first stage of the procedure, were tagged with ^{3}H reductant (specific activity >200 Ci/mol). The protein–iron mixture was adjusted to pH 9.0 and an aliquot of a freshly prepared, aqueous solution of the reductant was added to provide no less than one reducing equivalent per mole of amino acid residue. The mixture was stirred for 20 min at room temperature. During this time, the pH remained constant within about 0.2 pH unit. At the end of the period, the solution was acidified with trichloroacetic acid (final concentration 15%). After 5–10 min, gas evolution having ceased, the mixture was centrifuged briefly. The sediment was washed by resuspension in 10% trichloroacetic acid and centrifugation, three times. It was then transferred to a hydrolysis tube with a small volume of 95% ethanol and brought to dryness in a flash evaporator at about 40–45°.

The washed residue (representing about 2–3 mg of protein or polypeptide) was hydrolyzed (6 N HCl, *in vacuo*, 22 hr, 110°). The hydrolyzate was filtered and evaporated to dryness. The residue was taken up in water and dried, twice in succession. Finally, it was taken up in a precisely measured volume of 0.1 N HCl. Aliquots of this solution were used in the various analytical procedures. The solution was stored frozen when not in use. Recoveries from the entire procedure were generally about 60%.

The hydrolyzate was chromatographed by ion exchange, patterned on the standard procedure with the Beckman Model 120C amino acid analyzer (Spackman *et al.*, 1958). Acidic and neutral amino acids were separated on a column of UR-30 resin (0.9 × 55 cm), with 0.2 N sodium citrate buffer, pH 3.15, at 57°, with a flow rate of 1.13 ml/min. The same column was used for the fractionation of basic amino acids but, for this purpose, it was operated with a 0.35 N sodium citrate buffer, at pH 5.28. The analyzer was used in a split-stream mode: a constant proportion of the effluent was permitted to follow the usual route to ninhydrin analysis, while the balance of the effluent stream was diverted, in the form of 1-min fractions, to a fraction collector for purposes of radioactivity measurement.

Liquid scintillation counting of aliquots of the column effluent (0.5 ml or less) was carried out with 20-ml portions of a scintillation fluid composed of toluene and ethanol in a

TABLE I: Yield of Products Derived from Control Reaction Mixtures.

Expt No.	Reaction Mixture[a]	Relative Yield of Radioactive Product[b]						
		Total Hydrolyzate	Chromatographic Component					
			AN-49	B-105	B-123	B-128	B-144	B-ALK
1	Standard	1.0	1.0	1.0	1.0	1.0	1.0	1.0
2	$- O_2$	0.1	0.0	0.0	0.0	0.0	0.0	0.1
3	$- Fe^{2+}$	0.1	0.0	0.1	0.0	0.0	0.0	0.0
4	$- Fe^{2+} + Fe^{3+}$	0.1	0.0	0.2	0.0	0.0	0.0	0.1
5	$- Fe^{2+} + Mg^{2+}$	0.2	0.0	0.1	0.0	0.0	0.0	0.1
6	$- Fe^{2+} + Cu^{2+}$	0.2	0.1	0.1	0.0	0.0	0.0	0.2
7	$-$ Cyt^{3+} + Cyt^{2+}	1.1	1.1					

[a] The "standard" reaction mixture (no. 1) is analogous to the phosphate-buffered mixture described in the legend to Figure 1. All other reaction mixtures were prepared identically with the standard mixture except for the omissions or substitutions indicated in the table. Substitutions were made mole for mole. [b] Yields are given in terms of "control"/"standard" ratios, referring all results to those of experiment 1. This permits a direct comparison of different sets of experiments even if carried out with different lots of sodium borohydride which varied somewhat in specific radioactivity. Absolute radioactivity data underlying experiment 1 as represented in this table are comparable to those given in Figure 1. Component yields of less than 5% of the standard values were ignored.

volume ratio of 2:1, containing 2,5-diphenyloxazole (2.7 g/l.) and 1,4-bis[2-(5-phenyloxazolyl)]benzene (32 mg/l.). The fluid was supplemented with silicone dioxide (Cab-O-Sil; Cabot Corp.) at a concentration of 3%. Measurements were made with Beckman and Packard liquid scintillation instruments (Models LS-150 and Tri-Carb 2420). Data were corrected for efficiency and quenching losses (based on calibration with a [^{3}H]water standard) and the resulting values of disintegrations per minute (dpm) were referred to unit weight (milligrams (mg)) of protein (based on amino acid analysis). This normalization of results in terms of corrected dpm/mg values was necessary to render data comparable even when obtained with different counting instruments and referring to reaction mixtures containing slightly varying concentrations of protein. Standard amino acids, labeled with ^{14}C, were added to each sample before chromatography for calibration of effluent positions. Radioactivity data obtained with the ^{3}H "window" were corrected for ^{14}C "spill-over." In effluent regions where radioactivity was at peak levels (actual counts of the order of 10^2–10^4), the counting error was less than 5%. Very low levels of counts (about 2× background) were ignored. Yields of radioactivity associated with chromatographic components were calculated by numerical integration of radioactivity data across the eluted bands.

Results

The several proteins and polypeptides used in this study yielded results which were sufficiently similar to permit giving full details for one of them, relating to it results obtained with the others. Cytochrome *c* was chosen as the "reference material."

Ferrous Ion–Ferricytochrome c Reaction in Phosphate and Tris Buffers. Upon reaction with ferrous ion, in the presence of air, cytochrome *c* becomes reducible by sodium borohydride. The nature of the functional groups which are reduced by the metal hydride depends markedly on the nature of the buffer. This is revealed by ion exchange chromatographic fractionation of the acid hydrolyzate of the reaction product (Figure 1).

In phosphate buffer (upper panels), the protein yields a principal product eluted exactly in the position of threonine (P-AN-49[1]). A lesser but appreciable amount of radioactive material is found in the alkaline wash of the "basic" column (P-B-ALK). Its affinity for the column is greater than that of arginine. This material and the putative threonine together account for nearly one-half of the total isotope content of the protein hydrolyzate.

In Tris buffer (lower panels), total borohydride-reducible product formation is much less extensive than in phosphate—about one-fourth—and the nature of the product is also different. In this instance, too, there is a single dominant product. It is eluted exactly in the position of ε-*N*-monomethyllysine (T-B-179) and represents about half of the total incorporated isotope. This material is formed in phosphate buffer in a trivially small amount. Conversely, the Tris-buffered reaction yields not even a trace of the component eluted in the threonine position after phosphate-buffered reaction.

The two patterns differ sharply even in terms of minor components. In Tris, in contrast with phosphate, acidic and neutral derivatives are produced in barely significant amounts. In phosphate, basic derivatives are formed which have no counterparts among the products derived from Tris-buffered protein.

"Control" Experiments. Results in Table I help define the nature of the reaction. These results are expressed relative to those of the "standard" reaction involving ferricytochrome *c*, ferrous ion, and phosphate buffer, in the presence of air (experiment 1). The exclusion of oxygen (experiment 2) abolishes the reaction: no labeled products are formed except some material which adheres firmly to the columns. The omission of

[1] Chromatographic fractions of hydrolyzates of the reaction products are designated according to the respective reaction medium from which the products originated (P, phosphate; T, Tris), the nature of the ion exchange column used for the fractionation of amino acids of a given type (AN, acidic–neutral; B, basic), and the position of elution (fraction number). For example, P-AN-49 denotes the component which is derived from a phosphate-buffered reaction mixture and is eluted from the "acidic–neutral" column centered in fraction 49.

TABLE II: Yield of Products Derived from Various Proteins.[a]

Expt No.	Protein[b]	Buffer	Relative Yield of Radioactive Product: Total Hydrolyzate	Chromatographic Component: AN-49	B-105	B-123	B-128	B-144	B-ALK
1	Cyt^{3+}	P_i	1.0	1.0	1.0	1.0	1.0	1.0	1.0
2	CTgen	P_i	0.7	0.6	0.0	0.2	0.2	0.2	0.4
3	RNase	P_i	0.5	0.3	0.7	0.3	0.3	0.4	0.4
4	Mb	P_i	0.6	0.2	0.7	0.3	0.1	0.4	0.5
5	PV	P_i	0.7	0.1	0.2	0.6	0.6	0.4	0.5
6	PV	Tris	0.3	0.1	0.2	0.4	0.3	0.3	0.4
				B-115		B-169	B-179		B-ALK
1′	Cyt^{3+}	Tris	1.0	1.0		1.0	1.0		1.0
2′	CTgen	Tris	0.9	1.0		0.7	0.3		
3′	RNase	Tris	0.7	0.2		1.1	0.3		0.6
4′	Mb	Tris	1.2	2.3		0.4	1.1		0.7
5′	PV	Tris	0.8	0.0		0.0	0.0		1.8

[a] For details, see footnotes under Table I. [b] Cytochrome *c* (Cyt^{3+}), chymotrypsinogen (CTgen), ribonuclease (RNase), myoglobin (Mb), and phosvitin (PV) were treated identically by the procedure used in the "standard" experiment depicted in Figure 1. All protein concentrations were the same on a weight basis.

Fe^{2+} (experiment 3) has a similarly inhibitory effect. Ferrous ion cannot be replaced by Fe^{3+} (experiment 4), Mg^{2+} (experiment 5), or Cu^{2+} (experiment 6). The replacement of *ferri*cytochrome *c* by *ferro*cytochrome *c* in the initial reaction mixture is without consequence: the reaction product incorporates isotope (experiment 7) similarly to the product of the standard reaction (experiment 1).

Analogous control experiments with Tris-buffered reaction mixtures yielded similar results (data not shown). Both O_2 and Fe^{2+} are required for the generation of the major isotopically labeled product and no reaction occurs with Fe^{3+}, or with Fe^{2+} in a nitrogen atmosphere. Also, ferrocytochrome *c* is fully as reactive as the oxidized heme protein.

Completion of the oxidative phase of the reaction is assured under the conditions of these experiments. Prolonging the time of reaction between protein and Fe^{2+} from 30 min to 20 hr makes essentially no difference. Indeed, a reaction time of only a few minutes was found to be sufficient to yield results similar to those given in Figure 1.

The reductive phase of the overall reaction, the reaction with sodium borohydride, was also essentially complete under these conditions. Neither a doubling of the borohydride concentration nor an extension of the reaction period from 20 min to 3 hr made an appreciable difference in either the nature or the yield of the reduced components.

An appreciable fraction of the amount of isotope which appears at the "front" and "tail" of the elution profiles of the AN and B columns, respectively, is an artifact derived from the reducing agent. The amount of this artifact varies with the particular lot of [^{3}H]sodium borohydride. The reducing agent by itself does not contribute, however, any radioactivity to the resolving elution range of these columns.

The reaction with ferrous ion appears to cause no appreciable change in the cytochrome molecule other than the reduction of the heme iron (Taborsky, 1972) and the apparent oxidation of some amino acid side chains. In particular, no significant cleavage of the protein is noticeable under the conditions of these experiments. When a reduced reaction product is passed through a column of Sephadex-G-25, the elution of heme protein and the elution of radioactivity coincide. Also, the amino acid content of the reaction product derived from a ferricytochrome *c*–ferrous ion reaction mixture, after repeated washing with trichloroacetic acid, is in essential agreement with the composition of the intact heme protein (Margoliash *et al.*, 1962), with the exception of methionine which is low.

Stoichiometry. Assuming the incorporation of one labeled hydrogen atom per molecule of protein and the absence of an isotope effect (Lustenberger *et al.*, 1972), the apparent yield of the quantitatively most significant labeled component is of the order of 0.1 mol/mol of protein. For example, in the experiment depicted in Figure 1, the radioactivity associated with the major product (P-AN-49) is equivalent to 0.08 mol/mol of cytochrome *c*. However, this estimate is misleadingly low because acid hydrolysis of the protein is accompanied by significant isotope exchange with the solvent. For example, in a typical experiment, about 30% of the radioactivity present at the end of hydrolysis was lost upon evaporation of the hydrolyzate to dryness. Subsequent, repeated dissolution of the residue in water and drying caused little further loss. In addition, it is possible that the multiplicity of labeled products is due, partly or wholly, to a partition of a unique, primary reaction product *via* several, secondary reactions. (The *total* amount of radioactivity incorporated in the experiment in phosphate buffer, depicted in Figure 1, corresponds to 0.31 mol/mol of cytochrome *c*, without correction for exchange losses.)

Reactions with Other Proteins. The scope of the generality of this reaction was explored with four additional proteins—chymotrypsinogen, ribonuclease, myoglobin, and phosvitin. They were treated and analyzed in the same manner as cytochrome *c*. After reaction in phosphate buffer, their chromatographic profiles—with one qualification—were congruent with the cytochrome profiles shown in Figure 1. The sole difference was that all additional proteins yielded one extra

TABLE III: Yield of Products Derived from Synthetic Polypeptides.[a]

Expt No.	Polypeptide[b]	Buffer	Relative Yield of Radioactive Product: Total Hydrolyzate	Chromatographic Component: AN-49	B-105	B-123	B-128	B-144	B-ALK
1	Cyt^{3+}	P_i	1.0	1.0	1.0	1.0	1.0	1.0	1.0
2	PGA	P_i	0.4	0.0	0.0	0.0	0.0	0.0	0.5
3	PL	P_i	2.9	0.1	6.8	6.8	5.2	5.9	5.3
				B-115		B-169	B-179		B-ALK
1′	Cyt^{3+}	Tris	1.0	1.0		1.0	1.0		1.0
2′	PGA	Tris	0.4	0.0		0.0	0.0		1.2
3′	PL	Tris	1.8	2.2		0.0	4.3		2.0

[a] For details, see footnotes under Table I. [b] Poly-L-glutamic acid (PGA) and poly-L-lysine (PL) were treated identically by the procedure used in the "standard" experiment depicted in Figure 1. All protein or polypeptide concentrations were the same on a weight basis.

product, P-AN-53. This product occurs in the elution position of serine. It may be noted that all proteins but cytochrome *c* include serine in their amino acid composition. It is noteworthy that the susceptibility of cytochrome *c* to this reaction appears to be markedly enhanced, compared with the other proteins. Lines 1–5 of Table II show that the total amount of incorporated isotope as well as the amount introduced into any of the six most significant chromatographic components is maximal in the case of the cytochrome.

In contrast, with Tris-buffered reaction mixtures (shown on lines 1′–5′ of Table II) none of the five proteins appears as a particularly distinguished reactant. Phosvitin is outstanding in that it yielded none of the dominant components typical of the Tris-buffered reaction products of the other proteins. This deviation from the "norm" has its probable explanation in the highly unusual nature of this protein, containing about 60 residue % of *O*-phosphoserine (Allerton and Perlmann, 1965). We assume that the typical difference between Tris- and phosphate-buffered reactions reflects a difference in the nature of the reactive species in the two buffers. In phosphate, this species is assumed to be a complex constituted from iron, phosphate, and the protein (see Discussion). In Tris, of course, such a complex could not form. But the profusely phosphorylated phosvitin molecule can be expected to be free of the influence of *inter*molecular interactions of this type: its phosphate-containing iron-binding sites are covalently built in. Indeed, product analysis of phosvitin reaction mixtures shows that the product pattern is independent of the buffer and is similar to the product pattern obtained with other proteins only in phosphate buffer (compare line 6 with lines 1–5 in Table II).

Reactions with Synthetic Polypeptides. Since the oxidative modification of carboxylic acid side chains was considered unlikely, the use of polyglutamic acid appeared desirable to test if isotope incorporation occurred into a presumably inert polypeptide. The data in Table III show that tritium incorporation into polyglutamic acid, after its exposure to Fe^{2+} and O_2, restricted to material which could be eluted only with strong base. Much of this isotope is ascribable to an acid-stable impurity in the reductant.

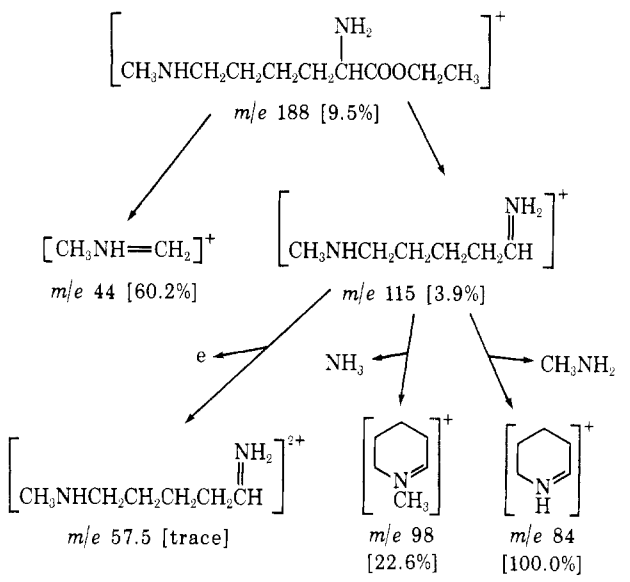
The basic nature of a number of the labeled products derived from the several proteins suggested that they might be derivatives of basic amino acids. Experiments with polylysine narrowed this possibility to lysine residues. The elution patterns obtained with polylysine matched those produced in the protein reactions as far as the dominant basic components were concerned (Table III). Compared with cytochrome *c*, polylysine in phosphate buffer (line 3) gives all of the significant basic products in good yield, but only a trivial proportion of the isotope appears in the position of the major acidic–neutral product derived from the cytochrome and the other proteins (P-AN-49). In Tris buffer also, polylysine produces all but one of the significant protein products (line 3′).

Identity of Products. The major products (P-AN-49 and T-B-179, in Figure 1) appear to be identical with the amino acids with which they coincide in the effluent patterns: threonine and ε-*N*-methyllysine. This inference drawn from their chromatographic behavior has been corroborated by additional evidence.

The putative threonine has been exposed to hot alkali overnight: an aliquot of the acid hydrolyzate of a phosphate-buffered, cytochrome *c* containing reaction mixture was treated with 4 N $Ba(OH)_2$ at 110° for about 20 hr. The Ba^{2+} was then precipitated as the carbonate (a negligible amount of radioactivity precipitated in this step). A major portion of the radioactivity associated with the original acid hydrolyzate became exchangeable with solvent and was removed upon evaporation of the alkali-treated solution to dryness (loss of tritium about 40%). Upon rechromatography of this solution, radioactivity was found in the threonine position only to the extent of about one-third of the original amount. The results of this experiment are consistent with the view that the material eluted in the threonine position of the chromatogram obtained with the original reaction product is largely if not wholly threonine itself.

The material was shown to be identical with authentic threonine also by thin-layer chromatography on silica gel (Silica Gel G plates; Quantagram) with three different solvent systems. The product P-AN-49 was isolated by ion exchange chromatography of a pooled sample of the hydrolyzates of several typical reaction mixtures. A column of Aminex A-5 resin (Bio-Rad; 0.9 × 25 cm) was used, operated with 0.1 M pyridine–acetate, pH 2.9, at 52°. Under these conditions, the putative [^{3}H]threonine was essentially fully resolved from

SCHEME I



[^{14}C]glutamic acid which had been added to the hydrolyzate as a marker. The ^{3}H-containing effluent was collected, combined, dried in the flash evaporator, and was taken up in water and dried again, three times. The final residue was dissolved in a small volume of 0.1 N HCl and used for thin-layer chromatography.

The solvents were chosen to yield R_F values for threonine over a wide range (about 0.2–0.8), based on data summarized by Brenner *et al.* (1969). With a *n*-butyl alcohol–acetic acid–water mixture (80:20:20), authentic threonine and the "unknown" migrated with indistinguishable, low R_F values of 0.30 in parallel lanes of the chromatogram. Threonine was visualized with a ninhydrin spray; the "unknown" was located by the position of isotope (counting scrapings of the thin layer mixed with scintillation fluid). With two other solvent systems, the "unknown" was mixed with authentic threonine before its application to the chromatographic plate. After development, it was found that ninhydrin reactivity and radioactivity coincided whether the solvent system permitted migration of threonine with an intermediate R_F (*n*-propyl alcohol–ammonium hydroxide, 70:30) or with a high R_F (chloroform–methanol–ammonium hydroxide, 40:20:20). In these experiments, samples were applied to the thin layer in different amounts; the small but significant variations in R_F based on ninhydrin reactivity were noted to be matched by corresponding variations in R_F in terms of isotope position.

The putative ε-*N*-methyllysine was subjected to mass spectrometry. For this analysis, a Tris-buffered reaction mixture of cytochrome *c* was hydrolyzed as usual and chromatographed on an Aminex MS column, operated with 0.5 M pyridine acetate buffer (pH 5.5). The radioactive material which appeared after lysine (*cf.* Figure 1, panel T-B, which is comparable with the elution pattern obtained with the Aminex column except that the latter column does not resolve the small satellite peak at position 169 from the major peak at 179) was further purified by ascending paper chromatography (Whatman 3 MM; ethanol–*tert*-butyl alcohol–formic acid–water in proportions of 60:20:5:15), and by paper electrophoresis (Whatman 1; 1.0 M acetic acid, 30 min, 30 V/cm). It was then esterified with 10% ethanolic HCl (2 hr, 80°, in a closed vial), evaporated to dryness, and converted to the free base with a solution of NH_3 in CH_2Cl_2, removing the resulting NH_4Cl by filtration. The mass spectrum of the free ester was recorded on an AEI MS-9 instrument (source temperature 130–150°, 70 eV, direct sample insertion probe fitted with a capillary tube containing the sample).

The fragmentation pattern resembled very closely the mass spectrum of ε-*N*-methyllysine ethyl ester obtained by Murray (1964). The most significant fragments included all key fragments on which Murray's structural interpretation rested. They are summarized in Scheme I, indicating their mass units and their relative abundance as measured in our experiments (fragment 84 being the "base peak"). They include the molecular ion (188), the amine fragment (115), and the cyclized products (98 and 84) derived from the amine fragment by elimination of NH_3 and CH_3NH_2, respectively (*cf.* Biemann *et al.*, 1961).

Assuming that ε-*N*-methyllysine arises in our experiments by reduction of a Schiff base formed from lysine and formaldehyde (*cf.* Means and Feeney, 1968; Page and Benditt, 1970; see also Discussion), it would be expected that hydrogen from the metal hydride would be incorporated into the methyl group of the reduced derivative. This hydrogen atom would be retained in fragments 188, 115, and 98, but not in 84 (*cf.* Scheme I). This expectation was fully borne out when we compared two mass spectra, obtained with identically prepared derivatives except that the reduction was carried out in one case with [^{1}H]sodium borohydride, in the other case with [^{2}H]sodium borohydride (deuterium content 98%). (Both reducing agents contained a negligible amount of tritium for tracer purposes.) The two spectra were nearly identical except for a single mass unit difference between several major fragments, including the pairs 188–189, 115–116, and 98–99. In both spectra, fragment 84 was the base peak; no shift to 85 occurred upon deuteration.

A fragment of mass 44 (see Scheme I) is generally not useful for diagnostic purposes because it is also the molecular ion of a common contaminant, CO_2. In this case, however, it is significant that in the spectrum of the deuterated derivative the abundance ratio 45/44 was 5.6 while in the spectrum of the "normal" derivative, the same ratio was only 0.2. Since an "α cleavage" (*cf.* Budzikiewicz *et al.*, 1964) might be expected to occur in the methyllysine skeleton with respect to both amino nitrogens, fragments 44 or 45 might be expected to occur also, depending on the mass of the hydrogen isotope in the reducing agent. Clearly, the large increase in the abundance ratio 45/44 upon deuteration is consistent with this interpretation.

Additional corroboration of this structural interpretation came from the presence of a fragment with the nonintegral *m/e* ratio of 57.5. Evidently, this fragment is a doubly charged ion of mass 115. The large amine fragment has this mass and, in view of the presence of two nitrogen atoms in its structure, it is reasonable to expect it to have a propensity for the loss of a second electron. No fragment with this nonintegral *m/e* ratio could be seen in the spectrum of the deuterated derivative and none should be expected if our interpretation is correct.

Finally, it should be noted that when the esterified derivative was subjected to chromatography on the "basic" column, it could not be eluted except in the alkaline wash fluid. However, after acid hydrolysis of the derivative, the material reappeared in the same effluent position as before esterification. This behavior is consistent with the view that the substance is a basic amino acid.

Discussion

These results suggest that protein oxidation in the presence of Fe^{2+} and O_2 may be a general phenomenon.[2] The potential oxidizability of ferrous ion–protein systems should be of interest because of the participation of Fe^{2+} in many enzymic reactions and the not infrequently observed instability of such enzymes. A recent example is the rapid irreversible loss of activity by tyrosine hydroxylase when incubated with Fe^{2+} in air (Petrack *et al.*, 1972).

The ultimate oxidant is undoubtedly O_2 since the reaction does not occur in its absence. Whether electrons move directly to O_2, perhaps within a coordination complex involving protein side chains and oxygen as ligands (*cf.* Kaden and Fallab, 1961), or whether some already partially reduced oxygen derivative (such as H_2O_2)—produced at the expense of Fe^{2+}—serves as the electron acceptor is yet to be discovered.

Although the multiplicity of reaction products suggests that the reaction is not a simple one, the predominance of single products is an indication of a relatively "clean" reaction. If the dominant product of the phosphate-buffered reaction is threonine, it could arise after initial oxidation of a threonine side chain to the corresponding ketone from which borohydride would be expected to regenerate the alcoholic side chain.[3] If, in Tris-buffered reactions, ϵ-*N*-methyllysine arises from the reductive alkylation of lysine residues (as noted earlier), then the source of the Schiff base forming formaldehyde is yet to be identified. Presumably, it is generated by an oxidative reaction involving a protein side chain. This appears to be the only possible carbon source: in the presence of tris(hydroxymethyl-^{14}C)aminomethane—the potential alternative source of carbon—no incorporation of ^{14}C into the product could be noted. The suggestion that the source of the formaldehyde is some side chain of the protein is not inconsistent with the finding that the amino acid composition of the cytochrome *c* product is essentially unchanged—with the exception of methionine which is lowered—compared with the composition of the intact protein. Some loss of any of several side-chain types, in amounts consistent with the yield of the reaction products, would not significantly affect the amino acid analytical data.

Among other products, the material P-B-ALK represents a relatively appreciable amount of isotope. It is not known if this material is a single product or a mixture. Its elution with a variety of elution programs occurred only under "extreme" conditions (strong alkali in the case shown in Figure 1) when resolving capability would be expected to be low. This material as well as some of the other, minor products could represent further degraded stages of the primary reaction products.

The extensive agreement between product profiles obtained with the several proteins (Table II) and with polylysine (Table III) suggests that most, if not all, basic components observed in these chromatograms are derived from lysine. In terms of their effluent positions, the relatively minor components appear to correspond to the spontaneously produced lysine derivatives observed in collagen (Bello and Bello, 1967; Schiffman and Martin, 1970; Deshmukh *et al.*, 1971). Should their identity be established, then the nonenzymic oxidation of polylysine might serve as a model for the lysine oxidase reaction by which, presumably, biological cross-link formation in collagen is initiated (Kivirikko and Prockop, 1972).

The striking difference between phosphate- and Tris-buffered reactions must reflect an essential difference in the manner in which protein, ferrous ion, and oxygen interact in the two buffer systems. Whenever phosphate is available, the reactive species may be a complex of protein, iron, phosphate, and oxygen. In Tris then, the interaction would necessarily take a different course (except in the special case when protein-bound phosphate obviates the need for added phosphate and eliminates the buffer dependence of the reaction).

Regarding the enhanced reactivity of the cytochrome in phosphate buffer, it may be argued that the enhancement is more apparent than real because the comparison was made between cytochrome *c* and the other proteins on a weight basis and not, more appropriately, on the basis of susceptible amino acid residue content, such as lysine. Without a thorough view of the mechanism of the reaction it is difficult to counter this argument because this problem, in its essence, is inherent in any gross comparison between proteins. However, had we constructed Table II on the basis of the lysine content of the several proteins, it would still show that the production of components B-123, B-128, and B-144 (that is, all but B-105 among the basic products; *cf.* Figure 1, panel P-B) is significantly enhanced in the cytochrome reaction relative to the others, except phosvitin. Per lysine residue, phosvitin's susceptibility is as good or better than that of the cytochrome, and polylysine is very similar to cytochrome *c* in this respect. It is suggestive to consider that lysine residues are known to be adjacent to phosphoserine residues in phosvitin (Belitz, 1965), juxtaposing perhaps the oxidizable side chain with the iron-binding side chain. In polylysine, the lysine side chains might serve both functions once phosphate is bound electrostatically by some of them. Cytochrome *c* might provide such a phosphate binding site as well, in view of the known clustering of lysine side chains on its surface (Dickerson *et al.*, 1971).

A particularly efficiently constructed binding site in cytochrome *c* could account for its enhanced reactivity. Since phosphate also enhances the reduction of the cytochrome *c* heme group by ferrous ion, it seems reasonable to speculate that both enhancements may be consequences of the particular nature of the cytochrome–iron–phosphate complex.[4] This hypothesis suggests an approach to the problem of electron entry to the heme iron (*cf.* Dickerson *et al.*, 1971; Morton *et al.*, 1970; Chance *et al.*, 1968; Winfield, 1965). Since the oxidative side chain modification would be expected to occur in spatial proximity to the postulated binding site, it would leave a chemical trace near the site from which electrons moved toward the "buried" heme group. If such a postulated site existed, in fact, location of the modified side chains would help define the site where electron transfer began. This approach is conceptually similar to Kowalsky's (1969) who studied the interaction of cytochrome *c* with chromous ion except in that case the metal itself was intended to provide the trace.

[2] In an earlier paper (Grant and Taborsky, 1966), we referred to some of the numerous reports which attest to the autoxidizability of both metal ion and ligand group, in a variety of ferrous complexes. These reports deal mostly with complexes of low molecular weight. Proteins appear not to have been studied extensively in this regard.

[3] This reaction is analogous to the oxidation of serine residues observed in a study of phosphoprotein oxidation under similar conditions (Grant and Taborsky, 1966; Rosenstein and Taborsky, 1970).

[4] The hypothesis of a reactive cytochrome–iron–phosphate complex stems from our earlier observation that even very low concentrations of phosphate can effectively enhance the reduction of the cytochrome heme iron by ferrous ion (Taborsky, 1972). Phosphate binding by cytochrome *c* has been shown to occur (Barlow and Margoliash, 1966), and functional significance has been attributed to such binding (Schejter and Margalit, 1970; Margoliash *et al.*, 1970).

The possibility of artifacts arising from the use of borohydride must be considered. Some small peptides have been shown not to undergo reductive cleavage (Yonemitsu *et al.*, 1968) but fragmentation of some proteins has been reported (Crestfield *et al.*, 1963; Paz *et al.*, 1970). However, neither of the major products in our experiments appears to have arisen by reductive peptide bond cleavage. Collagen which is known to be subject to reductive cleavage (Paz *et al.*, 1970) yields no appreciable amounts of any α-amino *alcohol* which would co-chromatograph with threonine (Gallop *et al.*, 1968; Tanzer and Mechanic, 1970). The alkali lability of the material which does co-chromatograph with threonine on an ion exchange column and on thin layers developed with three different solvent systems is consistent with its tentative identification with the amino *acid*. The other major product, eluted in the position of methyllysine, is also most unlikely to be an amino alcohol. Since it is practically certain that this compound is a lysine derivative (note that it can be obtained from polylysine in good relative yields), were it the reduction product of a lysyl peptide, lysinol, it would not be expected to be eluted from the "basic" analyzer column at all, except with alkali (Gallop *et al.*, 1968). Its esterifiability also argues against its being an amino alcohol, and so does the incorporation of only a single hydrogen atom (from borohydride) per molecule—as shown by the experiment with [^{2}H]borohydride, done in conjunction with the mass spectrometric analysis of this product. Finally, the inhibition of the formation of the major products of our reactions when O_2 or Fe^{2+} is omitted, even when other cations are present, argues for the supposition that their formation requires an oxidative reaction for the production of borohydride-reducible material.

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References

Allerton, S. E., and Perlmann, G. E. (1965), *J. Biol. Chem. 240*, 3892.

Barlow, G. H., and Margoliash, E. (1966), *J. Biol. Chem. 241*, 1473.

Belitz, H.-D. (1965), *Z. Lebensm.-Unters. Forsch. 127*, 341.

Bello, J., and Bello, H. R. (1967), *Biochim. Biophys. Acta 147*, 272.

Biemann, K., Seibl, J., and Gapp, F. (1961), *J. Amer. Chem. Soc. 83*, 3795.

Brenner, M., Niederwieser, A., and Pataki, G. (1969), *in* Thin-Layer Chromatography, Stahl, E., Ed., 2nd ed, New York, N. Y., Springer-Verlag, p 740.

Budzikiewicz, H., Djerassi, C., and Williams, D. H. (1964), Interpretation of Mass Spectra of Organic Compounds, San Francisco, Calif., Holden-Day, p xiii.

Chance, B., Lee, C.-P., Mela, L., and DeVault, D. (1968), *in* Structure and Function of Cytochromes, Okunuki, K., Kamen, M. D., and Sekuzu, I., Ed., Tokyo, University of Tokyo Press, p 475.

Crestfield, A. M., Moore, S., and Stein, W. H. (1963), *J. Biol. Chem. 238*, 622.

Deshmukh, A., Deshmukh, K., and Nimni, M. E. (1971), *Biochemistry 10*, 2337.

Dickerson, R. E., Takano, T., Eisenberg, D., Kallai, O. B., Samson, L., Cooper, A., and Margoliash, E. (1971), *J. Biol. Chem. 246*, 1511.

Gallop, P. M., Blumenfeld, O. O., Henson, E., and Schneider, A. L. (1968), *Biochemistry 7*, 2409.

Grant, C. T., and Taborsky, G. (1966), *Biochemistry 5*, 544.

Joubert, F. J., and Cook, W. H. (1958), *Can. J. Biochem. Physiol. 36*, 399.

Kaden, T., and Fallab, S. (1961), *in* Advances in the Chemistry of the Coordination Compounds, Kirschner, S., Ed., New York, N. Y., Macmillan, p 654.

Kivirikko, K. I., and Prockop, D. J. (1972), *Biochim. Biophys. Acta 258*, 366.

Kowalsky, A. (1969), *J. Biol. Chem. 244*, 6619.

Lustenberger, N., Lange, H.-W., and Hempel, K. (1972), *Angew. Chem. 84*, 255.

Margoliash, E., Barlow, G. H., and Byers, V. (1970), *Nature (London) 228*, 723.

Margoliash, E., Kimmel, J. R., Hill, R. L., and Schmidt, W. R. (1962), *J. Biol Chem. 237*, 2148.

Means, G. E., and Feeney, R. E. (1968), *Biochemistry 7*, 2192.

Morton, R. A., Overnell, J., and Harbury, H. A. (1970), *J. Biol. Chem. 245*, 4563.

Murray, K. (1964), *Biochemistry 3*, 10.

Page, R. C., and Benditt, E. P. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 9*, 49.

Paz, M. A., Henson, E., Rombauer, R., Abrash, L., Blumenfeld, O. O., and Gallop, P. M. (1970), *Biochemistry 9*, 2123.

Petrack, B., Sheppy, F., Fetzer, V., Manning, T., Chertock, H., and Ma, D. (1972), *J. Biol. Chem. 247*, 4872.

Rosenstein, R. W., and Taborsky, G. (1970), *Biochemistry 9*, 649.

Schejter, A., and Margalit, R. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 10*, 179.

Schiffman, E., and Martin, G. R. (1970), *Arch. Biochem. Biophys. 138*, 226.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.

Taborsky, G. (1963), *Biochemistry 2*, 266.

Taborsky, G. (1972), *Biochemistry 11*, 729.

Tanzer, M. L., and Mechanic, G. (1970), *Biochem. Biophys. Res. Commun. 39*, 183.

Winfield, M. E. (1965), *J. Mol. Biol. 12*, 600.

Yonemitsu, O., Hamada, T., and Kanaoka, Y. (1968), *Tetrahedron Lett. 32*, 3575.