FORMATION OF INSOLUBLE GELS AND DITYROSINE BY THE ACTION OF PEROXIDASE ON SOLUBLE COLLAGENS[1]

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A probable peptide crosslinking moiety, dityrosine (formed by a biphenyl linkage between two tyrosine residues), has been identified in resilin, a rubber-like protein from insects (Andersen, 1965), and evidence has been presented for its presence in vertebrate elastin (LaBella et al., 1967). The identification of traces of dityrosine in some, but not all, preparations of mammalian collagen (see below) led us to propose that this aromatic substance may be concerned with physiological crosslinking reactions in collagen, also. Purified preparations of neutral-salt soluble and acid soluble collagen consist mainly of long-chain polypeptides in either monomer or dimer form, the proportions of the two depending upon the tissue source. These "soluble" collagens are readily converted into aggregates and gels of varying stabilities by a wide variety of physical and chemical procedures. Peroxidase has been shown to catalyze the formation of dityrosine from free tyrosine but in only a very limited number of protein substrates (Gross and Sizer, 1959; Andersen, 1966). In the present investigation, incubation of highly purified soluble collagens with peroxidase, in the presence of H_2O_2, resulted in dramatic and rapid gel formation, as well as in the formation of readily detectable amounts of dityrosine.

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MATERIALS AND METHODS

The skin from 12, 28, or 56 day old male rats of a hooded strain was used as the source of collagen. Neutral-salt soluble (NS) collagen was prepared by extraction with cold 0.4 M NaCl and purified by repeated precipitation with concentrated NaCl and dissolution with 0.4 M NaCl. Acid soluble (AS) collagen was extracted from the residue with 0.5% acetic acid and purified by repeated precipitation with concentrated NaCl and dissolution with 0.05% acetic acid. "Insoluble" (IS) collagen was prepared from the residue by the Nishihara technique (Steven, 1965). Amino acid analyses of NS and AS collagens indicated as high a state of purity as would be expected. H_2O_2 (0.4 ml of a 0.1% solution) and 0.4 mg of horseradish peroxidase (Type II, Sigma Chem. Co.) were added to 10 ml buffer containing 10 mg of collagen, and the pH of the solution was adjusted to 7.4 for NS collagen and 3.5 for AS collagen. Incubations were carried out at 37° for NS collagen and at 23° for AS and IS collagen. Controls included incubation mixtures containing H_2O_2 but no peroxidase and others in which both H_2O_2 and enzyme were absent. Amino acid analyses of protein hydrolysates were carried out on a Technicon amino acid analyzer (LaBella et al., 1966), and the column effluent was monitored for fluorescence with an Aminco-Bowman photofluorometer equipped with a flow-through cuvette. Two amino acid separation systems were used: a "short", 6-hour system using a 0.63 x 75 cm column at 60° or 40°, and a "long", 18-hour system using a 0.63 x 140 cm column at 65°. At the acid pH of the buffers used in the amino acid separation, optimal fluorescence activation and omission wavelengths for dityrosine are 290 and 410 $m\mu$, respectively. Authentic dityrosine was prepared enzymatically (Gross and Sizer, 1959) and purified as previously described (LaBella et al., 1967).

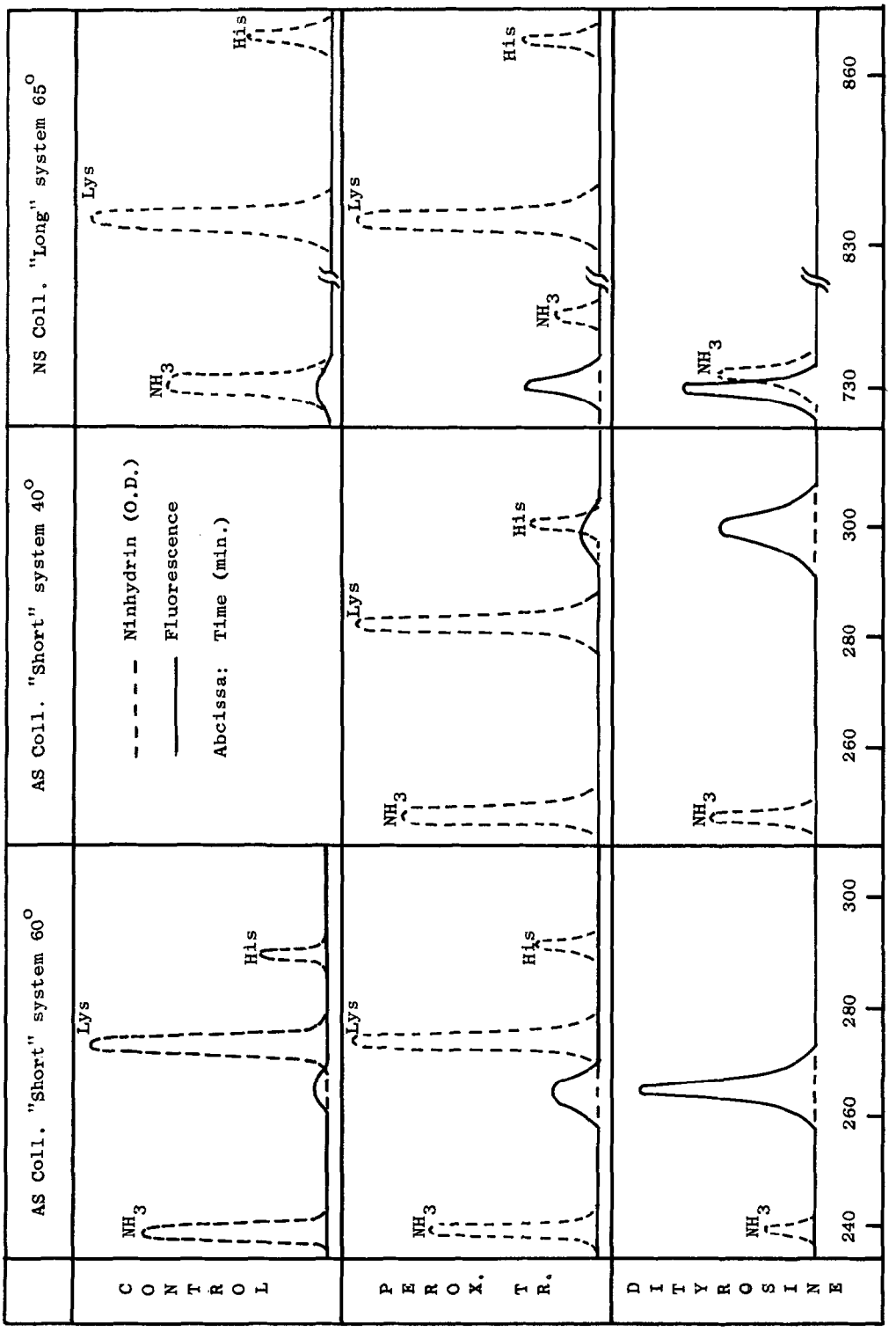
RESULTS

The addition of H_2O_2 and peroxidase to a test tube containing 0.1%

solution of AS or IS collagen at 23° resulted, within a few minutes, in the formation of a clear, solid gel. The uniformly dispersed gel was very rigid and not displaced when the test tube was inverted. Incubation of NS collagen with H_2O_2 plus peroxidase at 37° resulted in the formation of a gelatinous precipitate within 30 minutes; a smaller, flocculent precipitate developed much later in the control tube lacking the enzyme. Treatment of AS collagen with pepsin to remove telopeptides (Rubin et al., 1963) prevented gel formation by peroxidase. The gels or precipitates formed in the presence of peroxidase were extremely resistant to dissolution (Table). Dityrosine in collagen samples was identified on the basis of its fluorescent properties and its mobility in the three chromatographic systems, in comparison to authentic dityrosine (Figure). Not all preparations of untreated collagens showed the presence of dityrosine. However, dityrosine was readily detected in all collagen preparations which had been treated with peroxidase and was increased in amount in those preparations which already contained it (Figure). The reason for the inconsistent detection of dityrosine in purified, untreated collagens is unknown, but it does not appear to be age differences among animals. No detectable differences in the amino acid composition were found between peroxidase-treated and untreated collagens, including differences in the concentration of tyrosine

EFFECT OF PEROXIDASE ON SOLUTIONS OF NS, AS AND IS COLLAGENS

	Preparation	Result
NS	Control	Ppte. forms over several hours; completely soluble at 4°.
NS	Peroxidase	Ppte. forms within 30 min.; insoluble several hours at 4°; 5% soluble in 0.05% HAc and 45% soluble in 0.5% HAc after several hours at 23°.
AS	Control	No gel or ppte. formed.
AS	Peroxidase	Rigid gel forms in a few minutes; insoluble in 0.5% HAc after several hours at 23°.
AS	Pepsin-treated, dialyzed; then peroxidase	No gel or ppte. formed.
IS	Control	No gel or ppte. formed.
IS	Peroxidase	Rigid gel forms in a few minutes; insoluble in 0.5% HAc after several hours at 23°.


AS Coll. "Short" system 60°
AS Coll. "Short" system 40°
NS Coll. "Long" system 65°
Ninhydrin (O.D.)
Fluorescence
Abcissa: Time (min.)
CONTROL
PEROX. TR.
DITYROSINE
NH3
Lys
His
240
260
280
300
730
830
860

(from which dityrosine is derived). The amount of dityrosine present in the collagens, even after exposure to peroxidase, was insufficient to yield a ninhydrin-positive peak, consequently, the amino acid was identified in the chromatogram by its fluorescence. From the fluorescence yield of known amounts of authentic dityrosine, it was estimated that the dityrosine, in those preparations of the native collagens which contained it amounted to about 1 residue/100,000 total residues; this value increased by as much as twenty fold after treatment with peroxidase i.e. to 1/5,000.

DISCUSSION

Peroxidase, in the presence of H_2O_2, is known to produce dityrosine when reacted with tyrosine or with some proteins (Gross and Sizer, 1959; Andersen, 1964; 1966). It appears probable that, in the present study, the changes induced in the physical properties of the soluble collagens by the action of peroxidase are at least partly due to the result of dityrosine formation between pairs of tyrosine residues, each member of the pair situated on a separate peptide chain. Removal of the tyrosine-rich telopeptides, shown to be concerned with crosslinking in collagen (Rubin *et al.*, 1963), prevented these peroxidase-induced changes from taking place. The number of dityrosine crosslinks is quite small; differences in tyrosine content, as well as other amino acids, between control and peroxidase treated collagen are within the limits of error in the method of determination. (The increase in dityrosine produced by the action of peroxidase amounts to only about 0.1 residue/1000 total residues in the protein, i.e. a decrease of only 0.2 residues of tyrosine/1000 residues.)

The products resulting from the action of peroxidase on NS and AS collagens are apparently covalently-crosslinked, high molecular weight polymers, as determined by measurements of viscosity and sedimentation as a function of temperature and by solubility characteristics described above. Furthermore, electronmicroscopy of the ATP-precipitated products of peroxi-

dase action indicates the presence of longer segments than are seen in precipitates of control collagen, presumably resulting from peroxidase-catalyzed end-to-end fibril association. (Details of these physical parameters of peroxidase-treated collagens will be presented elsewhere.)

The detection of minute amounts of dityrosine in some preparations of purified, untreated NS and AS collagen is an important finding, but it must be established that the presence of this potential crosslink in trace amounts is not artifactual. We have examined hydrolysates of many different proteins and have found dityrosine in preparations of collagen and elastin only, suggesting a physiological crosslinking role for this amino acid in the fibrous connective tissue proteins. Resilin, a rubber-like protein from certain insects, is the only other known natural source of dityrosine. Dityrosine formation through the action of ubiquitous peroxidases may represent one type of crosslinking process concerned with polymerization and maturation of collagen.

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