Enhancement of Collagen Aggregation by Catecholamines and Related Polyhydric Phenols*

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ABSTRACT: Catecholamines and related polyhydric phenols retard the dispersion of collagen fibers induced by cooling. At concentrations of 5–50 μM, epinephrine, norepinephrine, 3,4-dihydroxyphenylalanine (dopa), and 3,4-dihydroxyphenylethylamine (dopamine) inhibit collagen solubilization to about the same extent as does ascorbic acid. Catechol, hydroquinone, homogentisic acid, rutin, and caffeic acid also inhibit at these concentrations, but to a lesser extent. Tyrosine, gentisic acid, resorcinol, 2-methylnaphthoquinone, 3-methoxy-4-hydroxymandelic acid, and dehydroascorbic acid are ineffective. In the presence of epinephrine, low concentrations of ethylenediaminetetraacetate or arginine (but not urea) increase the dispersion rate. Tyrosinase lowers the dispersion of controls, and tyrosinase plus tyrosine lower the rate to about the same extent as the catecholamines. The presence of epinephrine or homogentisic acid significantly increases the time-dependent reduction in collagen solubility at 37°. Fibers formed in the presence of epinephrine possess a higher shrinkage temperature and increased resistance to enzymatic and nonenzymatic solubilization. Such changes characterize enhanced aggregation of collagen fibers and suggest a possible role for catecholamines in maturing and aging of collagen

Collagen fibers, newly formed by warming neutral tropocollagen solutions, gradually lose their capacity for redispersion in the cold. These *in vitro* time-dependent changes suggest a possible model for the study of *in vivo* age-dependent changes (Gross, 1958). The present report describes experiments in which low concentrations of several biologically important catechols and hydroquinones, including epinephrine, norepinephrine, rutin, and homogentisic acid, inhibit the redispersion of collagen and promote the time-dependent tendency toward aggregation.

Experimental

The collagen source was tail tendon from male Wistar rats weighing 150–200 g. The insoluble collagen, prepared as described previously (Grant and Alburn, 1960), contained 18.3% nitrogen, 13.5% hydroxyproline, and 0.18% hexosamine. Each gram was extracted twice with 50 ml of 0.5 N acetic acid for at least 24 hours. After centrifugation at 114,000 × *g* for 3 hours, the extract was dialyzed against 60 volumes of 0.13 M potassium phosphate buffer, *p*H 7.4, with two changes. It was then centrifuged at 25,000 × *g* for 1 hour. All steps were performed at 0–5°. The resulting extracts contained approximately 0.5% collagen and had relative viscosities of about 32 at 1°, as measured in an Ostwald-Cannon-Fenske viscosimeter with water-flow time of 7.6 seconds.

Fiber formation was carried out and measured mainly as described by Gross and Kirk (1958). One ml of cold neutral collagen solution was mixed with 4 ml of 0.13 M phosphate buffer containing the test agent and then incubated at 37°. Increasing turbidity was measured on a Klett-Summerson spectrophotometer with a 540-mμ filter. Rigid gelling and maximum turbidity usually appeared within 20 minutes. Additive controls, as well as collagen controls, were run in each experiment, and the formation of colored oxidation products, such as adrenochrome, was negligible. Except when warming time was varied, a standardized 1-hour warming preceded the redispersion in the cold. Extent of fibril-forming capacity was determined on a warmed control system by centrifuging at 114,000 × *g* for 30 minutes and assaying for hydroxyproline distribution; 95% of the amino acid was contained in the pellet under these conditions.

Redispersion was studied by placing the opaque, gelled system in an ice-water bath and measuring the clearing, as fall in turbidity, at various times. Changes were measured at 0.5-hour intervals for 2.5–3.5 hours and again after 17 hours. Liquefaction of the gel became noticeable when turbidity decreased about 50%. Kinetic calculations related to those for the renaturation of γ-gelatin (Drake and Veis, 1964) showed that typical redispersion data fit neither first- nor second-order kinetics; the data were therefore plotted directly as fall in turbidity.

Fibrils formed in the presence of test compounds were subjected to various analyses after separation by centrifugation, repeated washing with water, and air drying. Susceptibility to solubilization was determined in systems containing 5 mg of collagen, 1 mmole of $CaCl_2$, 0.5 mmole of L-arginine, and 50 μg of enzyme (when used) in 5.0 ml of 0.1 M Tris buffer, *p*H 7.4. After incubation for varying times at 37°, analyses were performed by weighing air-dried insoluble residues and by Lowry (Lowry *et al.*, 1951) assays of the filtrates.

Ester analyses were carried out by the hydroxamate

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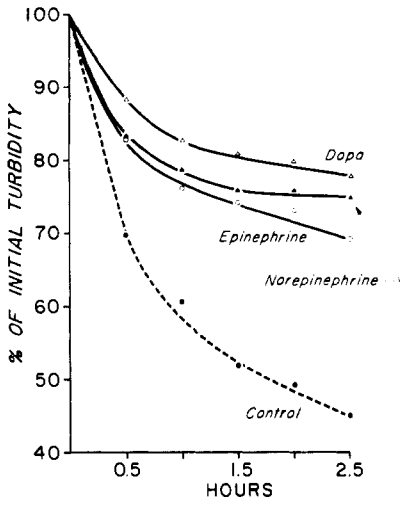

FIGURE 1: Effect of epinephrine, norepinephrine, and dopa on the cold dispersion of collagen. The additive concentration was 50 μM.

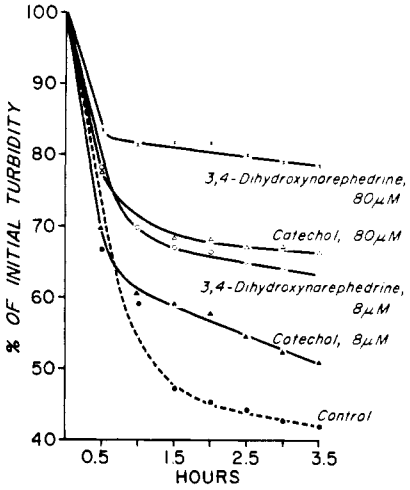

FIGURE 2: Effect of catechol and 3,4-dihydroxynorephedrine on the cold dispersion of collagen.

procedure of Blumenfeld and Gallop (1962) using a 90-minute incubation with hydroxylamine at 40°, followed, without dialysis, by the assay of Hestrin (1949). Free ε-amino groups were assayed by the fluorodinitrobenzene procedure of Solomons and Irving (1958), using a 19-hour reaction period.

Analyses for bound catecholamines were carried out by the spectrophotofluorometric procedure of Price and Price (1957). Samples (5 mg) of collagen pellets were suspended in 2.5 ml of water, heated in a boiling-water bath for 20 minutes, cooled, and diluted with 2.5 ml of water before assay.

The enzymes used were mushroom tyrosinase and crystalline trypsin, both from Worthington Biochemical Corp.; clostridiopeptidase A (collagenase), which was

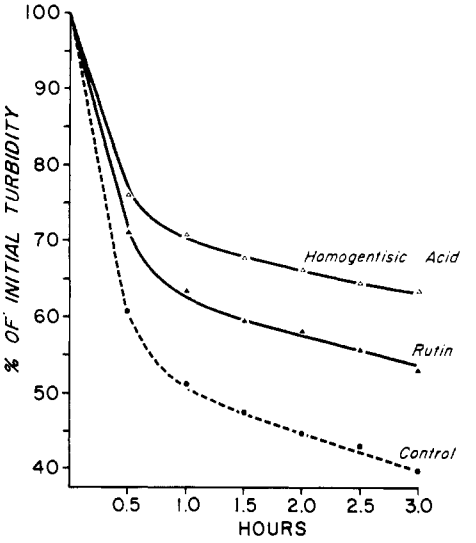

FIGURE 3: Effect of homogentisic acid and rutin on the cold dispersion of collagen. The additive concentration was 50 μM.

free of nonspecific proteinase activity after purification by chromatography on DEAE-cellulose (Grant and Alburn, 1959); and bovine testicular hyaluronidase, purified by ammonium sulfate and containing 200 USP units/mg (Alburn and Whitley, 1951).

Results

At concentrations of 5–50 μM, L-epinephrine, DL-norepinephrine, and DL-3,4-dihydroxyphenylalanine (dopa) significantly inhibited the rate and final extent of redispersion of collagen fibrils in the cold neutral buffer (Figure 1, Table I). Catechol, homogentisic acid, hydroquinone, and rutin were also effective at low concentrations, although less so than epinephrine (Figures 2 and 3, and Table I). Pyrogallol, at 50 and 10 μM, retarded dispersion to about the same extent as epinephrine. *N*-Isopropylnorepinephrine and 3,4-dihydroxynorephedrine also were as effective as epinephrine and norepinephrine, but caffeic acid and protocatechualdehyde (3,4-dihydroxybenzaldehyde), both of which retain the catechol grouping, were even less effective than catechol (Table I). Gentisic acid (2,5-dihydroxybenzoic acid) was ineffective despite its relation to hydroquinone and homogentisic acid. Vanillic acid (3-methoxy-4-hydroxybenzoic acid), which is not a catechol, displayed only a minimum borderline effect. A principal metabolite of catecholamines, 3-methoxy-4-hydroxymandelic acid, did not inhibit dispersion.

Ascorbic acid retarded dispersion about the same as epinephrine at concentrations of 10–50 μM; the two compounds together had an additive effect. Another enediol, dihydroxymaleic acid, retarded dispersion to about one-third the extent of ascorbic acid. Dehydroascorbic acid at 50 μM had no effect.

The ability of epinephrine and homogentisic acid to

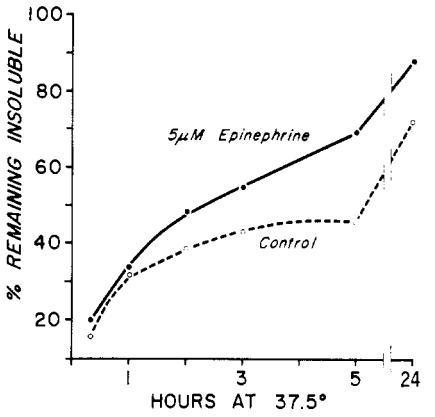

FIGURE 4: Influence of epinephrine on time-dependent solubility changes in collagen.

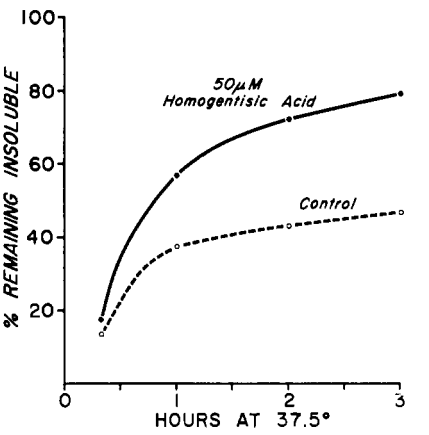

FIGURE 5: Influence of homogentisic acid on time-dependent solubility changes in collagen.

TABLE I: Activity of Various Compounds Relative to Equal Concentrations of Epinephrine.

Favor Aggregation		No Effect
Epinephrine	++++	Tyrosine
Dopa	++++	*p*-Hydroxy-α-methylphenethylamine
Dopamine	++++	
Norepinephrine	++++	2-Methylnaphthoquinone
3,4-Dihydroxynorephedrine	++++	3-Methoxy-4-hydroxymandelic acid
N-Isopropylnorepinephrine	++++	
Pyrogallol	++++	Gentisic acid
Ascorbic acid	++++	Dihydroxyacetone
Catechol	+++	
Hydroquinone	+++	Resorcinol
Homogentisic acid	+++	Glucose
Tolyhydroquinone	+++	
Rutin	++	Dehydroascorbic acid
Dihydroxymaleic acid	++	
Caffeic acid	++	
Vanillic acid	+	
Protocatechualdehyde	+	

affect time-dependent solubility changes of collagen was studied after various fixed periods of incubation at 37°. After each incubation the tubes were chilled to 0–2° and read 1 and 24 hours later. The patterns of results for the two cooling times were similar. Figure 4 compares 5 μM epinephrine with a control on the loss of dispersibility of collagen over a 24-hour period of warming. It is clear that this loss was significantly enhanced by a small amount of epinephrine. Figure 5 shows that 50 μM homogentisic acid similarly lowered the capacity of collagen for dispersion.

No evidence was found that changes occur at an earlier stage that could inhibit redispersion in the cold. At 0–1°, the viscosity changed 5% or less in the presence of 50 μM epinephrine, norepinephrine, dopa, catechol, homogentisic acid, and ascorbic acid. Norepinephrine, dopa, and catechol at 40 μM, and hydroquinone at 10 and 100 μM failed to alter either the lag period or the total amount of fibril formation.

Arginine is known to promote the solubilization of unreconstituted rat tail tendon collagen (Grant and Alburn, 1960, 1964) and to inhibit fibril formation from solutions (Gross and Kirk, 1958). In the presence of 10^{-4} M epinephrine, norepinephrine, or dopa, 0.02 M arginine counteracted the catecholamine effect, promoting dispersion, while 0.02 M urea had no such action (Figure 6). The arginine-induced dispersion was not evident in the absence of a catechol, but in the presence of epinephrine its effect was demonstrable at 10^{-4} M (Figure 7).

The possible formation of phenyl esters was examined by comparing the ester contents of collagen reconstituted by warming in the presence and absence of catechol, epinephrine, norepinephrine, and homogentisic acid. The results showed that no additional ester bonds were formed.

The ease with which catecholamines are oxidized points to the possibility that their oxidation products are the active agents. This equation was studied by imposing upon the catecholamines conditions which retard or promote oxidation or which may block the action of oxidation products. It was also studied by looking for possible decrease in the content of free terminal or ε-amino groups of collagen as a result of reaction with quinones. Ethylenediaminetetraacetate (EDTA) at 50 μM restored the dispersion rates almost to control levels in the presence of 50 μM epinephrine, norepinephrine, and catechol. Benzenesulfinic acid reduces *o*-benzoquinones to *o*-dihydroxydiphenyl sulfones (Bordner and Nelson, 1939), but its presence at 1.7×10^{-4} M

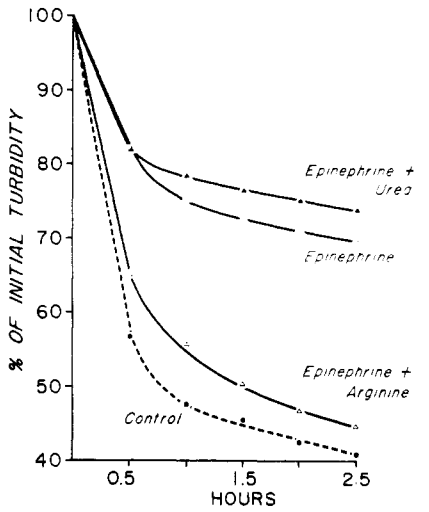

FIGURE 6: Effect of arginine and urea on epinephrine-retarded dispersion of collagen. The epinephrine concentration was 10^{-4} M, that of the other additives 0.02 M.

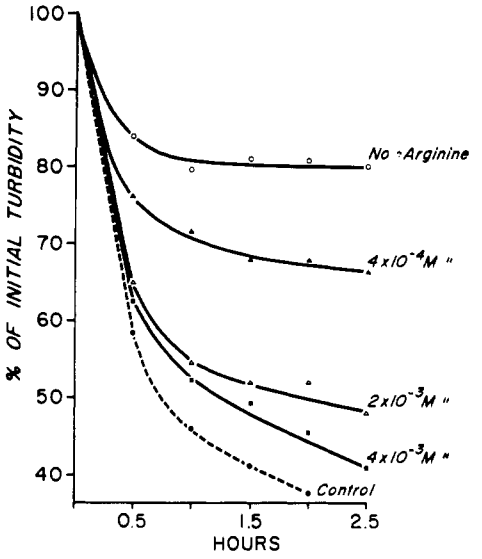

FIGURE 7: Effect of varying arginine concentration on epinephrine-retarded dispersion of collagen. The epinephrine concentration was 10^{-4} M.

failed to reverse the effects of 50 μM epinephrine or norepinephrine.

Nitrogen gassing was carried out with 50 μM epinephrine, norepinephrine, and ascorbic acid. It was done in two steps, using stoppered Klett tubes equipped with a gas inlet tube and a syringe needle for pressure release. Before the gelling procedure, nitrogen was bubbled through the cold solution for 5 minutes to displace dissolved air. At 37°, after completion of gelling, the gas space above the gel was similarly displaced by nitrogen and the system then was sealed and placed in the ice bath. With the catecholamines the rate of collagen solubilization was greater through the entire dispersion period for the systems under nitrogen. After 20 hours at equilibrium, the gassed epinephrine system had a turbidity 33% lower than the nongassed system, while the gassed norepinephrine system had a turbidity 19% lower than its nongassed counterpart. No acceleration of solubilization took place in the ascorbic acid or control systems when tested under nitrogen.

Pretreatment of collagen with either 10^{-5} M epinephrine or 10^{-4} M catechol before fibril formation and dinitrophenylation resulted in both cases in a 25% lower yield of dinitrophenyl products after acid hydrolysis, compared with untreated controls.

To determine the effect of tyrosinase on the redispersion, the enzyme solution was added immediately before the beginning of fibril formation. Reaction mixtures and collagen-free controls contained 40 μg of tyrosinase and 0.25 μmole of tyrosine or epinephrine. During fibril formation, neither the lag period nor the fibril aggregation rate was affected by tyrosine or epinephrine with or without tyrosinase. However, the enzyme significantly altered the rates and final (18 hours) extent of redispersion of each system. The control dispersion decreased from 71% to 50%, that with tyrosine from 71% to 20%, and that with epinephrine from 41% to 24%.

The epinephrine-induced changes in collagen solubility were reflected in changed hydration properties. Fibers were prepared by warming 6 ml of buffered tropocollagen solution with 24 ml of 50 μM epinephrine in the buffer. This precipitate and the control prepared without epinephrine were collected by centrifugation, thoroughly washed with water, blotted with filter paper, and air dried for 4 days. Treated samples, when wet, were visibly less swollen than controls, and they packed more firmly during short periods of centrifugation. They dried more rapidly at first and then more slowly than the control precipitate; the two ultimately reached the same weight.

These dry precipitates formed thin disks with an area of 1.0–1.5 cm^2. During determination of the shrinkage temperatures under constant load, measurable swelling occurred in the control sample at 35°, but not in the epinephrine-treated sample below 50°. The latter shrank at 58°, compared with 55° for the control.

When samples prepared and dried as described were subjected to solubilization by collagenase, trypsin, and the nonenzymatic combination of calcium ion and arginine (Grant and Alburn, 1960, 1964), the resistance of the epinephrine-treated collagen exceeded that of the control collagen in each case. Table II compares the solubilization rates over a 90-minute period.

In order to learn whether the catecholamines or derivatives become firmly bound to or incorporated into the collagen molecule, control precipitates and precipitates prepared by treatment with 10^{-4} M epinephrine or norepinephrine were assayed spectrophotofluorometrically. The amount of either catecholamine detected was

TABLE II: Susceptibility to Additional Solubilization Procedures.[a]

	Rate (mg dissolved per hour)[b] Epinephrine Treated	Control
Collagenase	1.9	2.7
Trypsin	1.4	2.2
Nonenzymatic	0.7	1.3

[a] Composition of reaction mixtures described under Experimental. [b] Gravimetric method.

insignificant (≪1% of the calculated weight in contact with the collagen sample).

When gelatinized solutions of norepinephrine-treated collagen and control collagen were diluted with 0.2 M phosphate buffer (*p*H 8.0) or 0.2 N NaOH instead of water, ultraviolet absorption spectra disclosed no qualitative differences. In 0.1 N NaOH, both curves had peaks at 292 mμ, and in 0.1 M phosphate buffer both had shoulders at 260–277 mμ, and no peaks at higher wavelengths. In each case, the absorbance throughout the range observed was about 50% higher for the catecholamine-treated sample, possibly resulting from the deposition of traces of pigmented oxidation products.

We also considered the possible influence of minute amounts of mucopolysaccharide on fibril formation and dispersion in the presence and absence of epinephrine. Solutions containing 3 ml of buffered collagen were mixed with 6 ml of 0.13 M phosphate buffer containing 0.3 mg of hyaluronidase (60 units) and stored for 65 hours at 2°. One set of controls contained no enzyme and another was stored at 23°. Three ml from each set was mixed with 3 ml of either 10^{-4} M buffered epinephrine or buffer, and rates of fibril formation at 37° were measured. After 1.5 hours, rates of reversal in an ice-water bath were measured. The treatment with hyaluronidase, at a level in excess of that needed to hydrolyze any ground-substance polysaccharide that could be present, had no effect on (1) slow collagen precipitation at 23°, (2) rapid collagen precipitation at 37°, or (3) collagen dispersion at 0–2° in the presence or absence of epinephrine.

Discussion

Our principal finding is that epinephrine, norepinephrine, and related compounds confer on collagen fibers resistance to dispersion quite comparable to that induced by ascorbic acid. Epinephrine is known to induce changes in aortic connective tissue mucopolysaccharides (Lorenzen, 1961), but an interaction between catecholamines and collagen, either direct or indirect, has never been established. Ascorbic acid affects several facets of collagen formation, and in the present study we have confirmed the increased resistance to dispersion (solution) of fibers formed in the presence of ascorbic acid and dihydroxymaleic acid (Candlish and Tristram, 1963). No evidence has been adduced that the catechols, like ascorbic acid, participate in the formation of new collagen fibers. The data suggest participation in cross-linking aggregation reactions which lead to maturing and aging of fibers.

At least three mechanisms may be postulated for the aggregating action of the catecholamines and other effective polyhydric phenols. One is the formation of an array of collagen fibers cross-linked through intermolecular hydrogen bonding with the phenolic hydroxyl groups. The second is a process involving oxidation of the catechol or hydroquinone to the corresponding quinone, direct attachment of collagen amino groups to the quinone nucleus, and, finally, oxidative polymerization reactions. Such a process would resemble that which governs hardening of the cuticle in insects (Hackman and Todd, 1953). A third mechanism is suggested by possible analogy to radiation-induced aging; i.e., the formation of semiquinone-free radicals which, in the course of their necessary rearrangements, promote the cross-linking of neighboring collagen strands. This route to increased aggregation gains interest as the result of the finding that catecholamines form free radicals under the influence of ceruloplasmin (Walaas *et al.*, 1964).

The activity of catechol, pyrogallol, and hydroquinone in inhibiting collagen dispersion, compared with the inactivity of resorcinol, suggests the requirement for a form capable of conversion to a quinone or semiquinone structure. This is supported by the activity of homogentisic acid compared with the inactivity of gentisic acid, in which one hydroxyl is linked by a strong hydrogen bond to its neighboring carboxyl.

The restoring influences of a nitrogen atmosphere and a chelating agent, EDTA, point to participation of an oxidizing step in the actions of the catechols and catecholamines. The relation of this oxidation to free amino groups of collagen is indicated by the lowered yield of dinitrophenyl products. Calculations based on the analyses of Piez *et al.* (1963) indicate approximately 0.3 μmole of amino groups per mg of rat tail tendon collagen. The experimental level of 10^{-4} M epinephrine would therefore provide approximately one molecule of agent for each three amino groups.

Two pieces of evidence fail to support directly a mechanism based upon oxidation of the catecholamine followed by incorporation into the collagen molecule. The inability to detect bound catecholamines or their oxidation products spectrophotofluorometrically indicates (1) that they were in fact not bound; (2) that polymerization reactions proceeded to products insensitive to the assay; or (3) that interfering compounds or groups prevented detection. Restoration of the dispersion rate by benzenesulfinic acid would have added strong support to a mechanism involving quinone formation. Failure to observe an increased dispersion rate may possibly be explained by Bordner and Nelson's (1939) conclusions, in studying *p*-cresol oxidation, that a trace of quinone escaping the reaction of the sulfinic acid can participate in an autocatalytic oxidation leading to polymerization.

If oxidation of the dihydric to the quinone structure were essential, then it appeared likely that tyrosine, which was inactive alone, would resemble the catecholamines when added with tyrosinase. This was the case, and the effectiveness of tyrosinase could further be shown without added tyrosine.

The observation that low concentrations of polyhydric phenols increase collagen fibril stabilization may well be related to the emerging ideas that relatively tyrosine-rich portions of the collagen molecule are involved in intermolecular and interchain cross-linkages (Bensusan and Scanu, 1960; Rubin *et al.*, 1963; Rojkind *et al.*, 1964; Schleuter and Veis, 1964).

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