Reports

Mucopolysaccharides of Costal Cartilage

Probably no other connective tissue has been investigated more frequently than hyaline cartilage. From the time of Boedecker in 1854 to the present, this tissue has been used for the preparation of chondroitin sulfate. In recent years it has been used in studies of the native protein complexes of mucopolysaccharides (*1*) and on biosynthesis of the carbon chain and the incorporation of ester sulfate (*2*). Meyer *et al.* have previously reported on the isolation of chondroitin sulfates from bovine tracheal cartilage and costal and sternal cartilage of newborn children (3). On the basis of optical rotation and solubility of the Ca salts, the mucopolysaccharide fractions appeared to be mainly A (4-sulfate) with a minor C (6-sulfate) component (*4*).

In this report (*5*), attention is called to the isolation of keratosulfate from costal cartilage in two cases of Marfan's syndrome (for literature, see *6*) and from two 23-year-old males with apparently normal skeletal development.

Costal cartilage was obtained at autopsy from two males age 33 and 44 with typical Marfan's syndrome (*7*) who had died of ruptured aneurysm of the aorta and one 23-year-old male who had died suddenly after operation for removal of a brain tumor. The other died after an overdose of a drug. The cartilage was dissected free of extraneous tissue and of perichondrium and dehydrated in acetone. The air-dried tissue was homogenized in 0.1*N* HCl in a high-speed blender, digested with pepsin at *p*H 1.5 in the presence of toluene followed by tryptic digestion at *p*H 7.5. After deproteinization, the mucopolysaccharide fractions were obtained as calcium salts as described previously (*3*).

The total mucopolysaccharide fractions isolated represented 11 and 12 percent of the dry weight of the cartilage in the two cases of Marfan's syndrome and 16 to 18 percent for the controls (*8*). The former consisted of approximately equal quantities of chondroitin sulfate which was characterized as chondroitin sulfate C by analysis, solubility, and infrared spectra and of keratosulfate which was identical in analysis, rotation, and infrared spectrum with that isolated from bovine cornea (*9*). The hexosamine of the keratosulfate fraction was identified by paper chromatography as glucosamine and isolated from one fraction as the crystalline hydrochloride ($[\alpha]_D$ + 72°), and galactose was identified by paper chromatography, and as the crystalline α-methylphenyl hydrazone by melting point and infrared spectrum. The controls yielded chondroitin sulfate and keratosulfate in a ratio of 2.5 to 1 instead of the 1 to 1 ratios in the Marfan's syndrome (*8*).

Thus far keratosulfate, a polymer of unknown structure composed of equimolar amounts of N-acetyl glucosamine, galactose, and sulfate, had been isolated from bovine cornea (*9*) and nucleus pulposus (*10*) where it constitutes approximately one-half of the total mucopolysaccharides, and from the end pieces of the long bones of calf where it was only 1 percent of the total mucopolysaccharide (*3*). In cornea the other half of the mucopolysaccharides is made up of chondroitin sulfate A and chondroitin, while in nucleus pulposus the remaining half appears to be chondroitin sulfate C. Thus the mucopolysaccharide pattern of the costal cartilage in Marfan's syndrome resembles that of nucleus pulposus.

It appears possible that keratosulfate occurs more widely in connective tissue than believed heretofore and that it has been missed because of the greater alcohol solubility of its salts. The occurrence of glucosamine in articular cartilage has been reported by Kuhn and Leppelmann (*11*), who noted a large decrease in galactosamine and a small increase of glucosamine with increasing age. Similarly Shetlar and Masters (*12*) noted in costal cartilage a large decrease in uronic acid with increasing age, while the total hexosamine increased only slightly. These results were interpreted as indicating an increase of a "neutral mucopolysaccharide" in aging, but are readily explained by the absence of detectable amounts of keratosulfate in costal cartilage of newborn infants and the relatively large amounts found in mature costal cartilage. The indication from the rotation and solubility data that the chondroitin sulfate of the newborn infant costal cartilage was mainly A and of the mature costal cartilage mainly C was verified by the infrared spectra (*4*).

It appears from these data that the mucopolysaccharide pattern of hyaline cartilage is more complex than believed heretofore. The distribution of keratosulfate and of the types of chondroitin sulfate will have to be reinvestigated, with regard to age, species, and the source of the cartilage.

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Possible Role for Vitamin K in Electron Transport

Coupled oxidative phosphorylation in extracts of *Mycobacterium phlei* is dependent on a particulate and a soluble fraction (*1*). In an attempt to elucidate their respective roles, a study of electron-transport pathways in these fractions was undertaken (*2*). The characterization of some of the soluble electron-transport enzymes has been described in an earlier report (*3*).

Cells grown with continuous aeration

Instructions for preparing reports. Begin the report with an abstract of from 45 to 55 words. The abstract should *not* repeat phrases employed in the title. It should work with the title to give the reader a summary of the results presented in the report proper. (Since this requirement has only recently gone into effect, not all reports that are now being published as yet observe it.)

Type manuscripts double-spaced and submit one ribbon copy and one carbon copy.

Limit the report proper to the equivalent of 1200 words. This space includes that occupied by illustrative material as well as by the references and notes.

Limit illustrative material to *one* 2-column figure (that is, a figure whose width equals two columns of text) or to *one* 2-column table or to *two* 1-column illustrations, which may consist of two figures or two tables or one of each.

For further details see "Suggestions to Contributors" [*Science* 125, 16 (1957)].

were washed twice with distilled water and disrupted by sonic vibration at 10 kcy/sec for 4 minutes. They were then centrifuged at 20,000 *g* for 30 minutes. The cellular debris was discarded and the supernate, to be referred to as the crude extract, was centrifuged at 140,000 *g* for 90 minutes. This yielded a straw-colored supernate and a red pellet consisting of particles ranging in size from 20 to 180 mμ as previously described (*1, 4*). The supernate was withdrawn carefully and was recentrifuged at 140,000 *g* for 90 minutes to ensure a particle-free preparation. The particles, in the experiments to be described, were washed with 0.1*M* KCl buffered with tris(hydroxymethyl)aminomethane at *p*H 8 and recentrifuged at 140,000 *g* for 90 minutes.

The distribution of activities in the washed particles and recentrifuged supernate are compared in Table 1. As can be seen, the supernate did not catalyze the oxidation of DPNH (reduced diphosphopyridine nucleotide) with O_2 or vitamin K_1 (2-methyl-3-phytyl-1,4-naphthoquinone) as electron acceptor. However, electron transfer did occur in the presence of FMN (flavin mononucleotide), ferricyanide, menadione (2-methyl-1,4-naphthoquinone), dye (2,6-dichlorophenol indophenol), or mammalian cytochrome *c*. The particulate fraction catalyzed the oxidation of DPNH with O_2 as electron acceptor, and the rate was enhanced by ferricyanide or menadione but not by vitamin K_1 (the slight inhibition noted was due to the Tween 80 used to disperse the vitamin). The particles also catalyzed the reduction of dye, but not that of added cytochrome *c* or FMN.

It has previously been observed that light at 3600 A abolishes coupled oxidative phosphorylation in extracts of this organism and that activity could be restored by the addition of vitamin K_1 (*5*). It therefore was of interest to determine the effect of 3600-A light on the oxidation of DPNH by various electron acceptors in the presence of the supernate and particulate fractions. As can be seen in Table 1 (columns marked "Irradiated"), treatment of the supernate with light had little or no effect on the reduction of dye or cytochrome *c*, or on the oxidation of DPNH by the various electron acceptors. The catalysis of oxidation by the particulate fraction, however, with O_2 as electron acceptor, was completely inhibited. Restoration of activity could be partially obtained with ferricyanide or vitamin K_1, and fully with menadione. Although not shown, addition of vitamin K_1 to the ferricyanide reaction mixture resulted in an oxidation of DPNH equivalent to the sum of either alone. The addition of vitamin K_1 to the dye, however, significantly increased the rate of dye reduction. This increased rate was presumably a reflection of the nonenzymatic transfer of electrons from reduced vitamin K_1 to dye.

Table 1. Electron transport systems in particles and supernate. The reaction mixtures contained 500 μmole of potassium phosphate, *p*H 7.9, 0.42 μmole of DPNH, 0.5 mg of washed particles or recentrifuged supernate protein, and the electron acceptors as indicated, made up to a total volume of 3 ml with distilled water. Reactions were started with DPNH, and all readings noted at 340 mμ were recorded from 15 seconds to 3 minutes. The 600- and 550-mμ readings were made from zero time. Particles and supernate were irradiated in an ice bath in 1 ml amounts with a Gates Raymaster lamp model B at a distance of approximately 3.5 cm for 30 minutes.

Electron acceptor and amt. (μmole)	Particles						Supernate					
	$-\Delta E_{600}$ per 15 sec		ΔE_{550} per 2 min		$-\Delta E_{340}$ per 3 min		$-\Delta E_{600}$ per 15 sec		ΔE_{550} per 2 min		$-\Delta E_{340}$ per 3 min	
	Unirradiated	Irradiated	Unirradiated	Irradiated	Unirradiated	Irradiated	Unirradiated	Irradiated	Unirradiated	Irradiated	Unirradiated	Irradiated
Dye (0.09)	0.140	0.059					0.448	0.428				
Cytochrome *c* (0.06)			0	0					0.193	0.174		
O_2					0.513	0					0	0
FMN (0.03)					0.509	0					0.196	0.186
$K_3FE(CN)_6$ (0.51)					0.626	0.087					0.466	0.499
Menadione (0.51)					0.548*	0.529*					0.640*	0.636*
Vitamin K_1 (1.2)					0.457	0.111					0	0

* These are minimum values, for the initial rate of DPNH oxidation in the first 15 seconds, which was not accounted for, was very rapid.

Evidence that the catalytic oxidation of DPNH, with O_2 as electron acceptor, is dependent on the structural integrity of the particles was obtained by sonicating cells for various periods of time and testing the crude 21,000 *g* supernate for activity. With increased time of sonication, the ability to oxidize DPNH was reduced. When these extracts were centrifuged at 140,000 *g* for 90 minutes, most of the activity demonstrated in the crude extract was found in the remaining particles. The slight activity present in the supernate was eliminated by recentrifugation.

Spectroscopic examination of the particles revealed the appearance of the reduced bands of cytochromes *b*, *c*, and *a* after the addition of DPNH. After exposure to light at 3600 A, the addition of DPNH did not cause the appearance of these reduced spectra. On further addition of vitamin K_1, however, the reduced bands of cytochromes *c* and *a* appeared strongly and *b* only slightly.

Dicumarol, at a final concentration of $2 \times 10^{-4}M$, inhibited the particulate DPNH oxidase activity by 75 percent. In addition, as can be seen in Table 2, $10^{-3}M$ cyanide inhibited 75.6 percent of the oxidation in the untreated particles, and 81.2 percent of the vitamin K_1-restored oxidation in the light-treated particles. In contrast, although menadione and ferricyanide were able to act as electron acceptors, they were not on the cyanide-sensitive pathway of electron transport (Table 2). α-Tocopherol, which has been shown to be on the pathway between cytochromes *b* and *c* in mammalian tissue (*6*), could not replace vitamin K_1 in restoring DPNH oxidase activity to light-treated particles.

The already noted incomplete restoration of DPNH oxidase activity of irradiated particles by vitamin K_1 probably reflects multiple effects of this treatment. DPNH oxidase activity was also reduced by extracting the particles with iso-octane, according to the procedure of Nason and Lehman (*6*), and the activity could then be completely restored by the addition of vitamin K_1.

It is suggested that the catalysis of DPNH oxidation by the particles of *Mycobacterium phlei* proceeds through at least two enzymatic pathways. One, involving vitamin K_1, or some related

Table 2. Effect of cyanide on DPNH oxidase activity. The reaction mixtures contained 500 μmole of potassium phosphate, *p*H 7.9, 0.42 μmole of DPNH, 0.5 mg of washed particle protein, and 1.2 μmole of vitamin K_1, $K_3Fe(CN)_6$, or menadione made up to a total volume of 3 ml with distilled water. Cyanide was added in a final concentration of $10^{-3}M$. Reactions were started with DPNH. Light treatment was similar to that described in Table 1.

Addition	DPNH oxidized per 2 min (μmole)		Inhibition by KCN (%)
	Without KCN	With KCN	
	Untreated particles		
None	0.196	0.048	75.6
	Light-treated particles		
None	0.024		
Vitamin K_1	0.085	0.016	81.2
Menadione	0.247	0.248	0
$K_3Fe(CN)_6$	0.087	0.085	2.3

compound, proceeds through the *cyanide-sensitive* terminal respiratory pathway. The other, in which menadione or other electron acceptors can function, does not involve the *cyanide-sensitive* pathway, but rather a diaphorase type flavoprotein-catalyzed *cyanide-insensitive* reaction.

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Changes in the Perceived Color of Very Bright Stimuli

Abstract. When very intense stimuli in the long-wavelength region of the visual spectrum are viewed continuously, they change in hue from red, through yellow, to green. The relation of the time course of these changes to the intensity of the stimulus is reported.

In general, the perceived color of a visual stimulus is a function of its wavelength composition. It is well established, however, that the color of a stimulus of fixed wavelength varies somewhat as the intensity of the stimulus is changed. An extensive study of this phenomenon, the Bezold-Brüke effect, was reported by Purdy (*1*). He found, for example, that both red and yellow-green stimuli appear yellower at 1000 trolands than at 100 trolands. However, yellow stimuli (at about 575 mμ) were found to be "invariant"—that is, no color changes were observed as intensity was varied.

Our observations have shown that when a very intense stimulus in the long-wavelength end of the spectrum is fixated continuously, it appears red at first but rapidly changes to yellow and then to a deep, rich green. Intense yellow stimuli, at the wavelength found by Purdy to be "invariant," also turn green. Green stimuli may desaturate but do not turn red. Auerbach and Wald (*2*), while studying the effects of very bright stimuli on subsequent dark adaptation, also noted that intense reds turn green.

We have investigated the time course of these color changes at different wavelengths and light intensities. The observer was presented with a Maxwellian view of a bright, uniformly illuminated, circular field, 15 deg in diameter, with a set of cross hairs for fixation. The source was a 17-ca automobile headlight bulb, run at a color temperature of 2800°K from a direct-current power supply. The effective *f* ratio of the system was 3.0. Heat-absorbing glass protected the eye from excessive infrared radiation. The intensity was varied either by neutral density filters or by a variable on-off ratio flicker vane, run well above fusion frequency. The wavelength composition of the field was controlled by introducing color filters into the collimated part of the optical path.

The subject held three keys. He was instructed to press the first when the stimulus was first turned on, the second when it first turned yellow, and the third when it first turned green (*3*). The keys operated timers, so that the time of each color change was recorded. Each trial consisted of a single presentation of the stimulus at a particular intensity. At least 30 minutes elapsed between trials. For any given color filter, the order of intensity presentations was varied randomly.

In the first experiment a Wrattan No. 29 filter was used. This filter passes all wavelengths longer than about 615 mμ, and none shorter. Four subjects were tested under these conditions, and all showed similar results. The data for one subject are plotted in the upper portion of Fig. 1, which may be interpreted in the following way: At a given intensity —for example, 1×10^5 trolands—the stimulus was judged red for the first 7 seconds, then yellow for 9 seconds, and then green. No further color changes were observed, even though fixation was maintained for as long as 3 minutes. At lower stimulus intensities the color changes occurred later. For this particular filter, the stimulus remained red indefinitely at intensities below about 0.1×10^5 trolands. Each point on these curves is a mean of six judgments for one subject. The between-judgments variability for each of the subjects was surprisingly small.

These color changes are not restricted to broad-band stimulation. The red region of the spectrum, as far out as 640 mμ, was explored with a monochromator of 5-mμ spectral slit width, and similar color changes were found.

The lower portion of Fig. 1 shows a curve for a narrow band-pass interference filter at 5.75 mμ. Since this filter appears yellow initially, only the yellow-to-green curve can be plotted. This wave length is one which Purdy reported to be "invariant"—that is, its hue did not change with intensity. However, the highest intensity employed by Purdy was only 2000 trolands, or 0.02×10^5. At the higher intensities we used, this stimulus turns to a deep, rich green.

Our observations in the green and blue regions of the spectrum failed to reveal changes other than desaturation. Auer-

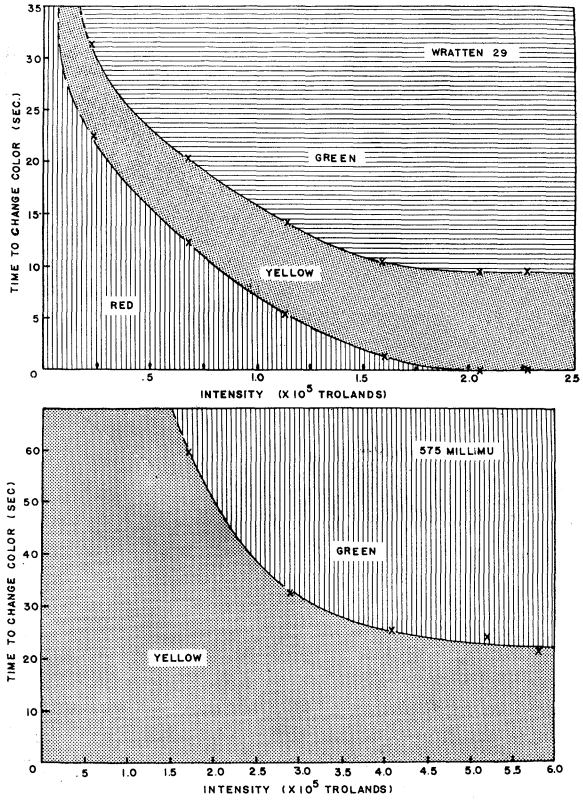

Fig. 1. Time course of changes in hue as a function of the stimulus intensity. The upper plot is for Wratten filter No. 29, the lower, for a narrow band-pass interference filter with a peak at 575 mμ.