CHEMICAL COMPOSITION OF ENZYME-FRACTIONATED AGED HEART TISSUE

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In research on aging the primary objective is to control the process of aging. An important intermediate object is to characterize the general cellular changes which occur over the period of a lifetime. One such cellular change, known to the pathologist for many years, is the accumulation of insoluble, fluorescing particles (lipofuscins) in aging nerve, heart, liver and other tissues (1). The validity of the age-pigment approach to the study of aging has been discussed by Heidenreich and Siebert (2), Strehler et al. (3), and others and need not be discussed here.

Two approaches have been used to isolate age pigments from cells, and both are based upon the extreme resistance of such particles to alteration during processing. Their resistance to physical disruption has been used as a basis for the isolation of pigment-rich fractions from cardiac tissues by Lang and Siebert (4), Hendley et al. (5), and Björkerud (6). We are not now concerned about the particular pigment-like physical entities, but rather with the sum total of all those substances in the aged heart which cannot be broken down by enzymes. The "age pigments" or "lipofuscins" are a significant part of these, but by no means the sum total.

Proteolytic enzyme hydrolysis has been used to isolate enzyme-resistant heart fractions (7–9). This method serves to remove extraneous contaminants and thereby obtain an inkling as to the basic chemical composition of the enzyme-resistant residuum accumulated in these old hearts over a lifetime.

The extreme resistance of age-pigment granules to solubilization by either enzymes, physical influences or hydrogen bond-splitting reagents (including anhydrous hydrogen fluoride) strongly suggests that the pigment molecules are highly cross-linked (10, 11).

In the research presented here, aged cardiac tissue was fractionated successively with 5 proteolytic enzymes, and the resultant insoluble hard core was subjected to chemical analysis.

EXPERIMENTAL

Materials and methods

Tissue preparation. Six human hearts from males whose ages ranged from 64 to 74 years, were obtained at autopsy. The hearts were trimmed of superficial fat and connective tissue and frozen at −20°C before being analyzed.

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One hundred-gram samples of heart muscle were diced and homogenized with a minimal amount of 0.25*M* sucrose solution, by means of a Waring Blendor. The resulting homogenate was further broken down in a Potter-Elvehjem homogenizer and then filtered through a single thickness of surgical gauze to remove large connective tissue elements.

The homogenate was centrifuged at high speed to separate the soluble compounds of the tissue. The residue was washed twice with 0.25*M* sucrose, followed by three washes with distilled water to remove soluble compounds. In each step, high-speed centrifugation (>16,000 × G) was used to effect the separation of cellular components.

The washed, insoluble heart residue was freeze-dried and then extracted for twenty hours with diethyl ether to remove free lipids. The lipid content of the samples varied from .44 to .73 per cent of the total dried weight of tissue. The dried, defatted, insoluble residue ranged in proportion from 5.9 to 6.22 gm per 100 gm of wet-weight tissue.

Enzymatic digestion-fractionation procedure

The dried, defatted heart residues were subjected to hydrolysis successively with pepsin, trypsin, papain, collagenase and elastase (obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio).

Conditions of hydrolysis: 100-mg quantities of the following 3 enzymes were used — crystalline pepsin (3X) at pH 2.0 in 500 ml of .02*N* HCl; trypsin (1:250) in Tris buffer [tris-(hydroxymethyl)aminomethane-HCl], ionic strength 0.05 and the suspension adjusted to pH 7.7; crystalline papain (2X) in Tris buffer adjusted to pH 7.0, and 10 mg each of ethylenediaminetetraacetic acid and cysteine added as activators. After each digestion step the residue was separated by centrifugation and the insolubles were washed three times with distilled water and once with the appropriate buffer for each subsequent enzyme.

The washed residue, after the papain step, was digested twice with 50-mg quantities of collagenase (from *Cl. histolyticum*) in Tris buffer at pH 7.4. $CaCl_2$ (10 mg) was added as an activator.

The collagenase residue was washed five times to expel nearly all of the calcium ions, and then washed once with Tris buffer (pH 8.8). The residue was suspended in Tris buffer and digested twice with 10-mg quantities of crystalline elastase (2X).

Each of the 5 enzymes was incubated with the heart tissue for twenty hours at 37°C, with toluene added as a preservative.

The final enzyme-insoluble residue was dialyzed against distilled water at 2°C for twenty hours, then dried with acetone and ether. The average dry weight of the residue was found to be 1.4 per cent of the original ether-extracted dry residue. The fractionation procedure is shown schematically in Figure 1.

Analytical procedures

Total nitrogen was determined on the enzyme-treated residue using the micro-Kjeldahl technique (12).

The residue was ashed and the mineral content was determined spectrographically using a Bausch & Lomb double-beam spectrograph.

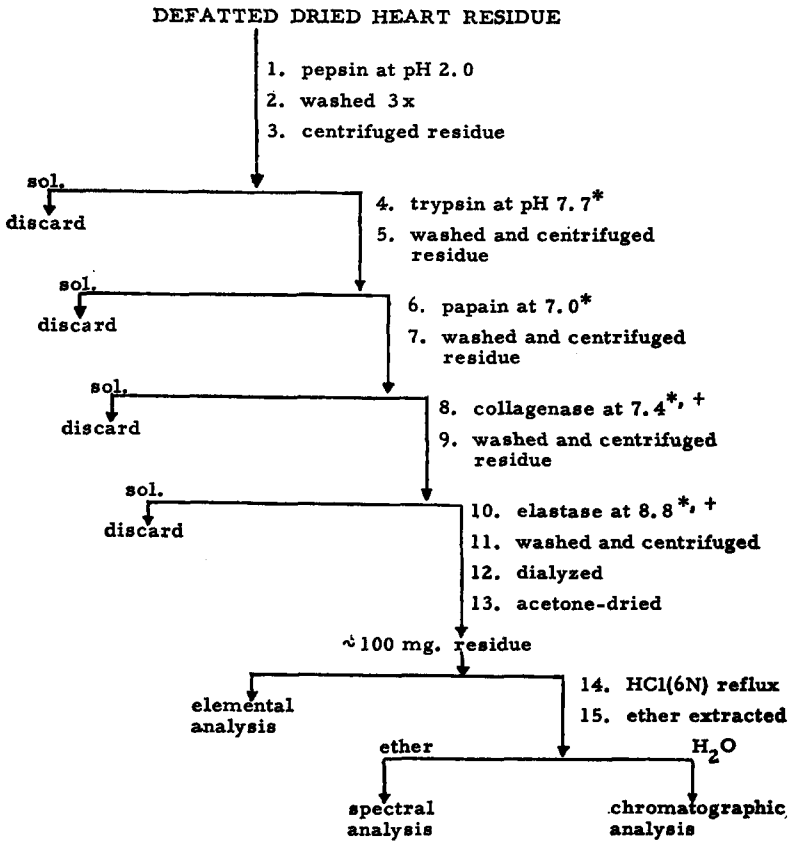
Carbon, hydrogen and oxygen determinations were made on the enzyme-insoluble residues by the Clark Microanalytical Laboratory, Urbana, Illinois.

The residue was subjected to acid hydrolysis with 6*N* HCl for twenty hours at 110°C. The hydrolysate was filtered, ether-extracted to remove lipids and aromatic compounds, and dried at 40°C under reduced pressure. The dried hydrolysate was stored in a vacuum over soda lime to remove residual HCl.

The amino acids from the acid hydrolysates were then determined by means of the ion exchange chromatographic method of Moore, Spackman and Stein (13).

Ether extracts obtained from these acid hydrolysates were shaken with 5 per cent

ENZYME HYDROLYSIS PROCEDURE



* Tris buffers, ionic strength 0.05 used throughout.

\+ Both collagenase and elastase hydrolyses were run twice.

FIGURE 1

NaOH to remove fatty acids and phenols from the neutral compounds in the ether layer. The NaOH solution was neutralized with CO_2 and again extracted with ether. The ether extract, which now contained the phenol (aromatic) compounds, was dried and studied spectrophotometrically. A Beckman D. U. spectrophotometer was used to obtain ultraviolet absorption spectra, and a Perkin-Elmer Infracord was used to obtain infrared spectra.

RESULTS

Elemental analyses

Proximate methods of analysis were used on the heart insolubles to gauge roughly the composition of the material. Total nitrogen determinations were

TABLE 1

Spectrographic Mineral Analysis of Human Heart Insolubles after Enzymatic Treatment in Tris Buffers

Element	Conc. (ppm)*
Potassium	950
Sodium	<1000
Calcium	118
Magnesium	<10
Silicon	210
Phosphorus	344
Iron	74
Aluminum	41
Manganese	2.3
Zinc	88
Boron	6.8
Copper	18.6
Molybdenum	<.10
Cobalt	6.1
Nickel	2.8
Chromium	3.4
Lead	2.7
Titanium	6.3
Total concentration	2884.1

* Parts per million.

run on 6 heart residues, and it was found that the enzyme-resistant residues consistently contained 4.2 to 4.65 per cent nitrogen. The values indicate that only 30 per cent of the residue was protein.

The ash content varied from 3.6 to 5.88 per cent, depending upon the individual preparation. The mineral content of the ash did not appear to be unusual; at least traces of 21 metals were found.

The mineral data given in Table 1 show that several potential cross-linking metals were present in appreciable quantities in the residue. These included iron, aluminum, calcium, copper, manganese and nickel. [Values for normal heart tissues have been reported by Underwood (14).] It is noteworthy that particularly the polyvalent cross-linking aluminum ion is present in the human heart at a level one hundred times higher than that reportedly found in dog heart.

Carbon, hydrogen and oxygen analyses were made on 2 heart residues—both the whole residue and the residue after boiling in 0.5*N* HCl for one hour. Preliminary data, shown in Table 2, reveal that very little change occurred in the basic material during dilute acid treatment. Because of the low hydrogen and oxygen content, it appears unlikely that the heart residue contained any insoluble polysaccharide.

TABLE 2
Combustion Analysis of Human Heart Insolubles after Enzymatic Treatment

Element Analyzed	Averaged Percentage of Element	
	Whole pigment residue	Residue after HCl extraction
Carbon	60.61	61.72
Hydrogen	7.66	7.94
Oxygen	22.00	17.99
Nitrogen	4.60	not done
Ash	5.88	1.34

TABLE 3
Amino Acid Composition of Heart Insoluble Residue

	Micromoles of Amino Acid Found in 100 mg of Dried Acid Hydrolysate*
Aspartic acid	29.2
Threonine	14.3
Serine	20.1
Glutamic acid	23.8
Proline	17.3
Glycine	28.0
Alanine	24.2
Valine	21.5
Methionine	17.2
Isoleucine	26.2
Tyrosine	6.06
Phenylalanine	12.6
Lysine	19.4
Histidine	11.6
Ammonia	112.6
Arginine	8.5

* Averaged values from 3 heart residues.

Amino acid analyses

Analysis for individual amino acids revealed that the protein of the heart insolubles contained 15 amino acids and ammonia. The data, summarized in Table 3, do not include a value for tryptophan since this amino acid is destroyed during the hydrolysis step.

The absence of hydroxyproline indicates that the material was not collagenous in nature, and probably not collagen-derived. The protein was rich in both cationic amino acids and ammonia, and apparently did not contain cysteine or cystine. At this point, our data are not sufficient to determine whether we are dealing with one distinct peptide or a mixture of several peptides covalently bonded together.

Spectrophotometric analysis

Ether extracts of heart insolubles after strong acid hydrolysis were analyzed by both ultraviolet and infrared procedures.

In ultraviolet light the ether extract absorbed light at 275 mμ, indicating that the heart residue contained an aromatic component. The absorption at 275 mμ could be destroyed by reduction with sodium borohydride ($NaBH_4$), indicating that the material might be a quinone. If it were a classical quinone, one would expect the spectrum to shift in such a way that absorption would occur at either 290 mμ or 312 mμ after reduction. A distinct spectral shift was not observed, however.

Since only minute amounts of the fluorescing compounds were extracted from the enzyme-treated residues, it was not possible to determine empirical formulae by standard combustion techniques. However, enough material was obtained from each heart residue for partial determination of certain of the reactive groups within this chromophoric molecule, using infrared techniques. Spectra of ether-soluble materials from 6 enzyme-fractionated heart hydrolysates were extremely uniform in the infrared region. A typical spectrum is shown in curve C in Figure 2. Two interesting absorption bands were found in the spectrum. One absorption band occurred at 5.9 microns, indicating that the extract contained carbonyl groups. Another strong absorption band occurred at 8.0 microns, indicating the presence of methoxy groups. Comparisons were made between the spectra of the extracted material and those of several known compounds which could conceivably be bound to cardiac age-pigment via an autoxidative mechanism.

Based on the results of infrared spectrum analysis, it is unlikely that the compound which causes fluorescence of age-pigment is either a carotene, vitamin E, cholesterol or an unsaturated fatty acid derivative, since none of these compounds gives a spectrum resembling those found for heart extracts. The possibility exists, however, that the chromophoric compound in cardiac age-pigments is an oxidative derivative of coenzyme Q. This coenzyme, which is a component of the electron transport system of mitochondria, exhibits strong absorption at both 275 mμ and 8.0 microns (15). The similarities of spectra between pure coenzyme Q_6 and ether extracts of heart-residue hydrolysates are shown in Figure 2. Curve B of the figure is a spectrum of defatted whole heart tissue which was subjected to hydrolysis successively with saturated barium hydroxide followed by 6*N* hydrochloric acid. It is noteworthy that firmly bound material quite similar to that of enzyme-treated residues was obtained in the ether extracts.

DISCUSSION

On the basis of these data it is apparent that in the aged heart there is a substantial fraction of substances which are not susceptible to hydrolysis by the proteolytic enzymes employed in this study. This fraction is predominantly composed of substances fluorescing in ultraviolet light with the same orange color as the lipofuscin age-pigments.

The fact that the protein component is free from hydroxyproline indicates that collagen is not involved in the formation of this material.

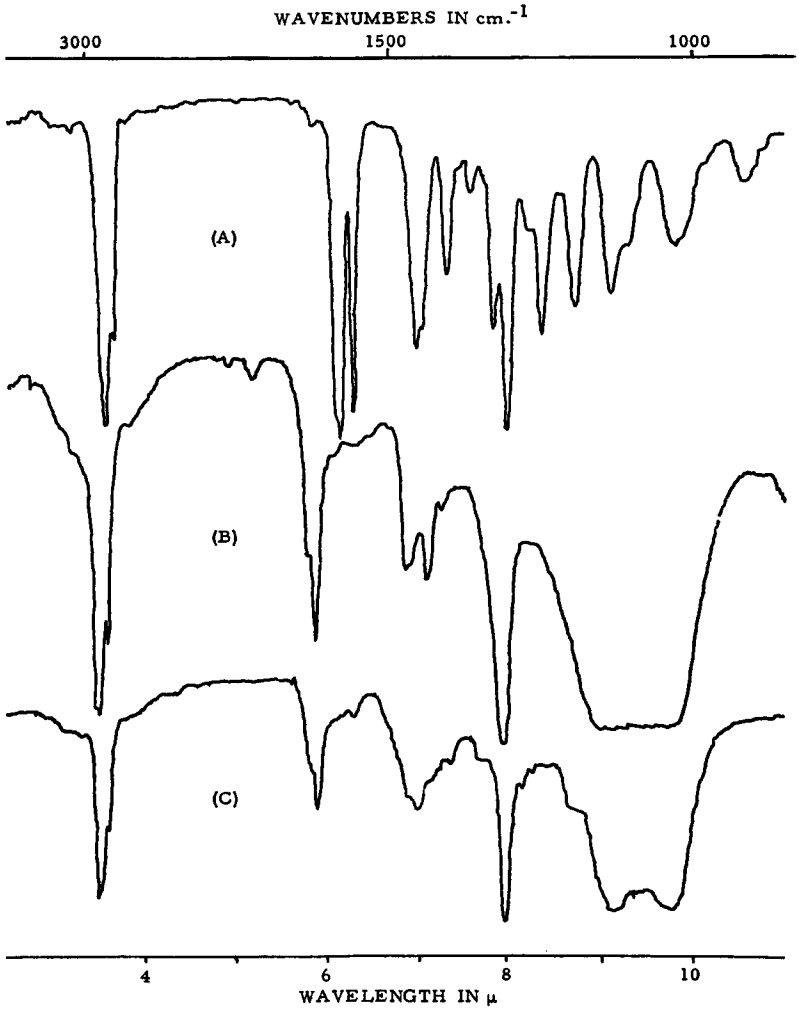


FIG. 2. Infrared spectra of ether-soluble neutral compound from human heart. (A) pure coenzyme Q_6; (B) ether extract of heart after chemical hydrolysis; (C) ether extract of heart after enzyme hydrolysis.

Insolubility in hydrofluoric acid indicates that this material is highly cross-linked (10). Attempts to prepare this fraction from young hearts failed to produce any measurable quantity of corresponding substance, beyond some microscopic specks of fluorescent material (9).

Material prepared from several different hearts by means of varying hydrolytic techniques consistently resulted in the isolation of 2–20 mg quantities of an organic substance, the infrared spectrum of which has been described. It would be consistent with the findings so far to assume that this substance might have been formed by an oxidative reaction involving coenzyme Q.

The strong aldehyde absorption band in the infrared spectrum would be consistent with oxidation of the isoprene side chain. Further work is under way to elucidate this point.

The low carbon:hydrogen ratio of the nonprotein material would point to material either very rich in tertiary carbons and aromatics, or highly unsaturated. The last mentioned possibility now appears unlikely. Further work is in progress.

It does not seem inconsistent with the findings to date to suppose that we are dealing here with a tightly knit conglomerate of ring or network structures formed over the years by random reactions involving the reactive groups of large molecules.

SUMMARY

Fractionation of the enzyme-nonhydrolyzable constituents of human heart muscle from persons 64–74 years old resulted in separation of a fluorescing fraction, insoluble in anhydrous hydrogen fluoride, and containing 4.6 per cent nitrogen (corresponding to 30 per cent protein). This fraction was free from hydroxyproline and is therefore not derived from collagen.

An aromatic aldehyde was consistently separated when the enzymatically nonhydrolyzable fraction was broken down by destructive acid hydrolysis. Infrared data indicate a structure having characteristics in common with coenzyme Q.

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