Studies on the Structure of Collagen III[1]. On the Presence of α-Keto Acids in Collagen

Several papers[2–5] mentioned the presence of carbonyl compounds in collagen. The only one giving some evidence about the structure of one of these carbonyl compounds is that of GALLOP et al.[4,5]. During our studies[1,6] on digestion of collagen by various enzymes (pronase, pepsin, trypsin, chymotrypsin), it became evident that several carbonyl compounds appear among the dialyzable breakdown products. The nature and amount of these carbonyl compounds are described in this communication.

Acid soluble and insoluble collagen were prepared according to the procedure of RUBIN et al.[7]. These collagens were digested by pepsin and pronase according to DRAKE et al.[8].

The carbonyl compounds were determined both in the undialyzable treated collagen and in low-molecular weight peptide mixture.

The peptide-carbonyl compounds mixture was separated by means of continuous paper electrophoresis in pyridine-acetate buffer (pH 6.3 $\mu = 0.1$). The carbonyl compounds were concentrated on the positive side of the sheet, where no peptides were present.

The carbonyl compounds were converted to corresponding 2,4-dinitrophenylhydrazones and were separated by means of TLC on silica gel. The following solvent systems were used: (1) For alumina layers: butanol-pyridine-acetic acid-water (30:20:6:24) (solvent A), and toluene-pyridine-ethylene chlorohydrine-0.8 *M* ammonia (100:30:60:60) upper phase (solvent B). (2) For silica layers: solvent A, solvent B, chloroform-benzylalcohol-acetic acid (70:30:3), *n*-butanol-pyridine (10:1), *n*-butanol-acetic acid (10:1), and *n*-butanol-acetone (10:1).

4 distinct spots have been separated and identified: α-ketoglutaric acid; α-ketoisovaleric acid; pyruvic acid; and an aldehyde of unknown composition, probably the same as described by ROJKIND et al.[3] (according to UV-spectra).

We measured the UV-spectra of 2,4-dinitrophenylhydrazones, *p*-nitrophenylhydrazones and *p*-bromphenylhydrazones of isolated carbonyl compounds. All those were in excellent agreement with spectra of model compounds.

The final evidence was obtained from reduction of 2,4-dinitrophenylhydrazones by tin and hydrochloric acid to corresponding amino acids. These amino acids were separated on an amino acid analyzer[9] and the presence of glutamic acid, alanine and valine corresponding to α-ketoglutaric, pyruvic and α-ketoisovaleric acids was confirmed.

[1] Communication II in this series: Z. DEYL, J. ROSMUS and S. BUMP, Biochim. biophys. Acta, in press.
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2,4-DNPH reactive substances in collagen

Sample	Collagen					Dialyzable peptides					Sum in the whole collagen molecule	
	aldehyde	keto-glutaric acid	pyruvic acid	keto-isovaleric acid	total keto acids	aldehyde	keto-glutaric acid	pyruvic acid	keto isovaleric acid	total keto acids	alde-hyde	keto acids
Soluble tropocollagen	1.75	–	–	–	2.50	–	–	–	–	–	(2)	(3)
	1.97 (2)	+	+	+	2.89 (3)	–	–	–	–	–		
	2.10	–	–	–	2.39	–	–	–	–	–		
Buffalo fish tropocollagen	1.59	0.78	0.60	0	1.85 (2)	–	–	–	–	–	(2)	(2)
	1.68 (2)	0.83 (1)	0.91 (1)	0	–	–	–	–	–	–		
	1.92	0.60	0.91	0	–	–	–	–	–	–		
Pepsin treated tropocollagen	0.79	–	–	–	0.69	0.49	0.60	0.60	–	–	(2)	(3)
	0.82 (1)	–	–	–	0.81 (1)	0.75 (1)	0.81 (1)	0.60 (1)	–	(2)		
	1.09	–	–	–	0.47	–	–	–	–	–		
Pronase treated tropocollagen	0.60	0	0	0	0	0.87	0.60	0.76	0.60	–	(2)	(3)
	0.77 (1)	0	0	0	0	0.80 (1)	0.73 (1)	0.78 (1)	0.43 (1)	(3)		
	0.88	0	0	0	0	–	–	–	–	–		
Pepsin treated insoluble collagen	1.20	–	–	0	1.15	0.93	0.60	0.78	–	–	(2)	(4–5)
	1.07 (1)	+	+	+	1.45 (2)	0.87 (1)	0.47 (1)	0.80 (1)	–	(2)		
	0.93	–	–	0	1.63	0.90	0.60	–	–	–		
Pronase treated insoluble collagen	1.20	0	0	0	–	1.05	1.95	0.88	1.68	–	(2)	(5)
	0.68 (1)	0	0	0	–	1.08 (1)	– (2)	– (1)	– (2)	(5)		
	0.90	0	0	0	–	–	–	–	–	–		

The values are expressed as moles of carbonyl compounds per tropocollagen molecule; molecular weight of tropocollagen is considered 265000. The values in parentheses are rounded values from experimental values; the experimental values are from 2–3 independent determinations and all respective values are presented in the Table.

Our further effort was to quantitate these results, and these are summarized in the Table[10].

Zusammenfassung. Aus dem nativen löslichen und unlöslichen Kollagen wurden drei Ketosäuren, und zwar Brenztraubensäure, α-Ketoglutarsäure und α-Ketoisovaleriansäure isoliert. Diese Ketosäuren häufen sich in den peripheren Gebieten des Kollagenmoleküls (in Telopeptiden)[7,8] an.

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Anthocyanins of *Dioscorea alata* L.

The greater yam, or water yam, *Dioscorea alata* L., is an important food crop in parts of South-East Asia, and has been introduced by cultivation to many other tropical countries, including West Africa. The flesh of the edible tubers of most cultivars is white, but some contain a purplish-red pigment. The whole tuber may be deeply coloured, or there may be only a thin s.c. coloured layer, the greater part of the tuber being faintly pink or white: the leaves and stems of the growing plant also are sometimes suffused with pigment. These types have been separated as distinct species, *D. atropurpurea* Roxb., *D. purpurea* Roxb. and *D. rubella* Roxb.[1], but are now generally regarded simply as varieties of *D. alata*[2]. The pigment is usually assumed to be an anthocyanin, but no information as to its composition appeared in the literature, and the authors are unaware of reports of anthocyanins in any of the Dioscoreaceae.

Tubers of a purple-fleshed form of *D. alata* grown on the University Farm, Legon, have been examined for the presence of anthocyanins. The pigments were extracted by homogenizing 20 g of peeled tuber in 100 ml of methanolic 1% HCl for 5 min. The suspension was left overnight, the extract filtered off and the whole process repeated. The combined extract was shaken with an excess of purified ether. The pigments were separated as an aqueous concentrate, washed successively with light petroleum and benzene, and then exhaustively extracted with ethyl acetate.

Further purification and separation of the anthocyanin pigments was undertaken by paper chromatography[3]. The purified extract was banded on several sheets of Whatman No. 3 paper and developed with 1:1 *n*-butanol-2*N* HCl. 3 distinct coloured bands, 1 being much stronger than the others, appeared.

The anthocyanin bands were cut out, eluted with 5% acetic acid in methanol and after concentration in vacuo at 30–35 °C, purified by re-running in *n*-butanol-acetic acid-water (62:12:26) and then in 15% aqueous acetic acid on washed paper. Aqueous solvent was used to remove free sugars[4].

The purified eluates were concentrated in vacuo and shaken with small amounts of 20% HCl in a large excess of ether. The pigment was transferred completely into the aqueous layer, which was separated, traces of ether and methanol removed in vacuo, and then heated on a boiling water bath for 3 min. The solution was quickly cooled, extracted with amyl alcohol, and the organic layer washed with water. Amyl alcoholic extracts of the aglycones were then applied to Whatman No. 1 paper. 4 solvent systems were used for development: (1) 'Forestal solvent' – water-acetic acid-conc. HCl (10:30:3 v/v); (2) Acetic acid-conc. HCl-water (5:1:5 v/v); (3) *m*-cresol-5,5*N* HCl-water (1:1:1 v/v); (4) *n*-butanol-2*N* HCl (1:1 v/v, top layer). The *n*-butanol: 2*N* HCl solvent mixture was kept, while the paper was equilibriated with the lower aqueous phase, for 24 h, before use.

On all the chromatograms of hydrolysed pigment extracts only a single anthocyanidin spot appeared; this spot was magenta in visible light and bright pink in UV-light. The Rf value of this anthocyanidin was compared with those of known anthocyanidins[4–8] (see Table I), and found to correspond with cyanidin. This was confirmed by co-running with a sample of cyanidin,

Table I. Rf values of the principal anthocyanidins and of *D. alata* anthocyanidin in different solvent systems

Anthocyanidin	Bu:HCl	'Forestal'	AcOH:HCl	*m*-cresol:HCl
Pelargonidin	0.80	0.68	0.55	0.82
Cyanidin	0.69	0.50	0.34	0.69
Peonidin	0.72	0.63	0.50	0.87
Delphinidin	0.35	0.30	0.22	0.52
Petunidin	0.45	0.45	–	0.75
Malvidin	0.53	0.60	0.43	0.90
Hirsutidin	0.72	–	–	–
Anthocyanidin of *D. alata*	0.69	0.50	0.35	0.70

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