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THERMOANALYTICAL STUDIES ON PROTEIN-POLYSACCHARIDE COMPLEXES OF CONNECTIVE TISSUES

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The Derivatograph has been found to be suitable for the investigation of biopolymers consisting of polysaccharides and proteins. It could be demonstrated that interactions between the macromolecules of the collagen-proteoglycan-glycoprotein complexes significantly influence the thermal stability of the individual components.

Studies concerning physiological and pathological changes in the composition and structure of connective tissues are at the focus of interest of biological and medical research.

The literature contains a great amount of information on the chemical composition of connective tissues, obtained by the analysis of fractions from various extraction-fractionation procedures. However, the extractability of the different tissue components is a function not only of the chemical composition of the tissue, but also of the original macromolecular structure (degree of intra- and intermolecular crosslinks) of the biological material. Thus, the yields and compositions of the fractions obtained by a given extraction method from different tissue samples may be characteristically different, depending on the source of the tissue (species and organ differences), the stage of development (age-related changes) and the condition of health of the individual (pathological alterations). However, these differences are often not reflected in the results of chemical analysis (hexose, hexosamine, hexuronic acid, hydroxyproline, etc. determinations). In our experiments a complex thermoanalytical method [1] was applied to study various collagen-proteoglycan-glycoprotein complexes prepared from different connective tissues by the method of Robert et al. [2]. Thermal analysis was successfully used in our previous investigations for the quantitative determination of polysaccharides [3], for the characterization of protein structure [4], and for the estimation of age-related [5, 6] and pathological [7–13] changes in proteoglycan- and collagen-containing biological tissues.

Materials and Methods

Fibrinogen (for the preparation of glycopeptides and S-sulfo-fibrinogen) and ovalbumin were purchased from Sigma (St. Louis). Purified bovine fibrinogen, fibrin and orosomucoid (Cohn fraction VI) for thermoanalytical studies were

obtained from Calbiochem (California). Bacillus cereus polysaccharide was kindly presented by Prof. Ivanovics (Szeged).

Glycopeptides were prepared by pronase-digestion, and purified on Sephadex G-50 columns [14]. Their purity was checked by thinlayer electrophoresis [15].

Poly-N-acetyl-glucosamine was prepared by the partial hydrolysis of chitosan, and reacetylation of the hydrolysis products with aqueous acetic anhydride in the presence of ion-exchanger (hydrocarbonate form) [16]. Chitosan was a product of Light (Colnbrook).

Extraction of the tissues. Corneal stroma was obtained from whitings and from calves. Mouse skin was obtained from two isogenic strains (C_3H and $C_{57}B$) in the laboratory of Dr. Friedman (Univ. of Créteil, France). The tissues were extracted 5–7 times with 1 *M* $CaCl_2$, buffered with tris and citrate, according to the method of Robert and Dische [2, 17]. For removal of the calcium salts, the extracts were dialyzed against a 2% versene solution, then against distilled water, and analyzed in a freeze-dried form.

From the extracts two main fractions could be separated:

1. the water-soluble fraction of the dialyzed extract (calcium-tris-citrate extract, "CTC");
2. the collagen-proteoglycan-glycoprotein coprecipitate obtained from the extract during the dialysis (crude soluble collagen, "CSC").

Chemical analysis. Hexoses were determined by the orcinol method [18], hexosamines by the Morgan–Elson method as modified by Blix [19], uronic acid by the carbazol method of Dische [20], sialic acid according to Warren [21], and hydroxyproline by the Newman–Logan method as modified by Bergman and Loxyley [22]. The proportion of hexoses and hexosamines was determined by thinlayer chromatography [23].

Thermal analysis was performed using a Paulik–Paulik–Erdey MOM Derivatograph. The material (approximately 20 mg) was weighed into a platinum crucible. The heating rate was 2°/min up to 900°.

Results and Discussion

1. *Polysaccharides, glycopeptides, glycoproteins*

In the first part of our experiments thermal decomposition of model compounds was studied in order to demonstrate the correlation between molecular structure and thermal stability.

Figure 1 shows the DTG curves of N-acetyl-D-glucosamine, poly-N-acetyl-D-glucosamine and chitosan. As seen in Fig. 1a, thermal decomposition of N-acetyl-D-glucosamine took place with maximum rate at about 200°, followed by a second process at 400–450°. Polymerization of the molecule resulted in an increase of thermostability, as demonstrated in Fig. 1b. In the DTG curve of poly-N-acetyl-

D-glucosamine the first peak, at 60°, corresponded to loss of the water content of the sample. The main thermal process took place at 275°, but as the sample contained some low-grade polymerization components, the 200° maximum was also detectable in the decomposition curve. In chitosan the 200° components were not present (Fig. 1c), and the main process took place at about 280°.

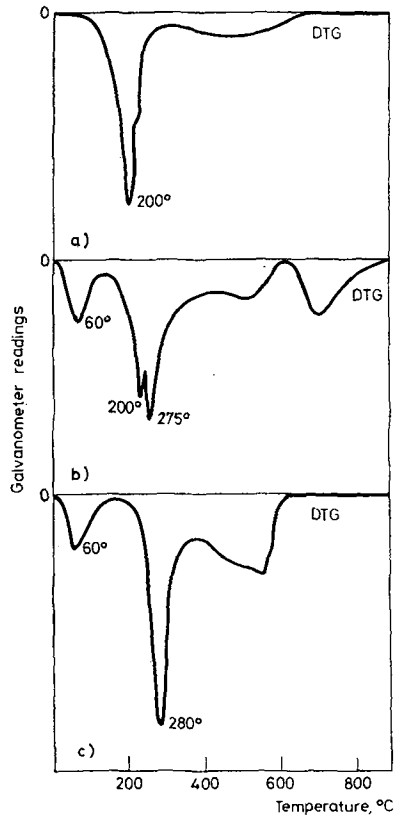

Fig. 1. DTG curves of N-acetyl-D-glucosamine (a), poly-N-acetyl-D-glucosamine (b) and chitosan (c)

Figure 2 and Table 1 present the results of thermal and chemical analysis of glycopeptides obtained by pronase-digestion from ovalbumin and from fibrinogen; of the capsular polysaccharide from Bacillus cereus; and of bovine α_1 acid glycoprotein. The main thermal decomposition process of the glucose-, galactose- and mannose-containing oligo- and polysaccharides took place with maximum rate at 260°, 220° and 260° resp., according to the DTG peak values (Fig. 2a–c). In the DTG curve of α_1 glycoprotein both characteristic polysaccharide peaks

could be detected. The DTG maxima at 300° and at temperatures above 500° correspond to the decomposition of the polypeptide components of the samples.

In Fig. 3 thermal decomposition curves of S-sulfofibrinogen, fibrinogen and fibrin demonstrate correlations between the thermal stability and the amount

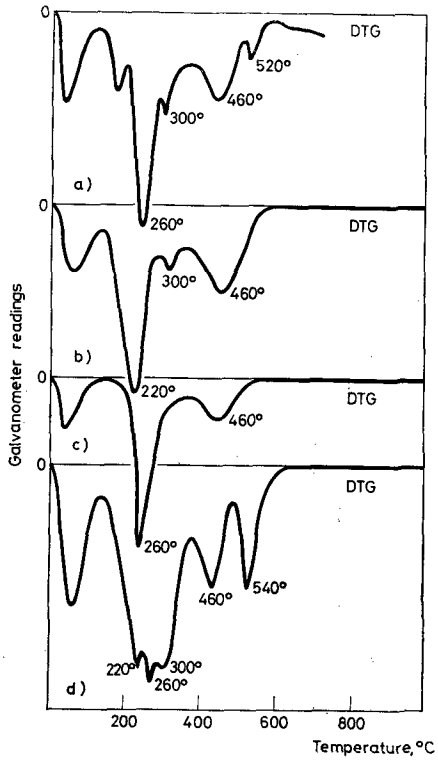

Fig. 2. DTG curves of ovalbumin glycopeptide (a), fibrinogen glycopeptide (b), capsular polysaccharide from Bacillus cereus (c) and bovine α_1-glycoprotein (d)

Table 1

Composition of glycopeptides and glycoproteins

	Glucosamine (%)	Mannose (%)	Galactose (%)	Sialic acid (%)
Ovalbumin glycopeptide	30.0	29.0	traces	—
Fibrinogen glycopeptide	16.0	2.0	15.0	10.0
Capsular polysaccharide from Bacillus cereus	30.0	—	48.0	—
Bovine α_1-glycoprotein	7.5	2.9	3.1	3.0

of covalent corsslinks present in the molecules of the investigated compounds. The main decomposition process in all three samples occured at 320°. The DTG peak, indicating the second process, was found in S-sulfo-fibrinogen at 520° (Fig. 3a). Structural differences caused by the presence of disulfide bridges in fibrinogen resulted in a shift of the DTG peak to higher temperature: 550° (Fig. 3b). As reported previously [4], the formation of highly crosslinked fibrin

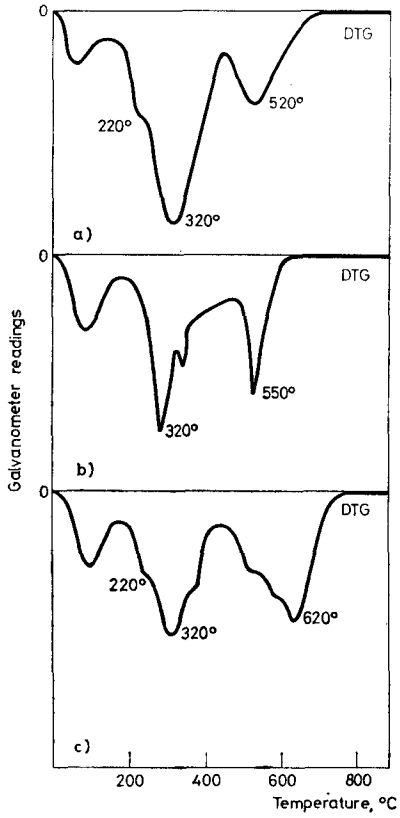

Fig. 3. DTG curves of S-sulfo-fibrinogen (a), fibrinogen (b) and fibrin (c)

fibres increases the thermostability of the molecule and the characteristic DTG peak was shifted to 620° (Fig. 3c). The characteristic decomposition peak of the carbohydrate components (220–260° region) could not be clearly distinguished on the DTG curves of these samples, probably due to their low concentration. However an inflexion at about 220° on the curves for S-sulfo-fibrinogen and of fibrin indicates the presence of small amounts of polysaccharides within the molecules.

2. *Cornea and skin extracts*

The compositions of the extracts and macromolecular complexes obtained from various tissues are presented in Table 2, and the results of thermal analysis in Table 3. As seen from the data in Table 2, the compositions of the CTC extracts and of the CSC complexes of different origin are different. The whiting corneas contain more extractable collagen, part of this collagen remaining soluble after dialysis. The CTC extracts of the calf corneas and of mouse skin contain only traces of collagen.

The insoluble CSC coprecipitate of the whiting corneas and of mouse skin is composed of collagen and non-collagenous structural glycoproteins or proteins, and chondroitin sulfate is absent [24]. The CSC fraction of the calf cornea is composed essentially of the same macromolecular constituents, but the ratio of

Table 2

Chemical compositions of the fractions of whiting and calf corneas and mouse skins

Fraction	Hexosamine %)	Hexose (%)	Uronic acid (%)	Chondroitin (%)*	Keratan sulfate+ neutral polysacch. (%)**	Hydroxyproline (%)	Gal	Glc	Man
Whiting intern. (scleroid) cornea CTC	2.8	3.4	0.6	1.2	5.8	5.6	1	0.4	0.25
Whiting intern. (scleroid) cornea CSC	4.5	7.3	traces	—	11.8	3.1	1	1	0.7
Whiting extern. (dermoid) cornea CTC	3.6	3.9	0.5	1.0	7.7	6.0	1	0.56	0.36
Whiting extern. (dermoid) cornea CSC	5.7	7.7	traces	—	13.4	3.4	1	1	0.6
Calf cornea CTC	5.4	6.1	1.9	3.7	9.6	traces	1	0.2	0.4
Calf cornea CSC	3.0	5.3	0.7	1.4	7.8	9.0	1	0.3	0.35
Mouse skin (C_3H) CTC	0.5	2.5	traces	traces	3.0	0.2	1	1.5	0.7
Mouse skin (C_3H) CSC	1.5	1.6	traces	traces	3.2	5.0	1	1.2	0.5
Mouse skin ($C_{57}D$) CTC	1.4	1.6	traces	traces	3.0	0.2	1	1.35	0.7
Mouse skin ($C_{57}D$) CSC	1.7	1.5	traces	traces	3.2	3.5	1	1.6	0.4

*.In the form of chondroitin sulfate, calculated on the basis of uronic acid content
** Calculated on the basis of hexose and hexosamine content

Table 3

Percentage weight loss of cornea and skin fractions at the characteristic decomposition temperatures
(calculated for dry, salt-free samples)

Fraction	DTG peak								
	210°	280°	320°	410°	480°	520°	540°	560°	580°
Whiting int. cornea									
CTC	3.0	12.5	30.5	—	—	31.5	—	—	22.5
CSC	—	25.0	34.0	—	—	18.5	22.5	—	—
Whiting ext. cornea									
CTC	2.0	13.5	32.5	—	—	30.0	—	—	22.0
CSC	—	28.5	32.0	—	—	18.5	21.0	—	—
Calf cornea									
CTC	3.0	20.0	16.0	25.0	36.0	—	—	—	—
CSC	2.0	18.0	29.0	—	—	27.5	10.5	13.0	—
Mouse skin (C_3H)									
CTC	2.0	6.6	41.0	—	13.0	37.0	—	—	—
CSC	—	8.0	35.5	—	9.0	20.0	17.5	7.0	—
Mouse skin ($C_{57}D$)									
CTC	2.5	6.0	38.5	—	20.0	33.0	—	—	—
CSC	—	9.5	44.5	—	—	29.0	11.0	6.0	—

hydroxyproline to neutral polysaccharides is different. In addition, calf cornea CSC contains chondroitin sulfate too [25]. The results of chemical analysis are confirmed by those of thermal investigations.

As described previously [3, 6] the 210° DTG peak is characteristic of the decomposition of acid glycosaminoglycans, while keratan sulfate and neutral polysaccharides are decomposed at about 270–280°. The percentage weight loss at these temperatures is proportional to the concentration of chondroitin, keratan sulfate and structural glycoproteins, respectively, and is in agreement with the chemical analysis data of Table 2.

The thermal decomposition of structural proteins takes place in two steps [4]: at 300° the partial cleavage of peptide linkages occurs, resulting in the formation of secondary products, the thermal stability of which depends on the original structure of the protein molecule. Within the second decomposition interval between 450 and 700°, the 520° maximum is characteristic of globular and soluble proteins, while fibrillar proteins of higher structural stability show higher thermostability as well.

If the thermoanalytical data in Table 3 are evaluated from this point of view, it can be concluded that the species differences of the corresponding fractions are also reflected in their thermal behaviour. The CTC extract of whiting cornea is composed of both collagen (characterized by the 580° DTG peak) and non-collagenous globular proteins (520° DTG maximum), while the CTC fractions of calf corneas and mouse skin contain only globular protein components (in good

agreement with the absence of hydroxyproline). The presence of the 540° and 560° DTG maxima in the insoluble CSC fractions indicates the higher crosslinked structure of the macromolecular network, probably due to the stronger association of the glycoprotein-proteoglycan-collagen complexes. The 580° maximum in some CTC extracts is probably due to the interaction of soluble collagen with calcium salts. These results seem to confirm the hypothesis, based on studies of the insoluble polymeric stroma (residue of 1 M $CaCl_2$ extraction) of fish [26] and mammalian corneas [26, 27], as well as their swelling behaviour following periodate oxidation, that the stabilization of the macromolecular network is realized in different species by different chemical means.

From the above results it may be concluded that thermal analysis is a suitable tool in the characterization of tissue fractions containing protein-polysaccharide complexes. The simultaneous quantitative determination of the proteoglycan and glycoprotein components of the complexes cannot yet be performed on the basis of the thermal curves, the decomposition temperatures of the neutral heterosaccharides of the glycoproteins being very close to those of the glycosaminoglycans of the proteoglycan molecules. However, interactions between the macromolecules of the collagen-proteoglycan-glycoprotein complexes significantly influence the thermal stability of the individual components, and thus thermoanalytical investigations usefully completed our knowledge on the structure of these important tissue fractions. The method can be further developed to study interactions between macromolecular complexes within various biological materials.

References

1. F. PAULIK, J. PAULIK and L. ERDEY, Z. anal. Chem. 160 (1958) 241.
2. L. ROBERT, B. ROBERT, E. MOCZAR and M. MOCZAR, Biologie et Pathologie, 20 (1972) 1001.
3. M. BIHARI-VARGA, Acta Biochim. Biophys. Acad. Sci. Hung. 6 (1971) 271.
4. M. BIHARI-VARGA, Acta Biochim. Biophys. Acad. Sci. Hung., 6 (1971) 265.
5. J. SIMON, M. BIHARI-VARGA, L. ERDEY and S. GERŐ, Acta Biochim. Biophys. Acad. Sci. Hung., 3 (1969) 273.
6. M. BIHARI-VARGA and T. BIRÓ, Gerontológia, 17 (1971) 2.
7. M. BIHARI-VARGA, M. VÉGH, J. LÉVAI and S. GERŐ, Clin. Chim. Acta, 22 (1968) 355.
8. M. BIHARI-VARGA, J. SIMON, J. FEHÉR and S. GERŐ, Acta Biochim. Biophys. Acad. Sci. Hung., 4 (1969) 279.
9. M. BIHARI-VARGA, T. BIRÓ and J. LÉVAI, Gerontológia, 17 (1971) 148.
10. T. BIRÓ and M. BIHARI-VARGA, Connective Tissue Res., 1 (1972) 305.
11. M. BIHARI-VARGA, J. FEHÉR, M. VARSÁNYI and S. Gerő, Acta Med. Acad. Sci. Hung., 29 (1972) 217.
12. M. BIHARI-VARGA and T. BIRÓ, Z. Alternsforsch., 26 (1972) 57.
13. T. BIRÓ and M. BIHARI-VARGA, Acta Chirurg. Acad. Sci. Hung., 14 (1973) 59.
14. E. MOCZAR and M. MOCZAR, Eur. J. Biochem., 13 (1970) 28.
15. E. MOCZAR, J. Chromatogr., 76 (1973) 417.
16. J. T. HOROWITZ, J. Am. Chem. Soc., 79 (1957) 5046.
17. L. ROBERT and Z. DISCHE, Biochem. Biophys. Res. Com., 10 (1963) 209.
18. J. MONTREUIL and G. SPIK, Microdosage des glucides Vol. I. p. 21 Monogr. du Laboratoire de Chimie Biologique de la Faculté des Sciences de Lille, Lille (1963).

19. G. BLIX, Acta Chem. Scand., 2 (1948) 467.
20. Z. DISCHE, J. Biol. Chem., 167 (1947) 189.
21. L. WARREN, J. Biol. Chem., 234 (1959) 1971.
22. I. BERGMANN and R. LOXYLEY, Analyst. Chem., 35 (1963) 1961.
23. E. MOCZAR and M. MOCZAR, In: Progress in thin layer chromatography and related methods., Humprey, Ann-Arbor, 1970 p. 183.
24. C. SEPULCHRE, E. MOCZAR and L. ROBERT, in preparation.
25. E. MOCZAR and M. MOCZAR, Exptl. Eye Res. in the press.
26. E. MOCZAR, P. PAYRAU and L. ROBERT, Comp. Biochim. Biophys., 30 (1969) 73.
27. E.MOCZAR and M. MOCZAR, Exp. Ann. de Biochimie Médicale, 30 (1970) 121.

RÉSUMÉ — Le "Derivatograph" s'est montré bien adapté à l'étude des biopolymères consistant en polysaccharides et protéines. On a mis en évidence une influence notable de l'interaction entre les macromolécules des complexes collagène-protéoglycane-glycoprotéine sur la stabilité thermique des composants individuels.

ZUSAMMENFASSUNG — Der Derivatograph wurde für die Prüfung von Polysaccharid- und Eiweiß-Biopolymeren als geeignet befunden. Es konnte nachgewiesen werden, daß Einwirkungen der Makromoleküle der Kollagen-Proteoglycan-Glycoprotein Komplexe die thermische Stabilität der einzelnen Komponenten bedeutend beeinflussen.

Резюме — Было найдено, что дериватограф может быть использован при исследовании биополимеров, состоящих из полисахаридов и протеинов. Показано, что взаимодействия, имеющие место между макромолекулами комплексов коллаген — протеогликон—гликопротеин, оказывают значительное влияние на термическую стабильность индивидуальных компонентов.