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STUDIES IN EXPERIMENTAL LATHYRISM—II
ON THE PROPERTIES OF COLLAGEN

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Abstract—Data are presented on the hydroxyproline and nitrogen contents of various collagenous extracts from the tissues of lathyritic rats.

Fresh lathyritic tissue adsorbed more chromium salts and slightly more water vapour than normal sample.

The physical chemical characteristics (among others intrinsic viscosity and sedimentation) were unchanged. The "α-component" was proportionally increased in the gelatinized collagenous extracts.

There was no change in free ε-amino-groups or ester linkages of soluble collagens in lathyrism.

IN THE previous paper[1] it was concluded that the defect in the fibre formation in lathyrism would be due to changes in collagen itself. Therefore, a further study on the properties of lathyritic collagen was desirable, especially since it would shed light on the mechanisms which normally regulate the tensile strength and solubility of collagen. It seems likely that the "nucleation" of new fibrils is not disturbed in lathyrism, but the fibres remain thinner than normal[2] and simultaneously the solubility of the surface collagen is increased.[3] The stabilization of collagen is thus impaired. β-Aminopropionitrile *in vitro* does not affect the thermal precipitation of collagen.[4]

In this paper we report analyses of lathyritic collagen with various physical and chemical means, which contribute to the evidence of a defect in stabilization. The presumed chemical change in collagen remained elusive.

EXPERIMENTAL

Collagen preparations

The preparation and analyses of the different collagen fractions, presented in Table 1, are described in our previous paper.[1] The amounts of collagen were calculated on the basis of hydroxyproline content, using a multiplication factor of 7·15.

Adsorption of chromium salts

According to Gustavson, the charge of the chromium complexes determines their adsorption to the different groups of collagen.[5] The compounds below were prepared following his descriptions:

I. 67% acid chromium sulphate, $Cr_2(OH)_2(SO_4)_2.Na_2SO_4$;
II. 67% acid chromium chloride, $Cr_2(OH)_2Cl_4.2NaCl$;
III. 33% acid chromium chloride, $Cr_2(OH)_4Cl_2.2NaCl$;
IV. Na-sulphito-sulphato-chromiate, $Cr_2(OH)_2(SO_4)_2.2{\cdot}0\ Na_2SO_3$;
V. Na-sulphito-sulphato-chromiate, $Cr_2(OH)_2(SO_4)_2.2\frac{1}{2}Na_2SO_3$.

Chromium complexes I and II are positively charged and selectively bound to anionic carboxyl groups of collagen. The compounds III, IV, V contain increasing proportions of non-charged chromium complexes and are adsorbed by keto-imide groups. The properties of the chromium complex preparations were controlled with paper electrophoresis[6] (in 0·1 M NaCl, buffered to pH 3·5, at 220 V for 3 hr).

The rat skins, stored 1–2 days at −15 °C, were cleaned mechanically of hairs and subcutaneous fat, and used as such or after defatting with chloroform–methanol (2 : 1, v/v) or after storage for about 1 month in −15 °C. To about 25 ml of chromium solutions (1 mEq Cr/l.), 1·0 g of wet skin (or 250 mg dry skin) was immersed and allowed to stand at room temperature or at +5 °C for 144–288 hr. The skins were rinsed thoroughly, first with tap water and finally with distilled water, dried at +105 °C and weighed. The organic material was combusted at +600 °C. At first, the chromium was estimated iodometrically after oxidation of the ashes with sodium peroxide at 600 °C. The ashes were almost totally of Cr_2O_3, and later the weight of the ashes was used as an estimate of the bound chromium.

Adsorption of water vapour

Bull's method[7] was adopted. The rat tail tendon fibres were kept at +25° ± 0·1 °C in a closed vessel, in which the partial pressure of water vapour was regulated by sulphuric acid of suitable strength in an open beaker. In a few days the fibres reached a constant weight and the sulphuric acid was titrated. The weight of the fibres was determined at ten different partial pressures of water vapour (from P/P_0 0·6–0·002). Finally, the dry weight (after 72 hr at +105 °C) was obtained and corrected for the ash. The expression $P/V(P_0 - P)$ was plotted against P/P_0 and the constant V_{max} (the first adsorbed layer) was evaluated statistically.

Sedimentation and viscosity

The sedimentation of collagen and gelatin was studied in a Spinco Model E analytical ultracentrifuge at 59,780 rev/min. A wedge cell was used for simultaneous run of two samples.

The sedimentation coefficient and intrinsic viscosity (with Ostwald viscosimeter) of collagen were determined at two concentrations (0·034 and 0·017 per cent) in 0·1 M citrate buffer at pH 3·8 and at +5·5 °C.

The ultracentrifuge study of the gelatinized collagen was carried out according to Piez *et al.*,[8] i.e. in 0·15 M acetate buffer pH at 37 °C after gelatinization of the sample at + 40 °C for 10–15 min.

Free ε-amino groups of lysines

The pH of the sample was adjusted to 8·0 with sodium hydroxide, and solid sodium bicarbonate was added up to 4 per cent concentration (pH 8·2). Dinitrofluorobenzene (5 per cent solution in ethanol) was added to a final concentration of 0·25 per cent and the mixture was shaken for 24 hr at room temperature. The precipitate was collected by centrifugation, washed with water, 0·1 N hydrochloric acid and ethanol, and finally air-dried. The DNP–collagen was hydrolyzed for 16 hr at +100 °C in 6 N HCl. The hydrolysate was extracted four times with ethyl ether. The water phase was diluted with water and the colour intensity was measured at 3600 Å.

Ester linkages

It has been demonstrated that hydroxylamine breaks the ester bonds in collagen,[9] which may form the stabilizing intermolecular cross-link. Various collagen solutions were studied as such and also after gelatinization in 3 M urea at +37 °C for 30 min. Aqueous hydroxylamine solution (3·8 M, pH 10) was added to the samples to final concentrations of 0·5 M and the pH adjusted to 9 with 6 N sodium hydroxide solution. The mixtures were kept for 1½ hr at +40 °C (some turbidity persisted) and dialysed against distilled water. The protein was precipitated with 10-fold volume of ethanol and the hydroxamate was estimated as nitrite produced by oxidation with iodine according to Bergmann and Segal[10].

Other experiments

Some X-ray diffraction diagrams were prepared on lyophilized rat tail tendon fibres by Professor M. Kantola (Department of Physics, University of Turku, Turku) and infra-red absorption was studied in Oy Keskuslaboratorio Ltd. (Helsinki) of samples pressed to pellets with 150-fold amount of sodium bromide. Since these preliminary investigations did not indicate changes in lathyrism, they are not presented in detail.

An attempt was made to study the rate of gelatinization at +39 °C from the decreasing viscosity of collagen samples, extracted from rat tail tendons with 0·4 per cent acetic acid. The "half-time" of the viscosity was shorter in lathyritic samples but more experimental work is necessary to settle this point.

RESULTS

Solubility of collagen

From Table 1 it is evident that the neutral salt-soluble collagen is much increased in lathyrism and that during re-extraction with citrate buffer, the precipitated collagen

TABLE 1. COMPOSITION OF THE COLLAGEN FRACTIONS FROM RAT SKIN AND TAIL TENDON
The figures are based on the original wet weight of the tissue: C, control, L, lathyritic sample. The preparations are explained in detail in the previous paper,[1] Fig. 1.

Origin and fraction	Collagen, measured as hydroxyproline		Total nitrogen		Hydroxyproline N in % of the total N	
	C	L	C	L	C	L
I. *Rat Skin:*						
Fraction A (combined)	0·287	2·184	0·499	1·003	0·861	3·3
Fraction A-1 (purified, alkali- and citrate-soluble)	0·082	0·964	0·020	0·222	6·2	6·5
Fraction A-2 (alkali-soluble, but citrate-insoluble)	0·175	1·186	0·041	0·228	6·4	7·7
Fraction A-3 (water-soluble)	0·011	0·010	0·079	0·092	0·20	0·16
Fraction A-4 (diffusible)	0·019	0·024	0·359	0·461	0·08	0·08
Fraction B (combined)	0·299	0·850	0·356	0·760	—	—
Fraction C (alkali-insoluble)	31·2	28·6	6·76	6·80	6·9	6·3
II. *Rat Skin:*						
Whole tissue	22·6	27·2	7·17	7·54	—	—
NaCl-extract	0·58	3·29	1·27	1·62	0·67	3·0
III. *Rat Tail Tendon:*						
NaCl-extract	3·6	10·7	0·99	2·47	5·4	6·5
citrate (pH 3·8) extract	9·6	9·7	1·67	1·46	8·7	9·9

of the lathyritic sample is more soluble and easier to purify from other nitrogenous substances. There is no indication of any differences in the amounts of water-soluble or diffusible hydroxyproline. The amount of citrate-extracted collagen fraction is the same in normal and lathyritic samples. The concentrations of saturated solutions of both normal and lathyritic neutral salt-soluble collagens are approximately the same.

Adsorption of chromium salts and water vapour

If the skins are treated fresh, the lathyritic specimens adsorb more chromium as indicated by Table 2. If the data on adsorption of complexes I–II (samples 3, 5–8) are

TABLE 2. ADSORPTION OF CHROMIUM COMPLEXES BY RAT SKIN COLLAGEN IN LATHYRISM
The values are expressed as % Cr_2O_3 of dry weight. C, normal, L, lathyritic sample.

Sample	Chromium complex (see the text)					Conditions
	I	II	III	IV	V	
C 1 stored	6·6	6·1	11·9	12·5	15·2	240 hr at room temperature
L 1 stored	6·5	5·8	10·0	13·1	16·2	
C 2 stored	8·9	6·5	12·2	14·2	15·5	
L 2 stored	8·3	6·6	12·4	13·6	14·7	
C 4 defatted	9·4	7·0	—	15·3	17·5	144 hr at room temperature
L 4 defatted	9·3	6·8	—	14·9	16·1	
C 3 fresh	9·1	7·4	—	14·2	16·2	
L 3 fresh	10·0	8·0	—	15·5	18·0	
C 5 fresh	7·8	7·1	13·9	11·5	12·6	288 hr at +5 °C
L 5 fresh	8·3	7·9	13·9	13·5	15·1	
C 6 fresh	7·8	7·4	13·7	12·3	13·3	
L 6 fresh	8·2	7·5	12·7	11·9	13·1	
C 7 fresh	9·3	7·5	14·1	14·3	15·9	144 hr at room temperature
L 7 fresh	9·7	7·8	15·4	—	18·2	
C 8 fresh	9·4	7·5	14·6	13·1	15·5	
L 8 fresh	9·5	7·6	14·9	14·5	16·5	

arranged for statistical evaluation as non-independent pairs, $P < 0{\cdot}005$, and in regard to complexes III–V, $P < 0{\cdot}01$. The increased chromium-binding capacity is not confined to either carboxyl groups or keto-imide groups. It seems rather that the structure of the collagen fibre allows a better access to chromium.

In the stored and defatted samples there seems to be no clear trend, indicating a lability in collagen which is manifested also in other respects, e.g. the solubility of collagen being lost and the thermal characteristics changed at storage.

The absorption of water vapour was slightly increased in lathyritic samples: V_{max} was 10·9, in control samples 10·6.

The physical properties of Na_2HPO_4*-soluble collagen*

Table 3 contains the data obtained by sedimentation and viscosity studies on Na_2HPO_4-soluble collagen.[1] The sedimentation gradient was hypersharp. A value of

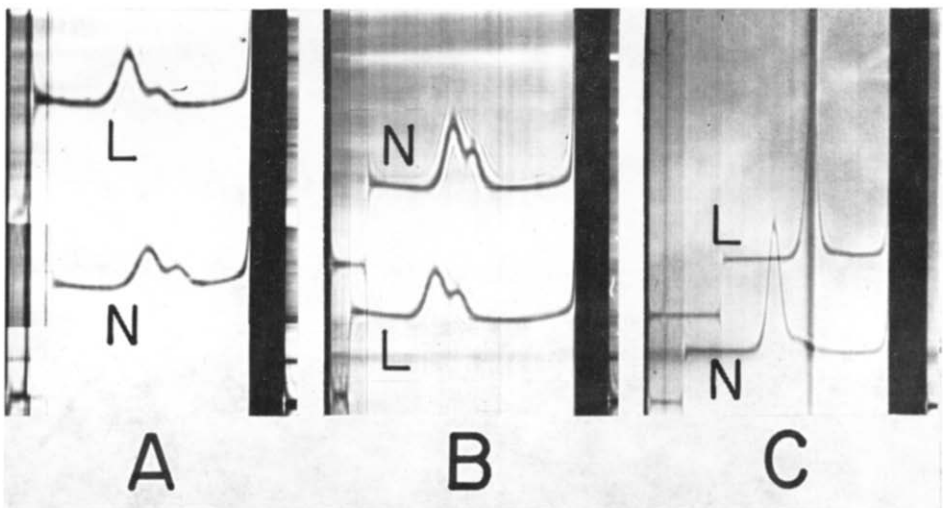

FIG. 1. Sedimentation patterns of gelatins from soluble rat tail tendon collagen in 0·15 M acetate buffer, pH 4·8. Temperature 37 °C. Sedimentation from left to right at 59,780 rev/min. L, lathyritic sample; N, normal sample. A, acetic acid-extracted (c_N = 0·24 per cent; c_L = 0·25 per cent), 100 min after reaching full speed. B, acetic acid-extracted after exhaustive NaCl-extraction (c_N = 0·41 per cent; c_L = 0·34 per cent, 94 min after reaching full speed. C, NaCl-extracted (c_N = 0·65 per cent; c_L = 0·68 per cent), 97 min after reaching full speed.

facing page 934

0·700 for the partial specific volume was used in the calculations and the equations of Simha[11] and Simha and Perrin[12] were applied. The results were similar to those given by Boedtker and Doty[13] for citrate-soluble collagen of carp swim bladder. The small difference in the physical constants between normal and lathyritic collagens is not considered as significant.

TABLE 3. THE PHYSICAL DATA ON THE SOLUBLE COLLAGEN FRACTION FROM SKINS OF LATHYRITIC RATS

Preparation A-1.

Determined	Normal sample	Lathyritic sample
Intrinsic viscosity ($c = g/100$ ml)	10·2	12·5
Sedimentation constant ($s^\circ_{20,w}$)	3·08	3·14
$d(1/s)/dc$	1·3	1·6
"Axial ratio" (J)	165	185
Parameter β	3·41	3·46
Molecular weight	254,000	282·000

Components of gelatinized collagens

Fig. 1 A shows a representative ultracentrifuge pattern of gelatinized acetic acid (3%) extracted rat tail tendon collagen showing the α- and β-components with $s^\circ_{20,w}$ of 3·09 and 4·46, respectively. The ratio of α- to β-component was clearly increased in lathyrism. When the Johnston–Ogston effect was eliminated by the use of the equation of Trautman *et al.*,[14] weight ratios α/β of 0·74 and 1·20 in the normal and lathyritic sample, respectively, were calculated.

When the tail tendons were first exhaustively extracted with 0·2 M NaCl in 0·15 M phosphate buffer, pH 7·4, with subsequent extraction of the residue with 3% acetic acid, the resulting acid extracts of both normal and lathyritic samples (Fig. 1 B) were similar with respect to their α/β-ratios (0·67 by weight in both). As expected,[15] the neutral salt-extracts consisted almost exclusively of α-component with traces of β in the normal sample (Fig. 1 C). Thus, the increase of α-component in lathyrism is due to the increase of the NaCl-extractable collagen.

The above results confirm the recently published work of Piez *et al.*[16] and can be considered as an additional indication of the accumulation of the neutral salt-extractable collagen fraction in lathyritic tissues.

Free ϵ-amino groups of Na_2HPO_4-soluble collagen

Only the "water-soluble" fraction of DNP-amino acid mixture of collagen was studied. A molar extinction coefficient of $17{\cdot}7 \times 10^3$ was used and following amounts of DNP-compounds were calculated:

control sample, 20·7 m-moles/100 g protein;

lathyritic sample, 21·3 m-moles/100 g protein.

This is in both cases rather near of the accepted amount of the free ϵ-amino-groups of lysines[17] in collagen.

Ester linkages (reactivity with hydroxylamine)

The amount of the bound hydroxylamine agreed with values cited in the literature[9, 18] (0·5–1·5 moles/10^5 g). The results varied in different preparations, but if there

is any decrease in lathyritic collagens at all, it must be in order of only 0·1–0·2 moles per 10^5 g of collagen.

DISCUSSION

The conclusion is reached in various ways that in lathyrism the collagen does not mature to a stable form. The evidence includes the increased amount of soluble collagen in the tissues, the above confirmed increase of α-components in the gelatinized collagenous extracts,[16] decreased tensile strength per cross-section,[2] decreased thickness of fibres[2] and their easy fragmentation,[3] decreased incorporation of labelled amino acids especially into the "β-component"[19] and the increased adsorption of chromium.

Two reasons can be speculated for this: (a) qualitatively changed collagen, or (b) defect in some extraneous factor which would be necessary for the stabilization. Although the possibility of some extrinsic factors should not be ignored, they are thus far poorly understood. Since even the lathyritic collagen precipitates and forms fibrous collagen, the defect cannot be very conspicuous and the assumed qualitative change in lathyritic collagen remains elusive.

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