Biochemical Pharmacology, 1962, Vol. 11, pp. 923–929. Pergamon Press Ltd., Printed in Great Britain.

STUDIES IN EXPERIMENTAL LATHYRISM—I
NON-COLLAGEN COMPONENTS OF TISSUES

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(*Received* 14 *April* 1962; *accepted* 13 *June* 1962)

Abstract—The amounts of soluble carbohydrates (containing hexosamine and uronic acid) in collagenous extracts from skin and tendon of lathyritic rats were not changed when expressed per wet weight of the original tissue. When the solubilized collagens were purified, less carbohydrate remained associated with collagen in samples extracted from lathyritic animals.

The amount of soluble non-collagenous proteins of skin was decreased in lathyrism in relation to the soluble collagen. The neutral fat fraction of skin lipids was decreased absolutely.

ALTHOUGH the sweet pea intoxication or lathyrism is manifested as a defect of the collagenous fibres,[1] it is not known what factors are responsible for the increased fragility. Although it has been amply demonstrated that the solubility of collagen from tissues is increased in lathyrism,[1, 2, 2a] the possibility that these changes were due to the absence of stabilizing effect of closely associated non-collagenous substances has not been excluded. It has been thought for long that the stability of collagen fibres is influenced by the non-collagenous matter,[3, 18] e.g. by carbohydrates. For this reason it seemed justified to study the non-collagenous components in tissues of lathyritic rats, especially since we believe that lathyrism is based on a general metabolic defect.[4]

EXPERIMENTAL

Preparation of the collagenous fractions

I. *From rat skin.* The material consisted of sixty-four skins of control rats and sixty-two of lathyritic rats which had been fed with standard food containing about half of sweet pea seeds. The skins originated from our feeding experiments on lathyrism.[5] The hair and subcutaneous tissue were removed mechanically. The fractionation (Fig. 1) was carried out at +5 °C. The centrifugations were performed with Wifug angle head centrifuge, 4300 rev/min, for 30 min, except the high-speed centrifugations with Spinco Model E centrifuge using preparative rotors. The main part of fraction A-1 was lost accidentally, and therefore the preparation B was made as also were the following two fresh preparations.

II. *From rat skin.* Two normal and two lathyritic skins were minced as above and extracted at +5 °C four times with 5-fold (v/w) volume of 0·2 M NaCl-solution, buffered to pH 7·4 with 1/15 M phosphate. The combined extracts were centrifuged for 60 min at 50,000 × *g*.

III. *From rat tail tendon.* The fibres were prepared from the tails with forceps and extracted at +5 °C three times for 24 hr with 30-fold volume (v/w) of 0·2 M NaCl-solution, buffered to pH 7·4 with 1/15 M phosphate. The residue was re-extracted four

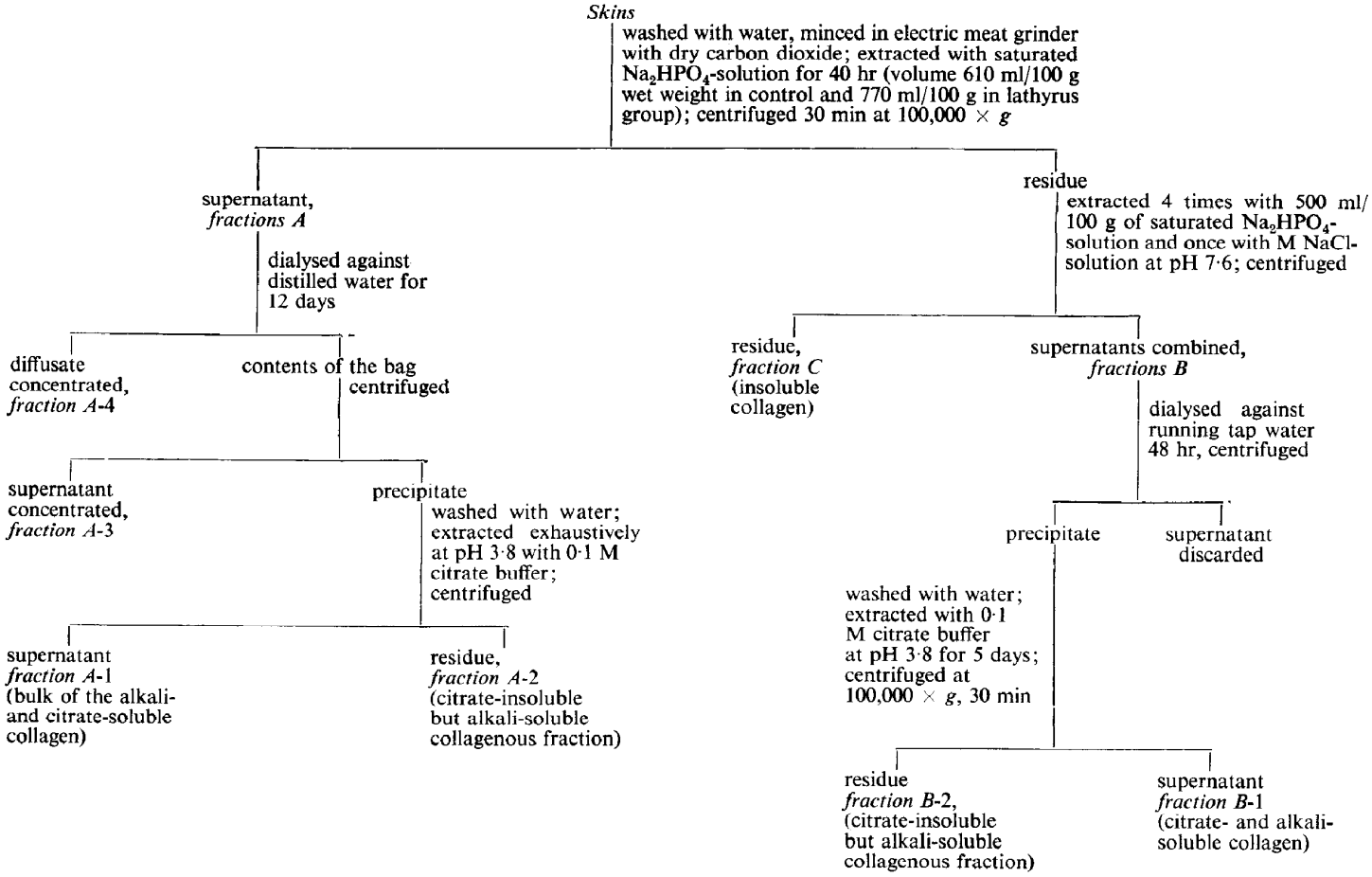

FIG. 1. Fractionation I of rat skins

times for 48 hr with 40-fold volume (v/w) of 0·1 M pH 3·8 citrate buffer. The combined NaCl-extracts and combined citrate-extracts were centrifuged for 30 min at 65,000 × *g*. The NaCl-soluble collagen was precipitated by addition of the equal volume of 32% NaCl-solution. The precipitate was collected by centrifugation and dissolved in 0·2 M NaCl-solution and precipitated finally by dialysis against distilled water. For analyses this preparation was dissolved into 0·2 M NaCl-solution. It was no longer completely soluble and the solution was cleared by centrifugation for 30 min at 100,000 × *g*.

Preparation of non-collagenous protein

Water soluble proteins were prepared from Na_2HPO_4- and NaCl-extracts of skins. The collagen was removed after precipitation either by dialysis or by heat (30 min at +30 °C) and the supernatant dialyzed against distilled water. The total contents of the bags were dissolved into 0·9% NaCl-solution.

Preparation of lipids

The lipid was extracted from wet skins at room temperature with 20 vols. (v/w) of 2 : 1 chloroform–methanol mixture. The crude lipid was purified according to Folch *et al.*[6] After evaporation of the solvent in a stream of nitrogen, the lipid was weighed, dissolved in light petroleum and fractionated in a silicic acid column according to Fillerup and Mead.[7]

Preparation of carbohydrates

The polysaccharides were prepared from some collagenous fractions. The samples obtained in collagen preparation I were dehydrated in acetone, digested with papain (in the presence of cysteine and EDTA) and the mucopolysaccharide fraction was recovered by precipitation with 10-fold volume of ethanol in the presence of 0·5% sodium acetate. This precipitation was repeated at pH 2·5 and pH 10. The neutral mucopolysaccharide was extracted from the total mucopolysaccharide with 90% aqueous phenol for 12 hr.[8]

The acid mucopolysaccharide residue was fractionated further using the precipitation of carbohydrate with cetylpyridinium chloride inside a cellulose column, which was eluted with solutions of stepwise increasing strength of magnesium chloride.[9] The procedures and results are not presented in detail here since the analyses revealed that the total and acid mucopolysaccharide preparations contained less than 10% of hexosamine and were therefore contaminated with proteins, presumably because of insufficient treatment with papain.

Analytical methods

Hexosamine was determined according to Blix[10], uronic acid by the carbazole method of Dische[11] and sialic acid according to Svennerholm[12]. Nitrogen was combusted according to Kjeldahl and measured as ammonia after distillation. Hydroxyproline was determined according to Neuman and Logan[13].

RESULTS

Carbohydrates in the collagenous fractions

The data are collected to Tables 1 and 2. It may be observed that neutral salt extracts contained almost the same concentrations of uronic acid and hexosamine

TABLE 1. CONTENT OF SOME CARBOHYDRATES IN COLLAGENOUS FRACTIONS OF RAT TISSUES

The designations of fractions refer to Fig. 1. The figures are expressed as percentage of the wet weight of the original tissue (except in the last column). C, samples from control rats, L, from lathyritic rats.

Origin and fraction	Hexosamine		Uronic acid		Sialic acid		Hexosamine in % of collagen	
	C	L	C	L	C	L	C	L
I. *Rat skin:*								
Fractions A (combined)	0·043	0·053	0·044	0·050	—	—	15·0	2·4
Fraction A-1 (purified, alkali- and citrate-soluble)	0·002	0·006	0·001	0·005	—	—	2·5	0·6
Fraction A-2 (alkali-soluble, citrate-insoluble)	0·006	0·013	0·005	0·010	—	—	3·5	1·3
Fraction A-3 (water-soluble)	0·022	0·021	0·019	0·019	—	—	—	—
Fraction A-4 (diffusible)	0·013	0·013	0·019	0·016	—	—	—	—
Fractions B (combined)	0·087	0·078	—	—	—	—	29·1	9·2
Fraction B-2 (alkali-soluble, citrate-insoluble)	0·013	0·008	—	—	—	—	4·1	1·3
Fractions A + B (all soluble fractions)	0·130	0·131	—	—	—	—	22·2	4·3
Fraction C (insoluble residue)	0·176	0·201	—	—	—	—	—	—
II. *Rat Skin:*								
Whole tissue	0·21	0·20	—	—	0·12	0·15	0·93	0·73
NaCl-extract	0·085	0·087	—	—	0·064	0·065	14·6	2·7
III. *Rat Tail Tendon:*								
NaCl-extract	0·120	0·120	0·070	0·070	—	—	3·3	1·1
Purificd NaCl-extract	—	—	—	—	—	—	0·21	0·11
Citrate extract (pH 3·8)	0·060	0·060	—	—	—	—	0·63	0·62

both in lathyritic and control samples, although the lathyritic samples contained collagen in 3–8-fold concentrations (Table 2).

When the collagenous extracts were purified, some carbohydrate remained associated with the collagen. From the last column of Table 1 it is evident that the proportion of hexosamine in collagen preparations is always smaller in lathyritic samples than in normal samples (with exception of fraction A-3, which contained very little collagen). The data on purified rat tail collagen agree with those of Moss[14]. When the crude neutral salt extracts of collagen are dialysed against water, the collagen precipitates almost quantitatively. The bulk of both the non-collagenous proteins and the carbohydrates remains in the solution, where there is no difference between the lathyritic and control samples.

TABLE 2. COMPARISON OF SOLUBILITIES OF TOTAL NITROGEN, HYDROXYPROLINE, HEXOSAMINE AND URONIC ACID INTO SOME FRACTIONS OF SKINS FROM LATHYRITIC VS. CONTROL RATS

The designations of fractions refer to Fig. 1 and Table 1. The amount (per wet weight) in fractions from control samples is taken as unity (=1·00).

Fraction	Total N	Hydroxy-proline	Hexosamine	Uronic acid
First Na_2HPO_4-*extract* (A combined)	2·0	7·6	1·19	1·15
Subfractions after dialysis:				
diffusate (A-4)	1·28	1·22	0·93	0·89
inside the bag:				
in solution (A-3)	1·17	0·93	0·93	0·98
as precipitate (A-1 + A-2)	7·5	8·3	2·3	2·5
citrate-soluble (A-1)	11·3	11·8	3·0	4·9
citrate-insoluble (A-2)	5·6	6·5	2·1	2·0
Following (2*nd*–6*th*) Na_2HPO_4-*extracts* (B combined)	2·1	2·8	0·90	0·93
All Na_2HPO_4-*soluble fractions* (combined)	2·1	5·2	1·01	0·96

A comparison of lathyritic vs. the normal samples is done in Table 2. For example the figure 7·6 on top in the hydroxyproline column means that 7·6-fold amount of hydroxyproline (per original wet weight) is present in the first Na_2HPO_4-extract of lathyritic skin in comparison to a similar extract of the normal skin. In the soluble fractions (A-3 and A-4) all the ratios are near unity. It seems that in the combined second to sixth Na_2HPO_4-extracts the solubility on carbohydrates is similar both in lathyritic and normal samples and the increased solubility of collagen in the lathyritic samples is no longer so marked as in the first extract.

When the collagen precipitates, which had been formed in dialysis of neutral salt extracts, were extracted with pH 3·8 citrate buffer, the lathyritic collagen dissolved better again. This purified sample of lathyritic collagen contained hexosamine only about a quarter the strength of that in the respective normal sample (Table 1).

We conclude: (1) That when the solubility of tissue carbohydrates is expressed per tissue weight, it is not changed in lathyrism. The amount of "free" carbohydrate in the tissue, both diffusible and non-diffusible, is the same. The "insoluble" carbohydrate (in fraction C) is increased very little, if at all. (2) That the carbohydrate content of the purified alkali-soluble collagen fractions is constantly smaller in samples prepared from lathyritic animals (see Discussion).

As mentioned, the acid and neutral mucopolysaccharides were isolated from this co-precipitating carbohydrate, and the acid mucopolysaccharide preparations were fractionated further with cetylpyridinium chloride into five subfractions. The distribution pattern of the whole acid mucopolysaccharide into its subfractions depended somewhat on the analytical test which had been used for quantitation. The chondroitin sulphate (1·5 N $MgCl_2$-soluble) fraction was clearly increased inside the acid mucopolysaccharide fraction from lathyritic A-2 sample in comparison of respective control sample. In hyaluronate fraction no change was observed. Since the lathyritic fraction A-2 contains less acid mucopolysaccharide per collagen than normal A-2, this small relative increase of chondroitin sulphate inside the mucopolysaccharide fraction does not seem significant. Since the data did not indicate that carbohydrates would be of importance in lathyrism we did not continue on this line.

Non-collagenous protein

Less non-collagenous Na_2HPO_4-soluble protein (per amount of collagen) was found in the extracts from lathyritic tissues (Table 2). During the purification this difference between normal and lathyritic samples disappeared. Although this finding did not suggest that the non-collagenous proteins were of importance for the condition of the collagen in lathyrism, a study with paper electrophoresis (pH 8·6, barbiturate buffer) was made. As expected, the proteins consisted mainly of albumin (presumably from blood) and very little of β-globulin. No clear difference was observed between the lathyritic and normal samples. These preparations were different from the non-collagenous skin proteins of Bowes *et al.*,[15] who used stronger alkali.

Lipids

The lathyritic skins contained less fat macroscopically, even if the animals were allowed to eat freely. An analysis was made on the fats from two samples and the results are presented in Table 3. The main difference was in the neutral fat fraction,

TABLE 3. LIPIDS OF SKIN OF NORMAL AND LATHYRITIC RATS

Figures given in percentage of the wet weight of the skins, which had been cleaned from hairs and subcutaneous tissue.

Solvent	Fraction	Normal samples	Lathyritic samples
Light petroleum	Nonpolar	0·24	0·38
Ether (1 %, v/v) in light petroleum	Sterol esters	0·66	0·23
Chloroform	Triglycerides, Cholesterol, Fatty acids	7·38	1·52
Methanol	Phospholipids	0·87	0·94
	Total	9·15	3·07

which was decreased to a fifth of the normal in lathyrism, although the subcutaneous fat was removed as thoroughly as possible. In another experiment the liver lipids were analysed similarly. No change was observed in lathyrism in the amount of the total lipids of the liver, but the percentage of the neutral fat was slightly decreased (from 37·2 to 27·5 per cent) and the proportion of phospholipids was increased accordingly.

DISCUSSION

These data on carbohydrates, lipids and non-collagenous proteins do not explain the changed state of collagen in lathyrism. They rather corroborate the belief that collagen itself is modified, even if acid mucopolysaccharides have been implicated in lathyrism.[16, 17] The possible changes may be interpreted rather as a part of reaction against mechanical dislocation of tissues in lathyrism. This conclusion is not meant as an argument against the idea of a "stabilizing" hexose in the collagen fibre.[18]

We suggest that the decrease in the co-precipitation of acid carbohydrate with collagen in lathyrism is not due to any changes in the carbohydrate or in the collagen, since extracts contained always less carbohydrate in relation to collagen. Then the precipitating collagen adsorbs proportionally less carbohydrate. This reasoning is confirmed by direct experiments of Nemeth-Csóha[19]. Extra proteins are easier to remove from soluble collagens than adsorbed acid carbohydrates.

Acknowledgements—This work has been supported first by the Sigrid Jusélius Foundation and later by the U.S. Department of Agriculture, Foreign Research and Technical Programs Division.

REFERENCES

1. C. I. Levene and J. Gross, *J. exp. Med.* **110**, 771 (1959).
2. L. Mikkonen, T. Tuominen and E. Kulonen, *Acta Chem. Scand.* **13**, 225 (1959); *Biochem. Pharmacol.* **3**, 181 (1960).
2a. W. Lasler, R. E. Stoner and R. V. Milliser, *Metabolism* **10**, 883 (1961).
3. D. S. Jackson, in *Nature and Structure of Collagen* (Edited by J. T. Randall). Butterworths, London (1953); *Biochem. J.* **54**, 638 (1953); *Ibid.* **56**, 699 (1954).
4. E. Kulonen, K. Juva, L. Mikkonen, T. Nikkari, A. Salmi and T. Tuominen, *Biochem. Pharmacol.* **6**, 56 (1961).
5. K. Juva, T. Tuominen, L. Mikkonen and E. Kulonen, *Acta path. microbiol. scand.* **51**, 250 (1961).
6. J. Folch, I. Ascoli, M. Lees, J. A. Meath and F. N. LeBaron, *J. biol. Chem.* **191**, 833 (1951).
7. D. L. Fillerup and J. F. Mead, *Proc. Soc. exp. Biol., N.Y.* **83**, 574 (1953).
8. E. Buddecke, *Z. physiol. Chem.* **318**, 33 (1960).
9. C. A. Antonopoulos, E. Borelius, S. Gardell, B. Hamnström and J. E. Scott, *Biochim. biophys. Acta* **54**, 213 (1961).
10. G. Blix, *Acta Chem. Scand.* **2**, 467 (1948).
11. Z. Dische, *J. biol. Chem.* **167**, 189 (1947).
12. L. Svennerholm, *Ark. Kemi* **10**, 577 (1956); *Acta Soc. Med. Upsal.* **61**, 75 (1956).
13. R. E. Neuman and M. A. Logan, *J. biol. Chem.* **184**, 299 (1950).
14. J. A. Moss, *Biochem. J.* **61**, 151 (1955).
15. J. H. Bowes, R. G. Elliott and J. A. Moss, in *Recent Advances in Gelatin and Glue Research* (Edited by G. Stainsby) p. 71. Pergamon Press, London (1958).
16. G. C. H. Bauer, A. Carlsson and B. Lindquist, *Acta path. microbiol. scand.* **37**, 407 (1955).
17. M. J. Karnovsky and M. L. Karnovsky, *J. exp. Med.* **13**, 381 (1961).
18. H. Hörmann, *Leder* **11**, 173 (1960).
19. M. Nemeth-Csóha, Abstract No. 9.22.641 of *The Fifth International Congress of Biochemistry*. Pergamon Press (1961).