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IN VITRO STUDIES ON THE EFFECT OF *IN VIVO* ZINC DEFICIENCY ON THE FORMATION OF GLYCOSAMINOGLYCANS IN RAT COSTAL CARTILAGE

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The effects of *in vivo* zinc deficiency and restricted food intake, on the *in vitro* synthesis of glycosaminoglycans of rib cartilage were studied in the rat. ^{35}S-sulfate and ^{14}C-glucosamine were used as precursors. The glycosaminoglycans were separated on microcolumns and specific radioactivities determined for the different fractions.

Chemical analyses showed that zinc deficiency or reduced food intake did not cause any qualitative or quantitative changes in the glycosaminoglycans. The radioassays indicated that zinc deficiency and reduced food intake, alone or combined, caused a somewhat lowered synthetic rate of chondroitin sulfate. In the discussion it is underlined that it seems difficult to determine conclusively the importance of zinc for the formation of the mucopolysaccharides through further *in vivo* deficiency studies, because of the difficulties to control and evaluate the inanition factor.

Zinc has been shown to be an essential nutrient for all avian and mammalian species so far investigated. This element has at least two biological functions It is a component of various metalloenzymes and plays a role in nucleic acid and protein metabolism.

The effects of zinc deficiency in experimental animals include loss of appetite, marked growth retardation, loss of hair and structural and functional abnormalities of gastrointestinal and male reproductive organs.

That zinc deficiency affects growth was first demonstrated by *Todd et al.* (1934). *Follis et al.* (1941) studied endochondral osteogenesis and reported that zinc deficiency caused a thinning of the epiphyseal plate in the rat. *Bergman et al.* (1970) investigated the early effects of zinc deficiency on

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Abbreviations used: CPC = cetylpyridinium chloride
KRB = Krebs-Ringer bicarbonate buffer
carbogen = 95 % O_2, 5 % CO_2

endochondral growth sites in the rat. These authors found a reduction in thickness of growth cartilage and evidence of reduced osteoblastic activity. Autoradiograms with ^{3}H-proline indicated that there was a reduced formation of collagen and a reduced rate of longitudinal growth and remodelling in the zinc deficient cartilage.

Nielsen et al. (1970) examined the effect of zinc deficiency on ^{35}S-Sulfate and hexosamine metabolism in the epiphyseal growth apparatus of the chick. Their data suggested a reduced synthesis of sulfated glycosaminoglycans in this deficiency.

Evidence for an impairment of wound healing in zinc deficiency was presented by *Sandstead et al.* (1970), and *Oberleas et al.* (1971).

The studies mentioned above point towards a rather general role of zinc in the formation of the intercellular substances of the connective tissues. To what extent zinc is engaged in the metabolism of the glycosaminoglycans has, however, not been conclusively demonstrated.

The present study is concerned with the *in vitro* synthesis of the glycosaminoglycans of rib cartilage from zinc deficient rats. Since, however, zinc deficiency is regularly accompanied by a reduced food intake, restricted fed controls were utilized. This work was of a pilot character, and limited material was used. ^{35}S-sulfate and ^{14}C-glucosamine were used as precursors.

MATERIAL AND METHODS

Animals. The rib cartilage was obtained from the rats used by *Bergman* (1970), *viz.* weaned, 21-day old, female rats of the Sprague-Dawley strain. The procedures for the housing of the animals and for the preparation of the zinc deficient diet and purified water etc. have been described by *Bergman et al.* (1970).

The animals were divided into four groups of 5 animals each:

A: experimental rats, which were given the zinc deficient diet *ad libitum*
B: paired-fed controls, which were given the zinc deficient diet plus zinc supplement
C: paired weight-fed controls, which were given the zinc deficient diet plus zinc supplement
D: controls, which were given a commercial rat diet *ad libitum*

The rats in group B were given an amount of the experimental diet equal to the mean food intake during the preceding day of the rats in group A.

For group C the food intake was restricted so that their mean weight curve closely followed that of the rats in group A.

Preparation of cartilage. After 18 days on the diets the rats were killed. Cartilage from ribs II—VIII was removed leaving a 2 mm wide zone of cartilage at the costo-chondral and sterno-chondral junctions. Rib cartilage was chosen because of the large amount available of homogenous cartilage. The cartilage pieces were freed from perichondrium. The cartilages from all 5 animals of a group were pooled and divided between two 50 ml Erlenmayer flasks, one for each of the two precursors used.

^{35}S-sulfate incubation. The cartilage was preincubated for 1 hour at 37°C in 10 ml of sulfate-free KRB supplemented with 10 mM glucose and 0.1 mM glutamine under carbogen atmosphere. The medium was then removed. Ten ml of fresh medium containing 5 μCi/ml of carrierfree $Na_2{}^{35}SO_4$, was added. After incubation for another two hours the flasks were chilled in an ice bath and the medium was immediately suctioned off. The cartilage was repeatedly rinsed with ice cold KRB containing non-labeled sulfate. Acetone was then added and changed several times over a period of 24 hours. The cartilage pieces were then air-dried.

^{14}C-glucosamine incubation. A similar procedure as described above was used. However, the KRB-medium used contained sulfate in the usual amount, 0.1 ml glucose and 20 mM sodium pyruvate. During incubation the medium contained 2 μCi/ml of D-glucosamine-1-^{14}C (specific activity 10.1 mC/mM).

Rinsing was performed with KRB solution.

Separation and analysis of glycosaminoglycans. The cartilage samples were digested with papain at 65°C for 3 hours according to *Scott* (1960). The glycosaminoglycans were then separated on microcolumns of CPC/cellulose (*Antonopoulos & Gardell*, 1963). Fractionation on microcolumns of ECTEOLA-cellulose (*Antonopoulos et al.*, 1967) was also performed in order to separate the glycopeptides from possibly occurring keratan sulfate. The solubility profile technique of *Antonopoulos et al.* (1964) was used for fractionation of chondroitin sulfates according to molecular weight.

Fractions were hydrolysed with 6 N HCl for 8 hours in a boiling water bath, with subsequent removal of the HCl *in vacuo*. The residues were dissolved in 1 ml of water; 0.5 ml thereof was taken for hexosamine determination, the other 0.5 ml was used for radio-assay. Hexosamines were determined by the Elson and Morgan reaction as modified by *Antonopoulos et al.* (1964). Separation of glucosamine and galactosamine was accomplished by chromatography on Dowex-50 columns (*Antonopoulos*, 1966).

For radio-assay 10 ml of scintillator fluid (4 g Omnifluor*, 1000 ml toluene,

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1000 ml 2-methoxyethanol) was added to each 0.5 ml sample. Activities were determined in a Packard liquid scintillation spectrometer. Quenching was determined with external standardization.

RESULTS

The results of the chemical analyses and the radio-assays on fractions from CPC/cellulose microcolumns (*Antonopoulos & Gardell*, 1963) are summarized in Table I. The eluting agents and the order of appearance of different glycosaminoglycans are given in the legend.

The hexosamine content of the costal cartilage in the normal controls (group D) was 2.9 percent of the dry weight. Ninety-three percent of that was galactosamine. About 80 percent of the hexosamines was derived from chondroitin sulfates, presumably mainly 4-sulfates. The cartilage did not contain any appreciable amounts of hyaluronic acid or dermatan sulfate. Neither did fractionation on ECTEOLA-cellulose show any detectable amounts of keratan sulfate.

As seen from the table, both the total amount of hexosamines and the distribution of the hexosamines between the fractions was essentially unaffected by either zinc deficiency (group A) or different degrees of restriction of the food intake (groups B, C). Similar results were obtained by fractionation on ECTEOLA-cellulose (*Antonopoulos et al.*, 1967). Nor did the solubility profile studies indicate any changes in molecular weight distribution of the chondroitinsulfates during zinc deficiency or restricted food intake.

Incorporation of radiosulfate. The radioassay of the glycosaminoglycan fraction of costal cartilage from group D showed that about 80 % of the total ^{35}S-activity was eluted with fractions 3 and 4. Somewhat lower figures for the incorporation into these two combined fractions were noted in groups A—C. The 'specific' activity in the last mentioned groups were 84, 90, and, 84 percent, respectively, of the normal controls (Table I). Fractionation on CPC/cellulose microcolumns according to *Antonopoulos et al.* (1964) did not indicate any differences between the groups A—D in the distribution of the labeling according to molecular weight of the chondroitin sulfates.

Incorporation of ^{14}C-glycosamine. The pattern of distribution of ^{14}C-activity differed markedly from that of the ^{35}S-activity. In normal cartilage about 75 percent of the ^{14}C-activity occurred in the 1 % CPC-fraction. This fraction represents free glucosamine and glycoproteins. Fractions 3 and 4 accounted for only about 18 % of the total ^{14}C-activity. As with radiosulfate the incorporation of ^{14}C-glucosamine into the latter two fractions was reduced

Table I.

Fractionation of the glycosaminoglycans of rat rib cartilage on CPC/cellulose microcolumns according to Antonopoulos and Gardell (1963)

Animal group	Fraction number	% Hexosamine of dry weight	CPM $^{35}SO_4 \times 10^{-3}$ per μ mole hexosamine	CPM $^{14}C \times 10^{-3}$ per μ mole hexosamine
A Experimental rats	1	0.46	94.9	215.3
	2	0.05	—	—
	3	2.28	116.0	6.5
	4	0.07	—	—
	5	0.07	—	—
	6	—	—	—
	Total	2.94	117.5	37.0
B Paired-fed controls	1	0.48	87.1	236.2
	2	0.05	—	—
	3	2.27	124.2	8.2
	4	0.10	—	—
	5	0.02	—	—
	6	—	—	—
	Total	2.92	124.7	42.0
C Paired weight-fed controls	1	0.44	85.1	230.2
	2	0.07	—	—
	3	2.35	116.1	8.5
	4	0.07	—	—
	5	0.02	—	—
	6	—	—	—
	Total	2.95	117.2	40.5
D Normal controls	1	0.44	95.7	225.6
	2	0.07	—	—
	3	2.28	138.1	9.0
	4	0.14	—	—
	5	0.02	—	—
	6	—	—	—
	Total	2.95	133.6	40.4

Given values are means of 6 microcolumns. Fraction 1 eluted with 1 % CPC (glycopeptides, keratan sulfate). Fraction 2: 0.30 M NaCl in 0.05 % CPC (hyaluronic acid). Fraction 3: n-propanol, methanol, glacial acetic acid,1 % CPC (40:20:1. 5:38.5; chondroitin-4-sulfate). Fraction 4: 0.75 M $MgCl_2$ containing 0.05 % CPC and 0.10 M HAc (chondroitin-6-sulfate). Fraction 5: 0.75 M $MgCl_2$ in 0.05 % CPC (dermatan sulfate). Fraction 6: 6 M HCl (keratan sulfate).

in groups A—C. Thus, the specific activity of the combined fractions 3 and 4 were 72, 91, and, 94 percent of that of the normal controls. The solubility profile technique of *Antonopoulos et al.*, (1964) did not show any changes in the distribution of the labeling according to molecular weight of the chondroitin sulfates in groups A—C as compared to group D.

Hexosamines of fractions 3 and 4 were separated on Dowex-50. In all four groups galactosamine constituted more than 98 % of the hexosamines. The specific activity of the galactosamine fraction of groups A, B and C, expressed as percentage of group D, corresponded well to the values given above for the original polysaccharide fractions.

DISCUSSION

A hexosamine value of 2.9 percent of the dry weight, presumably derived mainly from chondroitin-4-sulfate, was found for the costal cartilage of the normal controls. This is in agreement with earlier observations on rib cartilage, in the rat, by *Gross et al.*, (1960) and *Wastesson* and *Lindahl* (unpublished observations).

With both radioactive precursors moderate reductions in specific activity of the chondroitin sulfate were noted in groups A to C, as compared to the normal controls. Hence, it seems reasonable to conclude that zinc deficiency and reduced food intake both caused a lowered *in vitro* synthetic rate of chondroitin sulfate. On the other hand, no significant changes were observed in groups A to C with respect to total hexosamine content or polysaccharide profiles of the costal cartilage. This discrepancy may be explained as follows. The changes in synthetic rate were only moderate even after 18 days. Therefore, the period of appreciably reduced synthesis was presumably short as compared to the 8—16 day biological half-life of chondroitin sulfate in rat costal cartilage (*Boström*, 1952; *Gross et al.*, 1960; *Wastesson & Lindahl*, unpublished observations). Similarly, a reduced formation of chondroitin sulfate in guinea pig costal cartilage, without changes in polysaccharide content, was observed in experimental vitamin C deficiency (*Lohmander*, unpublished observations).

In consideration of the rather moderate reduction of synthetic rate of chondroitin sulfate in the zinc deficient group, as compared to the normal controls, it seems improbable that the growth retardation in this deficiency state is mediated, to any greater extent, by way of depression of the formation of the glycosaminoglycans.

The differences in synthetic rate of chondroitin sulfate between the zinc

deficient and restricted fed controls were small. Hence, inanition may well have contributed significantly to the reduction in mucopolysaccharide synthesis in the deficient group. On the other hand, normal function of cartilage is dependent on several nutritional and hormonal factors. Conceivably, disturbances of different kinds could, therefore, cause similar end results. Thus, it is difficult in deficiency studies of this type to asses to what extent the observed metabolic changes are brought about by the deficiency under investigation, or by the accompanying inanition. As pointed out by *Mills et al.* (1969) and *Chesters* and *Quarterman* (1970) it is also necessary to consider the possible metabolic effects of irregularities in feeding patterns which may occur in both deficient and restricted fed animals.

From the above discussion it is evident that further work is required to elucidate the eventual importance of zinc in the formation of the glycosaminoglycans of the connective tissues. We believe, however, that it is difficult to settle this question conclusively through further *in vivo* deficiency studies because of the difficulties to control and evaluate the inanition factor. Possibly, experiments utilizing simpler systems, such as cell cultures, could be more easily interpreted.

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