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Uridine Diphosphate Xylosyltransferase Activity in Cartilage from Manganese-Deficient Chicks

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The glycosaminoglycan content of cartilage is decreased in manganese deficiency in the chick (perosis). The activity of xylosyltransferase, the first enzyme in the biosynthetic pathway of sulphated glycosaminoglycans, was studied in the epiphysial cartilage of 4-week-old chicks which had been maintained since hatching on a manganese-deficient diet. Enzymic activity was measured by the incorporation of [^{14}C]xylose from UDP-[^{14}C]xylose into trichloroacetic acid precipitates. Optimal conditions for the xylosyltransferase assay were established and shown to be the same for both control and manganese-deficient cartilage. Assay of the enzyme by using an exogenous xylose acceptor showed no difference in xylosyltransferase activity between control and manganese-deficient tissue. Further, the extent of xylose incorporation was greater in manganese-deficient than in control cartilage preparations, suggesting an increase in xylose-acceptor sites on the endogenous acceptor protein in the deficient cartilage. ^{35}S turnover in the manganese-deficient cartilage was also increased. The data suggest that the decreased glycosaminoglycan content in manganese-deficient cartilage is due to decreased xylosylation of the acceptor protein plus increased degradation of glycosaminoglycan.

Xylosyltransferase (UDP-xylose–protein xylosyltransferase, EC 2.4.2.26) is the first enzyme unique to the biosynthesis of glycosaminoglycans (Stoolmiller *et al.*, 1972; Lindahl & Roden, 1965, 1966). The enzyme from various sources is activated *in vitro* by Mn^{2+} (Grebner *et al.*, 1966*b*; Robinson *et al.*, 1966). Other enzymes of glycosaminoglycan synthesis are also activated by Mn^{2+} *in vitro* (Leach *et al.*, 1969; Robinson *et al.*, 1966). The relationship between the Mn^{2+} requirement for glycosaminoglycan synthesis and the perosis produced by nutritional manganese deficiency has been the subject of several investigations (Leach & Muenster, 1962; Tsai & Everson, 1967). These investigations have demonstrated a decrease in glycosaminoglycan concentration and biosynthesis (as measured by [^{35}S]sulphate incorporation) in cartilage from manganese-deficient chicks. The various enzyme activities associated with glycosaminoglycan biosynthesis are either unchanged or increased in crude cartilage homogenates (Leach *et al.*, 1969) as a consequence of manganese deficiency.

These are bi-substrate reactions, with the endogenous acceptor (the second substrate) being present in the 'enzyme' preparation. Activity, as measured in crude extracts, is therefore a function of both enzyme and endogenous-substrate concentrations. Valid comparison of 'activities' in manganese-replete and -deficient cartilage must assess the contribution of endogenous substrate and show that the optimal concentrations of cofactors are identical for both preparations.

In the study presented here, it is demonstrated that an apparent increase in xylosyltransferase activity in manganese-deficient cartilage is a consequence of an increased concentration of acceptor protein, that the glycosaminoglycan turnover is faster in deficient cartilage and that the optimal concentrations of cofactors and exogenous substrate are identical for the enzymes from replete and deficient cartilage.

Experimental

Materials

UDP-[U-^{14}C]xylose (172.5 Ci/mol) and $Na_2{}^{35}SO_4$ (carrier-free) were purchased from New England Nuclear Corp., Boston, MA, U.S.A.; dithiothreitol was from Calbiochem, La Jolla, CA, U.S.A.; all other chemicals were reagent grade and were purchased from Fisher Scientific Co., St. Louis, MO, U.S.A. Crystalline bovine serum albumin was obtained from Sigma Chemical Corp., St. Louis, MO, U.S.A. The basal chick diet described by Leach & Muenster (1962) was prepared by Nutritional Biochemicals Corp., Cleveland, OH, U.S.A. The basal chick diet contained less than 4 p.p.m. of manganese by atomic

absorption analysis. The control diet contained 100p.p.m. of manganese, added as reagent-grade $MnSO_4$ to the basal diet.

The exogenous acceptor, prepared by Smith degradation of bovine chondroitin sulphate–protein complex as described by Baker *et al.* (1972), was kindly given to us by Dr. A. L. Dorfman (University of Chicago).

Hubbard chicks (1 day old) were given by Keith Smith Hatcheries, Hot Springs, AR, U.S.A. The chicks were placed in electrically heated brooders with raised stainless-steel floors and fed with either the control diet (basal diet plus manganese) and tap water or the basal (manganese-deficient) diet and distilled water *ad libitum*. The chicks were maintained on their respective diets until they developed obvious evidence of perosis between 3 and 4 weeks of age, characterized by short and thick long bones, bowing of the tibiotarsus and unsteady gait. The chicks were then killed and epiphysial cartilage was removed for the isolation of glycosaminoglycan and the preparation of enzyme.

Methods

Preparation of the enzyme. For the preparation of xylosyltransferase, epiphysial cartilage was shaved from the ends of femurs and tibias, placed in cold glass homogenizers and homogenized by using Teflon pestles in 0.2M-sucrose/1.0mM-dithiothreitol/50mM-Tris/acetate buffer, pH7.0 at 0°C. The homogenate was centrifuged at 10000*g* for 10min and the supernatant was used without further purification, except in studies using exogenous acceptor. Protein concentration was determined by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin as the standard.

To prepare the enzyme for the studies in which the Smith-degraded proteoglycan was used as an exogenous acceptor, the 10000*g* supernatant portion was made 1M with respect to KCl, subjected to six freeze–thaw cycles and then centrifuged at 105000*g* for 1h as described by Stoolmiller *et al.* (1972). The supernatant solution contained the enzyme, and the sediment contained the endogenous acceptor.

Enzyme assay. Xylosyltransferase was assayed by using a modification of the method of Robinson *et al.* (1966) (Grebner *et al.*, 1966*a*), with UDP-[^{14}C]-xylose as substrate (Elders *et al.*, 1973). After incubation with UDP-[^{14}C]xylose, an equal volume of cold 10% (v/v) trichloroacetic acid was added to stop the reaction. After standing for 30min in ice, the precipitates were collected by centrifugation (at 10000*g* × 10min) and washed three times with 5% trichloroacetic acid and once each with ethanol, ethanol/diethyl ether (1:1, v/v) and finally ether. The residue was dried, dissolved in 0.1ml of 1M-KOH and diluted to 0.5ml with water. Radioactivity was measured in the scintillation 'cocktail' of Bruno & Christian (1962) with a Nuclear–Chicago mark I liquid-scintillation spectrometer; 0.25ml samples were mixed with 10ml of the scintillation cocktail. Glycosaminoglycans were isolated from chick cartilage by a modification (Elders *et al.*, 1973) of the method of Antonopoulos *et al.* (1964), and uronic acid was determined by the method of Dische (1947).

For the estimation of the half-life of glycosaminoglycans in cartilage, 25μCi of $Na_2{}^{35}SO_4$ (carrier-free)/100g body wt. was injected intraperitoneally into 25 control and 25 manganese-deficient 4-week-old chicks. Two chicks were killed at various times during the following 96h and the specific radioactivity of the ^{35}S relative to uronic acid in glycosaminoglycans isolated from epiphysial cartilage was determined as described by Elders *et al.* (1973).

Results

Optimal assay conditions for xylosyltransferase

Optimal conditions for assaying this enzyme in epiphysial cartilage from growing chicks were determined. The time-course of transfer of [^{14}C]xylose into trichloroacetic acid-precipitable material was linear for 30min at 37°C in both control and manganese-deficient cartilage. The rate of incorporation of [^{14}C]xylose increased linearly with increasing amounts of enzyme.

The effect of Mn^{2+} on the enzymic activity in control and manganese-deficient cartilage is shown in Fig. 1. The concentration of Mn^{2+} which provided the optimal cofactor requirement for the assay of xylosyltransferase in both control and deficient chick cartilage was 6mM, and this concentration was used in subsequent assays.

The effect of varying the substrate concentration on xylosyltransferase activity in control and manganese-deficient cartilage is shown in Fig. 2. Enzyme preparations from control and deficient cartilage were incubated with 6mM-$MnCl_2$, 50μmol of Tris/acetate (pH6.5), and UDP-[^{14}C]xylose from 0.46 to 6.4μM in a final volume of 0.25ml. The Lineweaver–Burk plot shows that half-maximal rate of [^{14}C]-xylose incorporation occurred at a UDP-xylose concentration of 1.6μM in both preparations.

Specificity of bivalent-cation activation

To establish the best metal activator for xylosyltransferase, the reaction rate of the enzyme from control cartilage was compared in the presence of Mn^{2+}, Mg^{2+} and Ca^{2+} (Table 1). Mn^{2+} is the best activator under the conditions chosen, but Ca^{2+} and Mg^{2+} partially activated the enzyme.

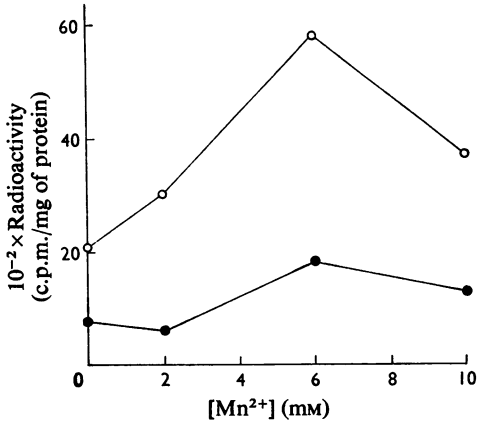

Fig. 1. *Effect of Mn^{2+} concentration on xylosyltransferase activity*

The reaction mixture contained 0.1 ml of enzyme from control (●) and manganese-deficient (○) cartilage, 50 μmol of Tris/acetate, pH 6.5, 4.6 μM-UDP-[^{14}C]xylose and the $MnCl_2$ concentrations shown on the abscissa, in 0.25 ml final volume. The samples were incubated at 37°C for 30 min.

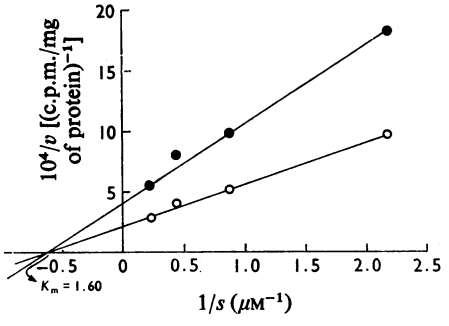

Fig. 2. *Lineweaver–Burk plot for xylosyltransferase from control (●) and manganese-deficient (○) cartilage*

The system contained 0.1 ml of enzyme, 50 μmol of Tris/acetate, pH 6.5, 6 mM-$MnCl_2$ and UDP-[^{14}C]xylose as indicated, in a total volume of 0.25 ml. The abscissa intercept is −0.625 ($-1/K_m$) for both plots, and the K_m for both enzyme preparations is 1.60 μM.

Effect of manganese deficiency on xylosyltransferase activity

With the optimal conditions for UDP-[^{14}C]xylose incorporation in the control and manganese-deficient cartilage preparations established, an effect of manganese deficiency on the apparent activity of the enzyme could be evaluated. Xylosyltransferase preparations were made from five control and five deficient chicks. Cartilage was homogenized in 0.2 M-sucrose/Tris/acetate buffer, pH 7.0, and centrifuged at 10000*g* for 30 min. The supernatant fluid (0.1 ml) was incubated at 37°C for 30 min in a mixture containing 4.6 μM-UDP-[^{14}C]xylose, 50 μmol of Tris/acetate, pH 6.5, in the presence and absence of 6 mM-$MnCl_2$. The results are shown in Fig. 3. The xylosyltransferase activity was increased twofold in the manganese-deficient cartilage. There is no overlap in the enzyme activities found in control and deficient chick cartilage when compared in either the presence or the absence of manganese in the assay system.

Table 1. *Bivalent cations and enzyme activity*

The reaction mixture contained 0.1 ml of enzyme, 50 μmol of Tris/acetate, pH 6.5, 4.6 μM-UDP-[^{14}C]xylose, and bivalent cations in the concentrations shown in 0.25 ml total volume.

Cation (mM)	Radioactivity (c.p.m./mg of protein)
None	1100
Mn^{2+} (6.0)	2540
Mg^{2+} (2.0)	1660
Mg^{2+} (4.0)	1680
Mg^{2+} (6.0)	1960
Ca^{2+} (2.0)	2350
Ca^{2+} (4.0)	2010
Ca^{2+} (6.0)	2270

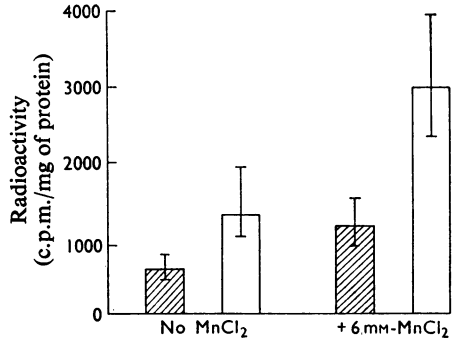

Fig. 3. *Effect of manganese deficiency on xylosyltransferase activity*

The assay mixtures consisted of 0.1 ml of enzyme, 50 μmol of Tris/acetate buffer, pH 6.5, 4.6 μM-UDP-[^{14}C]xylose and 6 mM-$MnCl_2$. The data represent individual assays on five separate chicks from each experimental group (■, control; □, manganese-deficient. The bars enclose the range of the data.

Table 2. *Xylose incorporation by manganese-deficient and control cartilage measured with and without exogenous acceptor*

Cartilage from manganese-deficient and control chicks was collected and assayed for xylosyltransferase activity. The 10000*g* supernatant contains both xylosyltransferase and the endogenous acceptor protein. Xylosyltransferase was solubilized by KCl and freeze–thawing as described in the text. The 105000*g* supernatant contains minimal acceptor protein. Both 10000*g* supernatant and 105000*g* supernatant were assayed for xylosyltransferase with and without the Smith-degraded proteoglycan. The reaction mixture contained: enzyme preparation, 150–300 μg of protein; Tris/acetate, 50 μmol; UDP-[^{14}C]-xylose, 1.2 nmol; 1.5 μmol of $MnCl_2$; final volume 0.25 ml, pH 6.5. When Smith-degraded proteoglycan acceptor was added (250 μg/tube), 5 nmol of unlabelled UDP-xylose was added to the reaction mixture.

Preparation	Enzyme source	Xylose incorporated (pmol/h per mg of protein)	
		Without acceptor	With acceptor
10000*g* supernatant	Mn-fed control	40.5	338.7
	Mn-deficient	59.5	398.5
105000*g* supernatant	Mn-fed control	6.0	210.2
	Mn-deficient	9.6	202.9

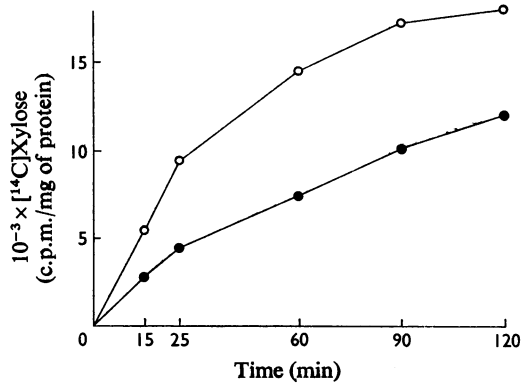

Fig. 4. *Extent of incorporation of* [^{14}C]*xylose by* 10000*g*-*supernatant preparations from control* (●) *and manganese-deficient* (○) *cartilage*

Excess of UDP-[^{14}C]xylose was added each 30 min during the incubation. For further details see the text.

Enzyme activity with exogenous acceptor

The experiments described above measured [^{14}C]-xylose incorporation into endogenous acceptor present in the cartilage preparation. Since this is a bi-substrate reaction (UDP-xylose plus acceptor), the increased incorporation by manganese-deficient cartilage could be due to increased acceptor rather than increased enzyme. To investigate this aspect of the problem, endogenous acceptor was removed from the enzyme preparation with KCl as described in the Experimental section. The enzymic activity was then measured by using Smith-degraded proteoglycan as the xylose acceptor.

The results of this experiment are shown in Table 2. With an enzyme preparation from control and manganese-deficient cartilage which contained endogenous acceptor, the addition of exogenous acceptor increased the incorporation 8.4-fold and 6.7-fold respectively. When the 105000*g* KCl-treated supernatant fraction was used as the enzyme source, very little endogenous acceptor was present and the rates of xylose incorporation into the control and deficient preparations were again of the same order with or without the Smith-degraded proteoglycan acceptor. These data suggest that comparable amounts of enzyme are present in the control and deficient cartilage and the apparent increased activity of xylosyltransferase in the crude 10000*g* supernatant fraction is due to increased endogenous xylose acceptor in deficient cartilage.

Xylose-acceptor sites in control and manganese-deficient cartilage

To estimate the relative number of available acceptor sites for xylose, the assay was performed as usual except that the time was extended to 2 h and the volume of the reaction mixture increased to 2 ml. The 10000*g* supernatant from control and deficient cartilage was used as the enzyme fraction. Excess of UDP-[^{14}C]xylose (1 nmol/0.25 ml of remaining reaction mixture) was added at 30 min intervals. Samples (0.25 ml) were removed from incubation tubes of both control and deficient cartilage at designated intervals and the [^{14}C]xylose incorporated into protein was determined. Controls for each time-point were included to ensure full activity of the enzyme. This was accomplished by incubating the enzyme without UDP-xylose for the same time-interval, then adding UDP-xylose 15 min before stopping the reaction, and measuring the initial rates. These varied by ±10% and there was no decline in the enzyme activity during the 2 h of incubation. The total incorporation of [^{14}C]-xylose was 18100 c.p.m./mg of protein in the deficient

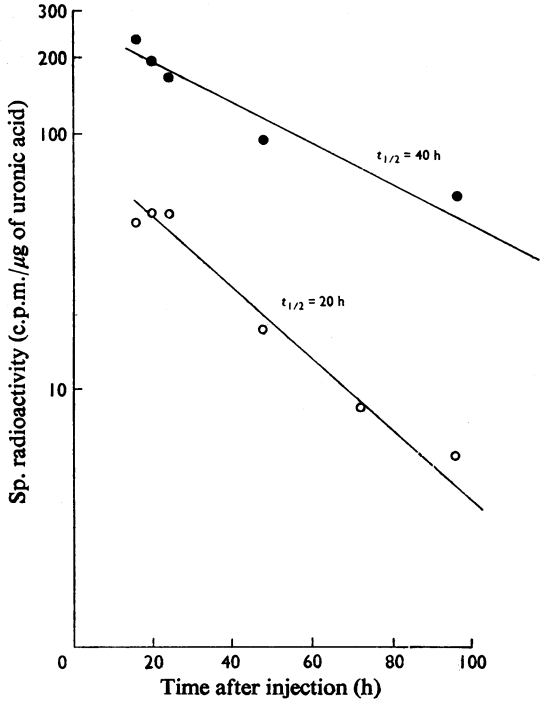

Fig. 5. *Glycosaminoglycan turnover in control* (●) *and manganese-deficient* (○) *cartilage*

The specific radioactivity of ^{35}S relative to the uronic acid content of isolated glycosaminoglycan is plotted semilogarithmically against time. For details see the text.

cartilage and 12000c.p.m./mg of protein in the control (Fig. 4), demonstrating an apparent increased number of xylosylation sites in the endogenous acceptor of deficient cartilage.

Glycosaminoglycan turnover in control and manganese-deficient cartilage

Normal or increased activity of xylosyltransferase with a decrease in glycosaminoglycan content of the manganese-deficient cartilage suggests that degradation occurs at a more rapid rate than synthesis. The uronic acid content (μg/mg dry wt. of cartilage) in control chicks is 60.9 (range, 54.4–66.3) after 4 weeks on the purified diet with manganese added, compared with 29.4 (15.5–43.4) in cartilage from chicks on the manganese-deficient purified diet.

Uptake of ^{35}S into cartilage reached a peak at 16h in both control and manganese-deficient cartilage, but maximum uptake was less in manganese deficiency. Data presented in Fig. 5 show the decline in ^{35}S radioactivity per glucuronic acid residue as a function of time in the cartilage of both control and deficient 4-week-old chicks. From this semilogarithmic plot, the half-life of glycosaminoglycans is estimated as 40h in the control group and 20h in the manganese-deficient group. It is firmly established that glycosaminoglycan is metabolized as a unit, i.e. sulphate, hexosamine and uronic acid have identical half-lives, and that essentially all sulphate in cartilage (and most other tissues) is present in glycosaminoglycan (Morrison, 1970). These data are compatible with the interpretation that an increased rate of turnover (degradation) of glycosaminoglycan occurs in cartilage in the manganese-deficient state.

Discussion

To produce manganese deficiency, chicks must be maintained on a low-manganese diet (less than 10p.p.m. of Mn^{2+}) from the time at which they are hatched (Leach & Muenster, 1962). The gross lesion (short and thick long bones and slipped tendons) appears after about 4 weeks. All manganese-deficient chicks used in these experiments had slipped tendons, definite shortening of the long bones, and decreased contents of uronic acid in epiphysial cartilage. On gross inspection of the epiphysial region of the femurs and tibias of these chicks, the layer of epiphysial cartilage was greatly decreased in width. These observations have been described previously by Leach (1969).

When xylosyltransferase was separated from the endogenous acceptor and exogenous acceptors was used to measure the activity, no appreciable difference was measurable in the enzyme activities in control and manganese-deficient cartilage (Table 2). Additional evidence that the difference in enzyme activity observed in the crude 10000*g* supernatant preparations from deficient cartilage is due to increased acceptor sites for xylose was provided by the experiment presented in Fig. 4. When the endogenous acceptor was xylosylated to a maximal degree by using crude preparations from control and deficient cartilage, the deficient preparation contained 1.5 times the number of sites present in the control cartilage. Leach *et al.* (1969) concluded that the incorporation of galactose into the Gal-Gal-Xyl–protein linkage region was diminished in manganese deficiency and that the xylosyl-protein accumulated in manganese-deficient cartilage. They found no difference in xylose incorporation between manganese-deficient and control cartilage.

Our studies differ from theirs in several respects. We used a much higher concentration of UDP-xylose and took precautions to assure that the enzyme remained active and UDP-xylose was not destroyed during the long incubation period. Other minor differences are in the Mn^{2+} and sucrose concentrations.

Under conditions *in vitro*, the galactosyltransferase is more sensitive to Mn^{2+}. However, our demonstration of increased xylose-acceptor sites in the

manganese-deficient cartilage suggests that the xylosyltransferase reaction can also become rate limiting under conditions *in vivo*. Even though other bivalent cations activate xylosyltransferase *in vitro*, they may not be significant *in vivo*. Other factors may also contribute to this step becoming rate limiting. The concentration of UDP-xylose *in vivo* would be expected to be dependent on the ATP production by the cartilage cell. Synthesis of xylose from glucose requires ATP and NAD^+. Any decrease in the cell's ability to maintain adequate steady-state concentrations of these compounds could decrease all UDP-linked substrates for glycosaminoglycan biosynthesis. Ultrastructural abnormalities of mitochondria have been demonstrated by Hurley *et al.* (1970) in the heart, liver, kidney and pancreas of manganese-deficient mice. Oxygen consumption by isolated liver mitochondria from deficient animals is decreased, but the P:O ratio is normal (Bell & Hurley, 1973). Indeed, this is a reasonable explanation for the decreased glycosaminoglycan synthesis in lathyritic chick embryos (Elders *et al.*, 1973).

Another effect of manganese deficiency is the increased degradation of glycosaminoglycan as evidenced by its decreased biological half-life in cartilage. Since glycosaminoglycan turnover is decreased in lathyritic chicks, increased glycosaminoglycan degradation in manganese deficiency need not be a consequence of impaired energy metabolism. These changes in manganese-deficient cartilage thus appear to be a manifestation of both decreased synthesis and increased degradation and provide further biochemical explanations for the observed gross and microscopic changes seen in manganese deficiency.

The biochemical manifestations of manganese deficiency in cartilage can thus be summarized as an increase in acceptor protein, a decrease in glycosaminoglycan content, an increase in the rate of degradation of glycosaminoglycan and no change in xylosyltransferase activity.

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