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Correlation of Vertebral Malformations With the Synthesis and Content of Mucopolysaccharides During Chondrogenesis

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Summary. Treating mice of strain C57BL/6Ffm on day 9 of gestation with 10 mg/kg of 5-fluoro-2′-deoxycytidine (FCdR) resulted in malformations of the thoracic vertebral column (ThVC) in 98% of near-term fetuses (Degenhardt et al., 1968). The spectrum of malformations was broad: fusion, dysplasia, cleft, aplasia and hypoplasia were all produced. Fusions of two or more segments represented more than half of all malformations (Bosse, 1978).

The alterations in embryonic precartilage and cartilage after FCdR-treatment were followed from day 11 to day 15 in a biochemical and histological study. Biochemically, the ^{35}S-uptake into embryonic mucopolysaccharides (MPS) and the content of total MPS and seven fractions of MPS in embryos or isolated ThVCs were analyzed. The histological variables studied were the types and incidence of malformations of the ThVC, ^{35}S-autoradiography of the ThVC, and the amount of alcian blue-stained cartilaginous matrix. The results showed that on day 11 the synthesis of embryonic MPS was not affected, on day 12 the synthesis of MPS was greatly reduced, on day 13 the synthesis of MPS was slightly reduced while the MPS-content was not affected. On day 13 aplasias were seen in the same percentage as at term, but no fusions were detected. By day 14 the MPS-content was greatly reduced; hyaluronate, condroitin 4-sulfate, and chondroitin 6-sulfate being principally involved; the first fusions were seen. On day 15 the MPS-content was slightly reduced (chondroitin 6-sulfate and heparan sulfate were involved), fusions were complete. The results are discussed in terms of disturbance of structure and function of the notochord and intervertebral discs with the production of fusions, the main type of vertebral malformation in these experiments.

Key words: Chondrogenesis – 5-fluoro-2′-deoxycytidine – Mucopolysaccharides – Vertebral malformations.

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Introduction

After treating B/6-mice with 10 mg/kg of 5-fluoro-2′-deoxycytidine on day 9 of gestation, Degenhardt and his coworkers (1968) found 98% of near-term fetuses had thoracic vertebral column malformations. The aim of this morphological and biochemical study is to examine the morphogenesis of malformations of the thoracic vertebral column during chondrogenesis, produced by the same compound. We have paid particular attention to changes in mucopolysaccharides. In a previous study Švejcar (1968) found, in whole mouse fetuses, a rise of the amount of mucopolysaccharides from day 13 to a peak at day 15. This correlates with the morphological appearances and the peak of chondrification in the thoracic vertebral column. Therefore, in the present study the morphological analysis of vertebral malformations at days 13 to 15 is compared with the content of mucopolysaccharides on these days, and the synthesis of mucopolysaccharides at days 11 to 13.

Materials and Methods

Mice of strain C57BL/6Ffm were bred as described by Degenhardt et al. (1968). The end of a 3 h-mating period was taken as onset of pregnancy, if a vaginal plug could be detected. Pregnant females were injected intra-peritoneally (ip) at day 9 (i.e., 216 h of pregnancy) with 5-fluoro-2′-deoxycytidine (FCdR) in a dose of 10 mg/kg maternal body weight, dissolved in 0.9% saline. Control animals received 10 mg/kg of physiological saline at day 9.

The uptake of ^{35}S into sulfated mucopolysaccharides (MPS) of whole fetuses was studied biochemically on days 11, 12, and 13. The ^{35}S-uptake into the cartilage of the thoracic vertebral column (ThVC) was measured in histological sections by autoradiography on day 13. In these experiments ^{35}S-sulfate (specific activity 5 Ci/mg S) was injected ip 24 h before in a dose of 5 μCi/g maternal body weight.

The MPS-content of whole fetuses was determined biochemically on days 13, 14, and 15, while MPS-content in isolated ThVCs was measured on day 15. The amount of alcian blue-stained cartilaginous matrix of the ThVC was determined in histological sections at days 14 and 15.

Malformations of the ThVC were analyzed in histological sections on days 13, 14 and 15. Data on near-term fetuses are from material previously published by Fränz and Degenhardt (1969), recently analyzed in detail in our laboratory by Bosse (1978).

Biochemical Procedures. Seven types of acid mucopolysaccharides were obtained by methods described in detail previously (Švejcar and Robertson, 1967): MPS of low molecular weight, hyaluronate, heparan sulfate + chondroitin, chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, and heparin + keratan sulfate. For ^{35}S-sulfate incorporation experiments the acid MPS were isolated and partly purified, as for biochemical analysis. The MPS powder was dissolved in 1 ml H_2O and 10 ml of dioxan-scintillator were added. The counts were measured with a Liquid Scintillation Spectrometer 21 days after removal of the fetuses.

Histological Procedures. After fixation (Bouin-Allen), dehydration and embedding the fetuses in paraplast, frontal sections of 5 μm were made. Sections of day 13 fetuses were stained with hematoxylin-eosin or lead (IV)-acetate-Schiff. In the autoradiographic experiment, with ^{35}S-sulfate given at day 12, slides (staining: hematoxylin-eosin) of day 13 fetuses were covered with Ilford G5-emulsion and exposed for ten days. Sections of day 14 and 15 fetuses were stained with alcian blue.

Quantitative data on histological sections were obtained using the Micro-Videomat (C. Zeiss), using methods described by Lang (1969) and Pera and Detzer (1977). Standard optical equipment was Photomicroscope II (C. Zeiss) with Planapochromat 25/0.65 and Optovar 1.6. Two types of determinations were done with this image analyzer.

(1) amount of ^{35}S-incorporation:
$$\frac{\text{(area of silver grains)} \times 100}{\text{(area of vertebral body)}}$$

(3) amount of cartilaginous matrix:
$$\frac{\text{(area of cartilaginous matrix)} \times 100}{\text{(area of vertebral body)}}$$

The measurements were carried out in the thoracic vertebral column. The amount of ^{35}S-incorporation (day 13) serves as an indicator of MPS-synthesis; the amount of alcian blue-stained cartilaginous matrix (days 14 and 15) gives some data on the MPS-content (Kvist and Finnegan, 1970a). For each fetus 40–60 measurements were found to be statistically sufficient, i.e., cumulative means then showed variations less than 3%.

For statistical analysis of Videomat-data the U-test was used, in the other experiments the t-test was applied.

Results

Day 11

The vertebral column is at the sclerotome stage. Švejcar (1968) found a very low content of mucopolysaccharides at this stage in whole fetuses. In this investigation it could be shown that the synthesis of sulfated mucoplysaccharides – measured as incorporation of ^{35}S into the isolated MPS of whole fetuses – is low (Table 1). The synthesis of sulfated MPS in fetuses of FCdR-treated litters was comparable with that of the control fetuses ($P > 0.1$).

Day 12

The fetal vertebral column shows precartilage, i.e., the early stages of the cartilaginous matrix can be identified microscopically. This morphological onset of chondrogenesis is correlated with alterations in MPS-synthesis. In comparison with the preceeding day the incorporation of ^{35}S into isolated MPS of whole fetuses rose dramatically. The mean showed a six-fold increase ($P > 0.001$) and in addition, the lower border of variability was clearly above the highest value for the day before (Table 1). Fetuses of FCdR-treated pregnancies also showed

Table 1. Uptake of ^{35}S into isolated mucopolysaccharides of whole fetuses. CPM/mg dry weight

Days of analysis	Experiment	n	$\bar{x}$	Variability
11	Control	26	217	49– 479
	FCdR	32	246	42– 580
12	Control	33	1,392*	772–2,358
	FCdR	24	1,131*	437–1,440
13	Control	24	1,206	99–2,015
	FCdR	16	1,258	102–1,785

* $P < 0.01$

Fig. 1. The image-analyzer Micro-Videomat (C. Zeiss) used for histological measurements

an increase of synthesis of sulfated MPS but the increase was lower than in the controls and the lower border of variability remained within the values of the preceeding day. Thus synthesis of sulfated MPS in fetuses of FCdR-treated litters was clearly inhibited ($P < 0.01$). The considerable increase of MPS-synthesis in control fetuses of day 12 does not – in accordance with the morphological features (precartilage) – result in a measurable increase of MPS-content on this day (Švejcar, 1968).

Day 13

The fetal vertebral column is at the stage of early cartilage. The cartilaginous matrix stains deeply with lead (IV)-acetate-Schiff, but only faintly with alcian blue. Enzymatic digestion with testicular hyaluronidase before staining with lead (IV)-acetate-Schiff results in a faint colour. This is in accordance with the biochemical findings of Švejcar (1968): the unsulfated hyaluronate at day 13 is the predominant mucopolysaccharide despite the high rate of synthesis of sulfated mucopolysaccharides on day 12.

The mean rate of synthesis of sulfated mucopolysaccharides in whole fetuses of the controls is comparable with that on the preceeding day ($P > 0.1$). However in one-third of those fetuses the rate of MPS-synthesis is below the lower border of variability of day 12. This results in a great variability of synthesis rates (Table 1). In contrast with controls, fetuses of FCdR-treated litters show a clear increase of synthesis of sulfated MPS from day 12 to day 13 on average. Nevertheless, the mean and upper border of variability of synthesis in the FCdR-series remain below the values for the controls of the day before. Within the FCdR-series some fetuses show lower rates as on the day before, apart from a general increase of synthesis.

^{35}S-incorporation into vertebral bodies of the thoracic vertebral column was measured autoradiographically. The quantitative analysis, using the Micro-Videomat, showed a 26% reduction of ^{35}S-incorporation in the FCdR-series

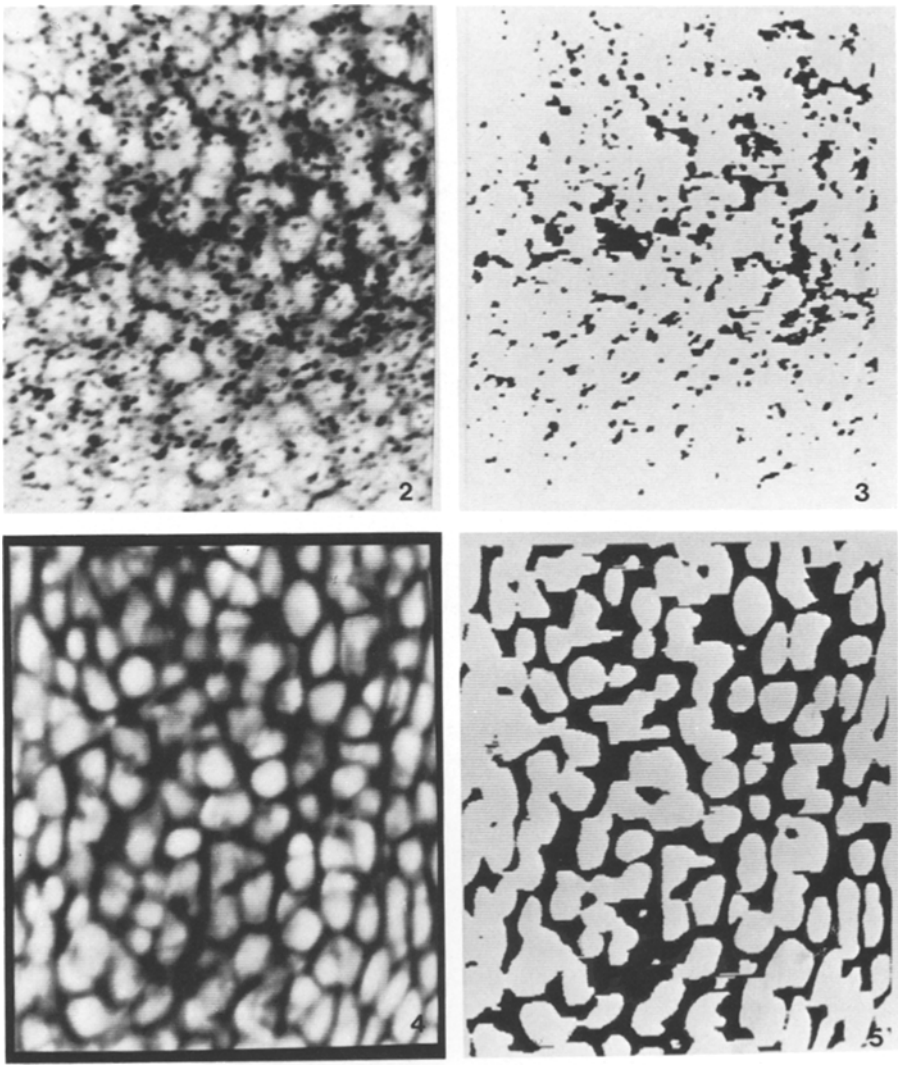

Fig. 2. Autoradiographic picture on TV screen showing ^{35}S-incorporation in vertebral bodies of the ThVC. Control fetus, day 13

Fig. 3. Discrimination with the Micro-Videomat for area of silver grains in Fig. 2

Fig. 4. Vertebral bodies of a day 15-fetus (Control) on TV screen

Fig. 5. Discrimination with the Micro-Videomat for area of cartilagenous matrix in Fig. 4

(Controls: $X = 6.2 \pm 2.0$ S.D., $n = 21$; FCdR-series: $X = 4.6 \pm 2.0$ S.D., $n = 13$; $P < 0.02$).

The distinct reduction of MPS-synthesis in the FCdR-series in whole fetuses on the preceeding day, seen here only in the thoracic vertebral column, does not result in alteration of the MPS-content of whole fetuses. Fetuses of the

Table 2. Content of mucopolysaccharides (MPS) in whole fetuses (13–15[1]) and isolated thoracic vertebral columns (15[2]). µg uronate/mg dry weight

Day of analysis	Experiment	Fetuses *n*	Total MPS	Fractions of MPS						
				1	2	3	4	5	6	7
13	Control	22	2.17	0.37	0.76	0.25	0.30	0.25	0.14	0.10
	FCdR	23	2.14	0.37	0.75	0.26	0.26	0.25	0.16	0.09
14	Control	23	3.58**	0.49	0.96*	0.52	0.85*	0.44*	0.20	0.22
	FCdR	23	3.13**	0.40	0.84*	0.48	0.71*	0.37*	0.20	0.13
15[1]	Control	25	4.77	0.66	1.13	0.78	1.29	0.54	0.23	0.14
	FCdR	25	4.91	0.68	1.17	0.85	1.33	0.55	0.20	0.13
15[2]	Control	17	7.97***	1.0	1.45	1.08****	2.48	0.97*****	0.46	0.53
	FCdR	13	6.78***	0.76	1.24	0.86****	2.27	0.71*****	0.44	0.50

MPS-fractions: 1 MPS of low molecular weight, 2 hyaluronate, 3 heparan sulfate, 4 chondroitin 4-sulfate, 5 chondroitin 6-sulfate, 6 dermatan sulfate, 7 heparin + keratan sulfate

Levels of significance: * $P<0.05$, ** $P<0.02$, *** $P<0.01$, **** $P<0.002$, ***** $P<0.001$

control series and of FCdR-treated pregnancies showed exactly the same amount of total MPS (Table 2). Shifting within the types of MPS was not observed.

Day 13 was the first stage at which malformations were sought. None of the 31 control fetuses showed malformations of the thoracic vertebral column (ThVC). In fetuses of FCdR-treated litters 48% (14/29) were malformed. Under the same experimental conditions (10 mg/kg FCdR at day 9) 98% of the fetuses showed malformations of the ThVC at term in the study of Degenhardt et al. (1968). In near-term fetuses fusions of two or more segments were predominant (Bosse, 1978). The low rate of malformation at day 13 when compared with that at term ($P<0.001$) is due to the absence of fusion at this early stage of chondrogenesis and signs of incipient fusion (i.e., disturbances of intermediate tissues) were also absent. The only malformations seen at day 13 were partial and total aplasias. Their incidence was the same as in near-term fetuses, where Bosse (1978) found 45% fetuses with aplasia.

Day 14

Condrification of the thoracic vertebral column has proceeded. The cartilaginous matrix is deeply stained with both lead (IV)-acetate-Schiff and alcian blue.

The content of mucopolysaccharides in whole fetuses was higher than day 13. This increase of total MPS was mainly due to an increase of the sulfated MPS chondroitin 4-sulfate and chondroitin 6-sulfate (Table 2). The increase in total MPS from day 13 to day 14 was higher in controls (64%) than in the FCdR-series (41%). The difference was statistically significant ($P<0.02$). The lower content of total MPS in fetuses of FCdR treated pregnancies is due mainly to a considerable reduction in hyaluronate ($P<0.001$). In the FCdR-series the content of hyaluronate at day 14 was the same as on the preceeding

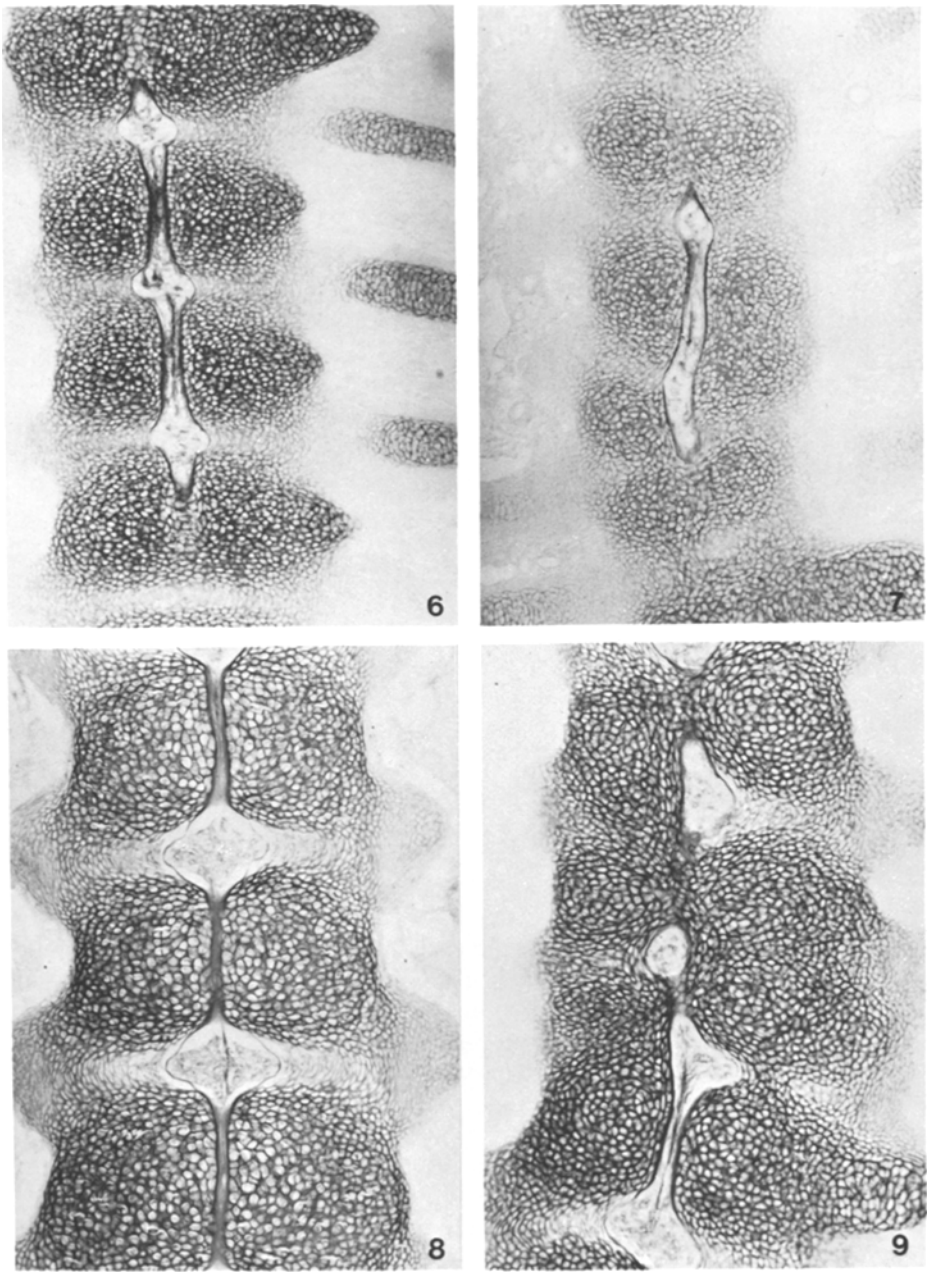

Fig. 6. Thoracic vertebral column at day 14. Control, alcian blue, × 116

Fig. 7. Thoracic vertebral column at day 14. FCdR-series, alcian blue, × 116. Bending of notochord. Beginning fusions in vertebral bodies

Fig. 8. Thoracic vertebral column at day 15. Control, alcian blue, × 116

Fig. 9. Thoracic vertebral column at day 15. FCdR-series, alcian blue, × 116. Fusion of vertebral bodies almost completed

Table 3. Amount of cartilaginous matrix of ThVC, measured with the Micro-Videomat

Day of analysis	Control		FCdR		Level of significance $P<$
	n	% ± S.D.	n	% ± S.D.	
14	17	28.2 ± 1.1	20	25.4 ± 0.9	0.001
15	14	38.3 ± 2.7	12	34.2 ± 4.0	0.02

day, while an increase of 25% was found in the controls. In addition, the content of chondroitin 4-sulfate and chondroitin 6-sulfate was significantly ($P<0.05$) lower in the FCdR-series. The latter is in accord with the reduction of synthesis of sulfated MPS found in the FCdR-series on the 13th day.

The amount of cartilaginous matrix in vertebral bodies of the alcian blue-stained ThVC was measured with the Micro-Videomat. A 10% reduction was found in the FCdR-series (Table 3). This difference in chondrification was highly significant ($P<0.001$).

In the control-series at day 14, none of the 20 fetuses was malformed within the ThVC. In the FCdR-series the malformation-rate was 70% (14/20 fetuses). This clear increase is due to fusions, which are first seen at this stage and are found in 55% of the fetuses. The morphological appearence of the fusions is highly variable at day 14, ranging from inception to fully completed lesions. The rate of aplasia at day 14 is unchanged. For the first time, pathological alterations of the notochord are found. These are malformations (bending) and/or retardation of development (persistently large diameter, cells not yet restricted to nuclei pulposi).

Day 15

Chondrification of the vertebral column is at its height. The cartilaginous matrix shows deep staining with alcian blue, while staining with lead (IV)-acetate-Schiff is very faint. This is particularly true in the thoracic and lumbar region. Over the whole vertebral column a cranio-caudal shift of development is observed. In the first cervical segments initial processes of calcification can be seen; the caudal vertebrae can be stained equally well with alcian blue and lead (IV)-acetate-Schiff. Thus, positive staining with lead (IV)-acetate-Schiff and no reaction to alcian blue is found in early cartilage, and mature cartilage is stained only by alcian blue.

The content of mucopolysaccharides in whole fetuses rose further (Table 2) and reached its peak. Parallel to the beginning of calcification on the next day, the MPS-content will decrease in whole fetuses (Švejcar, 1968). The new data at day 15 show a more pronounced increase of MPS-content in fetuses of the FCdR-series than in the controls. In this way, the differences in total MPS and in single fractions observed on the day before, disappeared. However, this holds true only for the MPS-content measured in whole fetuses. At day 15 good separation of the ThVC (including the ribs) from the surrounding tissues

was possible. Biochemical analysis of MPS in the isolated ThVCs from FCdR-treated pregnancies showed a 15% reduction in the content of total MPS (Table 2), due to a pronounced reduction of the content of chondroitin 6-sulfate (27%) and heparan sulfate+chondroitin (20%). The other fractions were not significantly different ($P > 0.05$) from the controls.

The amount of cartilaginous matrix in alcian blue-stained vertebral bodies of the ThVC was measured with the Micro-Videomat (Table 3). In both control fetuses and fetuses from FCdR-treated letters the amount of cartilaginous matrix was increased by 35% within the last day. Thus the difference between the controls and the FCdR-series remained constant (11% higher in the controls).

The malformation rate had increased further and by the 15th day was already comparable with that at term. Eightyeight percent (15/17) of the fetuses showed malformations in the ThVC (in near-term fetuses 98% of the fetuses are malformed in the ThVC, Degenhardt et al., 1968). The rate of fusion was now 59%. This is somewhat lower than in near-term fetuses, where 76% fetuses with fusions are found (Bosse, 1978). The fusions present were complete or nearly so. Aplasias are found in 41% of fetuses, comparable with the incidence at the other stages. As on day 14, bending of the notochord is often seen and the cells are not restricted to the nuclei pulposi. In addition the chorda sheet may be locally disaggregated. The 21 fetuses in the controls showed no malformations of the thoracic vertebral column.

Discussion

Chondrification of the thoracic vertebral column of mice (strain C57BL/6Ffm) starts between days 12 and 13. The total content of mucopolysaccharides of whole fetuses clearly rose between days 13 and 14. Some hours before the first signs of the cartilaginous matrix in the vertebrae could be detected, a marked recuction of ^{35}S-uptake into the MPS was found in the FCdR-series serving as an indicator of synthesis of sulfated MPS. On the next day (day 13) the reduction of ^{35}S-uptake was less marked; it could no longer be demonstrated by biochemical methods in whole fetuses, but was shown by autoradiographic measurements in the ThVC. At this developmental stage aplasias were the only anomaly seen. This type of malformation – under the same experimental conditions – represents not more than 3% of all malformations (ThVC) in near-term fetuses (Bosse, 1978). Thus, the severe reduction of ^{35}S-uptake cannot be due exclusively to the aplasia, but mainly to the fusions, which predominate at term. These malformations were seen first at day 14 and were almost completed at day 15. At day 14 the amounts of hyaluronate and, to a lesser degree, chondroitin 6-sulfate and chondroitin 4-sulfate, were lowered in whole fetuses. The reduction of the amount of chondroitin 6-sulfate was even more clear in isolated ThVC at day 15 (in addition there was a smaller reduction in the fraction heparan sulfate+chondroitin). Thus the reduction of ^{35}S-uptake in the preceeding days, was probably due in a large part to loss of chondroitin 6-sulfate. The main fraction of MPS dat day 15 (Švejcar, 1968) – chondroitin 4-sulfate – was not involved. It seems therefore to be unlikely that on day 9 FCdR has caused a general disturbance of primordia of MPS synthetizing cells.

In our experiments fusion in the ThVC were not preceded by fusion of somites, as has been found in some mutants (Grüneberg, 1963; Theiler, 1967). However, in vertebral bodies fusions resulted from absence or incomplete differentiation of the intervertebral discs. Unfortunately few biochemical data on the embryonic intervertebral disc are available. In adult cattle and in the whale, and in whale fetuses, chondroitin 6-sulfate is the main MPS of the intervertebral disc (Rosenberg et al., 1967; Ludowieg et al., 1972). If this finding is applicable to the vertebral column of mouse fetuses, the reduction of the amount of chondroitin 6-sulfate at days 14 and 15 would fit with the appearance of fusions at these days, and fusions would be due to a functional disturbance of intervertebral discs. There is another hypothesis which may explain the results. Normal development of the vertebral column requires a morphologically and functionally normal notochord. Fusions are often combined with malformations (retardation, bending) of the notochord in the region of intervertebral discs (Degenhardt und Kladetzky, 1955; Töndury, 1958), a finding confirmed by our experiments. The notochord synthetizes mucopolysaccharides which are necessary for the differentiation of the vertebral column. In the chicken embryo the MPS of the notochord consists of 40% chondroitin 6-sulfate, 40% chondroitin 4-sulfate and 20% heparan sulfate (Kosher and Lash, 1975). In our experiments on day 15 the ThVC showed a reduction of the amount of chondroitin 6-sulfate and heparan sulfate. This may be an indication of a functional disturbance of the notochord.

The non-sulfated MPS hyaluronate is found in small amounts only in the notochord and in the intervertebral discs. It may be that the reduction of hyaluronate in whole fetuses, as found on day 13, is not related to changes in the vertebral column. However it is known that chondrification is always combined with a reduction in the content of hyaluronate (Kvist and Finnegan, 1970a, b; Toole, 1972) and thus a relationship between the lowered hyaluronate content to the onset of fusion at day 14 is possible.

In summary all our biochemical, histochemical and histological data point to a correlation between fusion in the ThVC and a functional disturbance of intervertebral discs and/or the notochord.

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