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Malformations of the axial skeleton in cranioschisis aperta and exencephaly in rat fetuses induced after neural tube closure

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Summary. Single doses of cyclophosphamide were administered (IP) to groups of Wistar rats, on different days of gestation after neural tube closure (days 12–15) and fetuses were collected on day 20. A very large number of the fetuses treated during days 12–14 exhibited cranioschisis and exencephaly. Ethanol fixed, alizarin red stained specimens were observed for axial skeletal abnormalities. The exencephalic ones lacked ossified skulls. The basicranial bones were either under ossified or had undergone extensive fusion resulting in reduction in cranial volume. The basicraniovertebral angle was obtuse. The vertebral bodies and arches showed varying degrees of hypoplasia, fusion and/or agenesis. The development of ribs and sternebrae was also extensively affected. Treatment on day 15 did not induce exencephaly but the axial skeleton was hypoplastic. Wavy ribs were a remarkable feature of these fetuses. All exencephalic fetuses had subcutaneous haemorrhages; many of them were obviously oedematous. These data indicate that the susceptible period for induction of these anomalies does not stop at neuropore closure.

Key words: Malformations – Axial skeleton – Rat fetuses – After neural tube closure

Congenital malformations of the central nervous system such as cranioschisis aperta with exencephaly/anencephaly, and spina bifida aperta with myeloschisis are easily diagnosed at birth and are thus well documented. Since Forestus wrote a clear description of such monstrosities in 1590 (for details, see Ballantyne 1904), a series of papers have appeared purporting to describe them. Incidentally, most studies of these disorders stress their clinical relevance and symptomatic neurology (Barrow and Simpson 1966; Calviness and Evrard 1975; McLaurin 1977; Myrianthopoulos and Bergsma 1979; Diebler and Dulac 1983) or their curious association with the race, geogra-

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phy, parity, socioeconomic status of the parents (Lemire et al. 1978; Warkany 1971) and prenatal diagnosis (Sellers et al. 1974). Even those reports (Emery and Kalhan 1970) entirely devoted to the descriptive pathology of such defects have largely overlooked the fact that axial dysraphic disorders are characterised not only by symptomatic neurological abnormalities but also by important and clinically silent skeletal defects (Marin-Padilla 1980).

The embryogenesis of the open neural tube and underlying axial skeleton has remained controversial, possibly because of their complex interdependance in normal morphogenesis. Two theories are in vogue. According to Morgagni's hydromyelic theory (Morgagni 1769), the pressure of fluid descending from a hydrocephalic head through the spine presses the bones apart and hence the skeletal malformations. Von Recklinghausen's theory, proposed a little more than a century later (Von Recklinghausen 1886) states that open neural tube defects result from primary failure of the neural tube to close. Although both theories have had their supporters, in practice most clinicians and experimental embryologists seem to have taken side with Von Recklinghausen (Feller and Sternberg 1929; Giroud 1977; Marin-Padilla 1970, 1978; Warkany 1971) resulting in the widespread use of the term-dysraphia. Careful reconstruction studies on twin embryos (Muller and O'Rahilly 1984) clearly point to such a mechanism, but do not exclude the possibility of occurrence of such defects after closure. Occasional clinical reports suggest that a closed neural tube may reopen (Paget 1970; Ganchrow and Ornoy 1979; Gardner 1980), but experimental evidence is lacking. Recent data from our laboratories indicate that it is possible to induce this type of neural tube and axial skeletal malformations in the rat after closure of the neural tube (Padmanabhan and Singh 1983; Padmanabhan 1984). In this paper, the susceptible period for induction of cranioschisis aperta and exencephaly is reported and a spectrum of associated axial skeletal malformations is described. The data clearly indicate that the vulnerability of the axial skeletal primordia to gross defects does not stop with the closure of the neural tube.

Material and methods

Female rats of Wistar strain weighing 225 ± 20 g were mated with males of the same strain in the evening and pregnancy was confirmed by sperm positive vaginal smears observed on the following morning. The pregnant animals were caged separately and maintained on standard lab chow and tap water provided ad libitum. Single doses (15–20 mg/kg) of freshly prepared cyclophosphamide (CPA) in distilled water were administered intraperitoneally to groups representing successive days of gestation from day 12 to day 15 (sperm positive day = day 0 of pregnancy) i.e. well after the closure of the neural tube (Nishimura and Tanimura 1976; Wilson 1973; Edwards 1968. Our own observations). The controls received an equal volume of the vehicle only. Fetuses were collected on day 20 by hysterotomy under ether anaesthesia. They were blotted dry and the length of the umbilical cord, placental and body weights and malformations were recorded. Fetuses were fixed in 95% ethanol, processed for alizarin-red staining according to Hurley's technique (Hurley 1965) and stored in glycerol. Observations were made under a dissecting microscope. Only intact and well preserved preparations were included for data collection. Levels of significance were tested appropriately by either the Student t test or Chi square analysis. Statistical significances was assumed at $P < 0.05$ level.

Results

Administration of CPA in single doses to pregnant rats after neural tube closure resulted in significant reduction in fetal weight, placental weight, and umbilical cord length in all treatment groups when compared with the corresponding controls (Table 1). Treatment before day 12 resulted in extreme resorption, of some fetuses although the survivors showed exencephaly. Treatment on days 12–14 led to cranioschisis aperta and exencephaly in a large number of living fetuses. The associated anomalies included micrognathia, exophthalmia with or without cataract, cleft palate, subcutaneous and deep haemorrhages, oedema, low set ears, hydronephrosis, and digital and tail defects. These abnormalities were more pronounced in groups treated on days 12 and 13 than in those treated on days 14 and 15. Treatment on day 15 gave rise to meningoencephaloceles only in 11% of the living fetuses.

The skull bones

In most exencephalic fetuses the premaxilla and maxilla were very small, and the mandible was devoid of the ramus, presenting an obtuse angle at the symphysis menti. The frontal and parietal bones were frequently represented by only the orbital and squamous parts respectively. In many cases the parietal bones were missing (Table 2). No trace of a supraoccipital bone was found (Fig. 1). The exoccipitals were rudimentary and widely separated. The presphenoid-basisphenoid and the basisphenoid-basioccipital

Table 1. Effect of maternal administration of cyclophosphamide on the fetus and fetal milieu in the rat

Day of Treatment	Dose mg/kg	No. of animals	Implantations	Dead/ Resorptions	Fetuses alive	Fetal weight (gm)* Mean ± S.D.	Placental weight (gm)* Mean ± S.D.	Umblical cord length (mm)* Mean ± S.D.	Percentage of Neural tube anomalies
Control	–	11	109	4	105	4.00 ± 0.35	0.51 ± 0.07	22.92 ± 3.13	–
12	15	7	83	22	61	2.52 ± 0.63	0.24 ± 0.05	12.66 ± 3.46	100 CRE
13	20	9	82	4	78	2.11 ± 0.41	0.26 ± 0.06	13.65 ± 1.83	100 CRE
14	20	12	121	6	115	2.53 ± 0.43	0.35 ± 0.07	17.67 ± 3.48	90 CRE
15	20	10	98	1	97	2.85 ± 0.92	0.36 ± 0.10	19.97 ± 3.63	11 MEN

CRE = Cranioschisis aperta and exencephaly
MEN = Meningoencephalocele

* Significant at $P < 0.05$ when compared control vs experimental

Table 2. The extent of ossification of skull bones associated with cranioschisis and exencephaly induced on different days of gestation – percentage ossified

Treatment day	Dose mg/kg	No. of specimens observed	Premaxilla	Maxilla	Mandible	Zygomatic	Nasal	Frontal	Parietal	Squamous temporal	Tympanic ring	Supra occipital	Exoccipital	Basioccipital	Basisphenoid	Presphenoid	Hyoid
Control		40	100	100	100	100	100	100	100	93	100	95	100	100	100	100	93
12	15	43	28 (49)	26 (53)	44 (33)	14 (40)	21 (63)	5 (0)	0 (0)	0 (0)	60 (14)	0 (0)	12 (67)	33 (5)	91 (9)	93 (0)	9 (5)
13	20	40	5 (55)	5 (52)	2 (60)	2 (50)	8 (50)	0 (5)	0 (0)	0 (17)	38 (35)	0 (0)	75 (17)	80 (13)	80 (13)	80 (13)	22 (32)
14	20	31	3 (93)	3 (93)	3 (93)	6 (90)	6 (90)	0 (48)	0 (0)	3 (19)	13 (81)	0 (0)	68 (32)	68 (32)	68 (32)	68 (29)	13 (61)
15	20	30	73 (27)	73 (27)	73 (27)	70 (27)	70 (27)	53 (20)	10 (0)	50 (40)	87 (13)	23 (0)	97 (3)	93 (7)	93 (7)	80 (7)	50 (10)

Numerals in parentheses indicate percentage of rudimentary bones

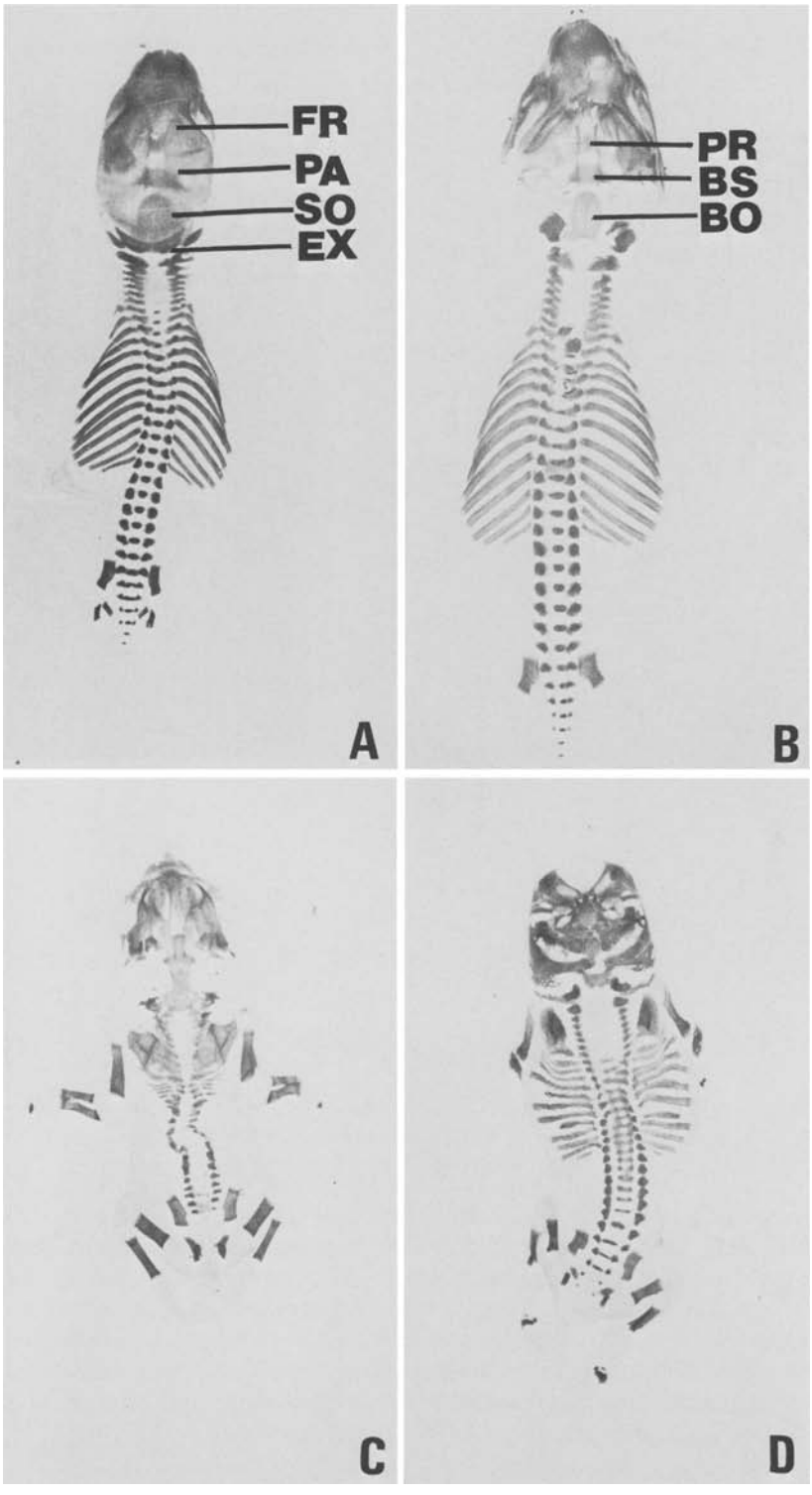

Fig. 1. **A** and **B** control skeletons. In **B** the skull vault has been removed to show the basicranium. Observe that the skull vault is missing and the basicranial bones have variously fused in exencephalic fetuses treated on day 12 **C** and day 13 **D**. Agenesis and hypoplasia of a number of ribs and vertebral bodies, scoliosis **C** and dilated cervical spinal canal **D** are also evident. *FR* = Frontal; *PA* = Parietal; *SO* = Suproccipital; *EX* = Exoccipital; *PR* = Presphenoid; *BS* = Basisphenoid; *BO* = Basioccipital

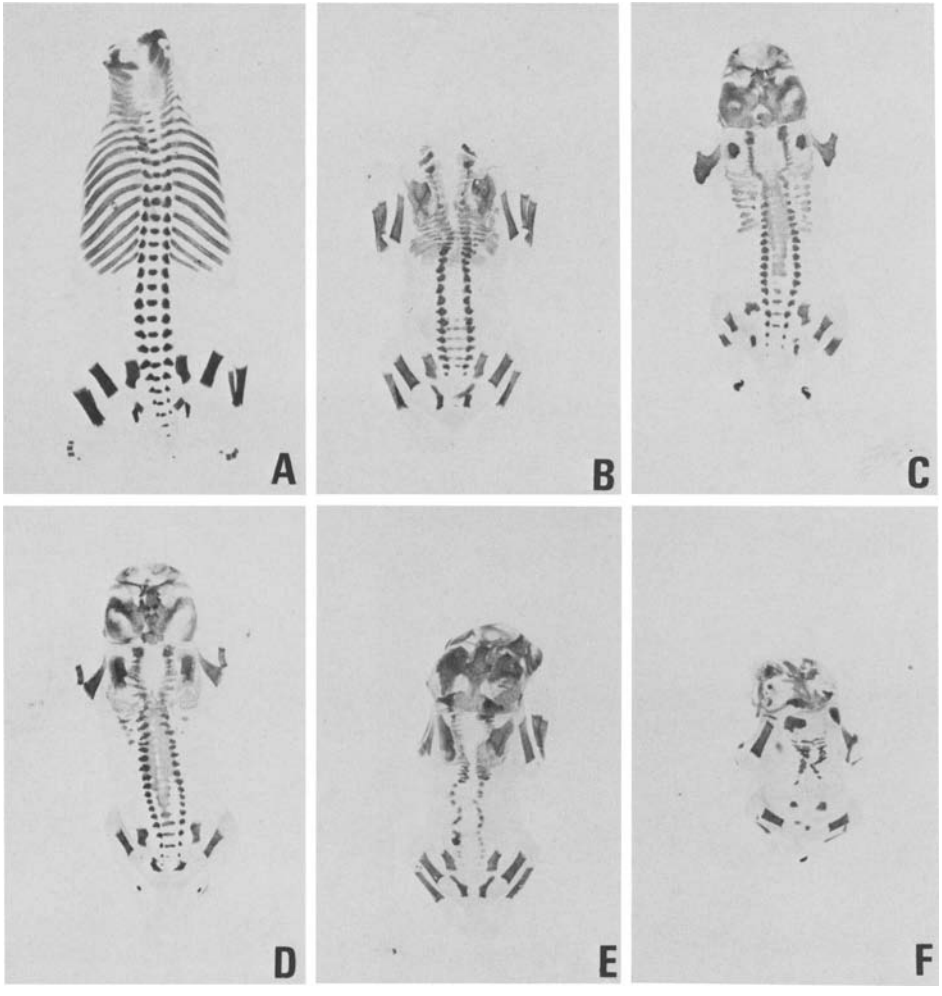

Fig. 2. **A** Control skeleton. **B–F** Exencephalic ones treated on day 12 **B, E, F** and day 13 **C, D**. In **B** the skull has been removed. Note the significant reduction in the number of ribs, vertebral bodies and arches, fusion of thoracic bodies **C, D** and scoliosis **E, F**

synchondroses had been obliterated by synostoses (Figs. 1, 2). The basiocciptal was located far posteriorly in the cleft between the exoccipitals thus widening the foramen magnum backwards. Also notable was the obtuse basicraniovertebral angle associated with the hyperextended neck (observed grossly before clearing). The vomer and ethmoid were poorly ossified. The hyoid was either absent or very small. In the nonexencephalic fetuses of the day 15 treatment group all bones of the cranial vault and cranial base were ossified to a significantly lesser extent compared to the controls.

The vertebrae

In all exencephalic fetuses the vertebral arches were small and in some, the lumbar and lower thoracic segments were under ossified. Absence of

Table 3. Extent of ossification of axial skeleton in cranioschisis and exencephaly – the vertebrae – percentage ossified

Treatment day	Arches					Bodies				
	Control	12	13	14	15	Control	12	13	14	15
No. of specimens observed	40	43	40	31	30	40	43	40	31	30
Cervical	100	100	100	100	100	45	9	0	0	0
Thoracic	100	100	98	100	100	100	67	0	87	100
Lumbar	100	95	93	94	97	100	93	20	81	97
Sacral	73	9	0	48	30	93	44	23	29	90
Coccygeal	0	0	0	0	0	3 (3)[a] 50 (2) 43 (1)	5 (2) 16 (1)	5 (3) 15 (2) 13 (1)	23 (2) 13 (1)	17 (3) 33 (2) 7 (1)

[a] Numerals in parenthesis indicate the number of coccygeal bodies

the arches was remarkable in the lower lumbar and sacral regions. The upper cervical arches were wide apart in line with the widely separated exoccipitals. Except in 4 cases of the day 12 group, cervical bodies were absent. The frequency of absence of bodies increased in the order of upper thoracic, sacral and coccygeal segments respectively (Table 3). Longitudinal fusion of the thoracic and lumbar arches and in the event of non-fusion varying degrees of scoliosis were noted in fetuses which were remarkably oedematous and stunted. Frequently such fusions involved the right and left halves of the bodies thus leaving a midline fissure (Fig. 2 CD). The number of vertebral bodies and arches involved in fusion did not follow any specific pattern. In the nonexencephalic day 15 treatment group, the absence of vertebral arches and bodies was confined to the lower lumbar and sacral regions. The cervical spinal column was as dilated as in the exencephalic fetuses.

The ribs

Thirty five percent of day 12 treatment group had 12 or less than 12 pairs (Table 4, Fig. 3). Of these, many were small or rudimentary and ossification did not extend beyond 2 mm from the angle. The least number of ossified ribs was 4 pairs in 10/40 cases in the day 13 group and 5 pairs in 3/43 cases in the day 12 group. Wavy ribs were observed predominently in exencephalic fetuses of day 14 group and the nonexencephalic fetuses of day 15 group. The last pair of ribs was wavy in one control fetus.

Sternebrae

A minimum of 3 (1/40) and a maximum of 6 (26/40) sternebrae were observed in control fetuses (Table 5). None of day 12 and day 13 groups and

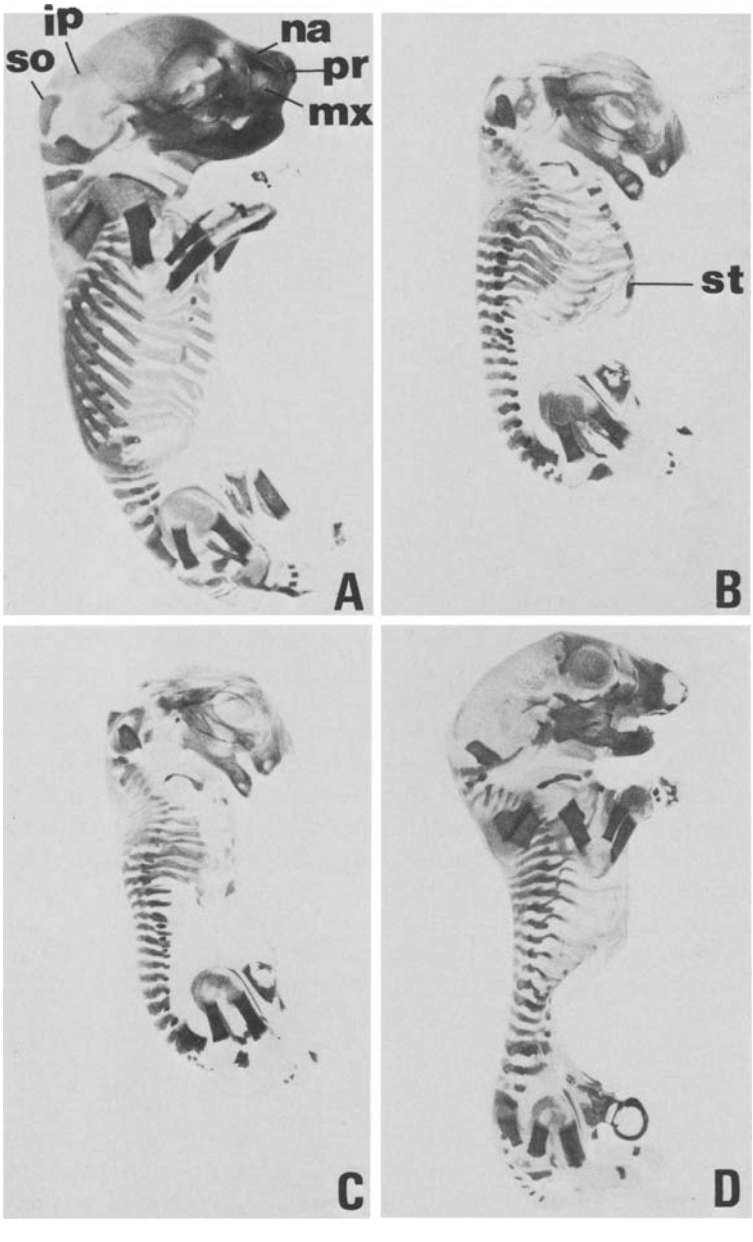

Fig. 3. Lateral view of skeletons showing the absence **B, C** and nonossification **D** of skull vault, of sternebrae **C, D** and wavy ribs **B, D**. **A** Control. **B, C** Exencephalic, treated on day 14 and 12 respectively. **D** Nonexencephalic, treated on day 15. *na* = nasal; *pr* = premaxilla; *mx* = maxilla; *ip* = interparietal; *so* = supraoccipital; *st* = sternum

Table 4. The extent of ossification of ribs in cranioschisis and exencephaly in rat fetuses – percentage ossified

	Control		12 D		13 D		14 D		15 D	
	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left
Normal	97.5	97.5	21	21	0	0	3	3	7	7
Deficient (4–12)	0	0	35	21	100	100	3	3	3	0
Rudimentary (1–13)	0	0	63	70	67.5	72.5	71	77	23	27
Wavy	2.5	2.5	9	12	0	0	90	90	93	90

D = Day of treatment

Table 5. The range of ossification of sternebrae in cranioschisis and exencephaly – percentage ossified

No. of sternebrae	0	1	2	3	4	5	6
Control	0	0	0	2	5	26	67
12 D	7	35	21	7	9	21	0
13 D	12	8	12	23	30	15	0
14 D	3	6	10	6	26	42	7
15 D	6	27	13	10	10	27	7

D = Day of treatment

7% of the day 14 and day 15 groups showed 6 sternebrae. In the rest, the number and extent of ossification and fusion of sternebrae varied widely (Fig. 4). Bipartite sternebrae were observed in those fetuses with ectopia cordis and in all oedematous ones. These were also the ones which possessed rudimentary ribs and scoliosis.

Discussion

Although Von Recklinghausen's theory of nonclosure of the neural tube is generally favoured to be the basic mechanism in dysraphic disorders, only a few have discussed the possibility of a closed neural tube reopening during critical stages of development (Hoshino 1971; Ganchrow and Ornoy 1979; Gardner 1966, 1980; Murakami et al. 1972). The studies of Hoshino (1971) and Murakami et al. (1972) are complicated by interference with neural tube development covering the preclosure period and not pertaining to skeletal malformations. Gardner's view was based primarily on his own clinical experience. Our earlier work on CF rats (Padmanabhan and Singh 1983) indicated that it was possible to induce axial skeletal malformations after neural tube closure. The data from the present investigation provides further evidence to the possibility of inducing cranioschisis aperta and exencephaly in another strain of rats and defines the maximum period of susceptibility (days 12–15). Injection of single doses of CPA on days 12, 13 and

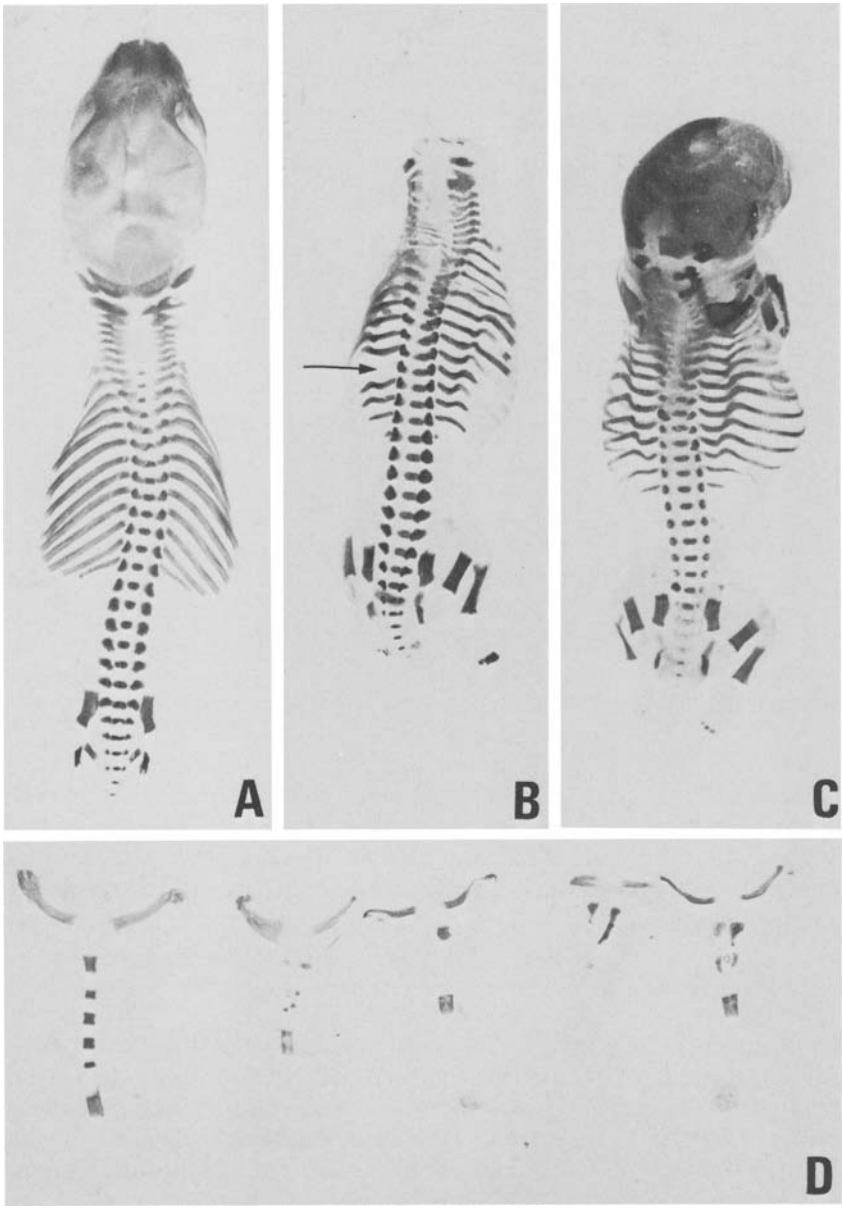

Fig. 4. **A** Control. **B, C** treated on day 15. The 10th rib on the left is missing in **B** (*arrow*). Several ribs are wavy in **B** and **C**. **D** Sternebrae, *left* control, showing 6 pieces of sternum. Observe the absence of different pieces of sternum and bipartite sternum in the experimental skeletons

14 i.e. well after neural tube closure resulted in cranioschisis aperta and exencephaly in a very large number of fetuses and injection on day 15 led to meningoencephalocele in a small percentage of fetuses. All these fetuses had several non-neural, particularly axial skeletal malformations. Taken together these data point to the fact that the susceptibility for neural tube abnormalities does not stop at closure of the neural tube but extends almost to the end of the period of organogenesis and that paraxial mesoderm from which the primordia of the axial skeleton are derived also remains equally vulnerable during this period.

The exencephalic fetuses had distended brain vesicles (with microscopically demonstrable pores) covered by a thin membrane and highly vascular and haemorrhagic mesenchyme (Padmanabhan 1984). Cleared specimens stained with alizarin revealed total absence of bony vault and hypoplasia and fusion of bones of the skull base. Premature closure of synchondroses of the skull base and the consequent reduction in cranial capacity coupled with the obtuse angularity at the basicraniovertebral junction possibly contributed to protrusion of the rapidly expanding hydrocephalic brain vesicles through the thin overlying mesenchyme. Our chronological and histological observations (Padmanabhan 1984) lend credence to such a possibility. Unlike the cranial end of the neural tube, the spinal part was not dilated at term. Still, the vertebral bodies and arches were extensively malformed. Absence and/or fusion of vertebral bodies and arches, loose intervertebral articulations and scoliosis were very remarkable. These malformations possibly result from the pressure exerted by the expanding neural tube on the precartilage mesenchyme and cartilage primordia. A fact worth noting here is that these defects very closely resemble the deformities of the thoracic and lumbar segments of the spine of human abortuses with hydrocephalus internus described in Werthemann's classical work (Werthemann 1955). These observations are in line with Morgagni's hydromyelic theory (Morgagni 1769), Gardner's (1980) hypothesis of over distension of the neural tube and Browne's experimental observations in chicken embryos (Browne 1970). The impressive association of the malformations with haemorrhage and oedema points to another factor contributing to the disturbance of these primordia, namely an altered fluid outside the neural tube. Such an oedematous environment is known to affect cell assembly and primordia formation (Grabowsky 1970; Poswillo 1975) depending on the stage of development. The antiproliferative activity of CPA and the consequent mesenchymal deficiency would be a contributing factor in the final outcome of the malformation (Padmanabhan 1984).

The high incidence of exencephaly and axial skeletal malformations and the consistency and regularity of associated anomalies in these experiments is of special significance because studies designed to investigate the pathogenetic mechanisms of such congenital malformations should be based on the confidence that every embryo that is handled in an early stage would be malformed (Fraser 1977) if allowed to go to term. The cellular basis of this malformation syndrome is currently being investigated in our laboratory.

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