18 Humphrey, R. R., Am. J. Anat. *76* (1945) 33.
19 Humphrey, R. R., and Fankhauser, G., J. Morph. *79* (1946) 467.
20 Humphrey, R. R., and Fankhauser, G., J. Hered. *40* (1949) 7.
21 Dawley, R. M., and Bogart, J. P., (Eds), Evolution and Ecology of Unisexual Vertebrates. New York State Museum Bulletin 466, Albany 1989.
22 Vrijenhoek, R. C., Dawley, R. M., Cole, C. J., and Bogart, J. P., in: Evolution and Ecology of Unisexual Vertebrates, pp. 19–23. Eds R. M. Dawley and J. P. Bogart. New York State Museum Bulletin 466, Albany 1989.
23 Vasil'ev, V. P., Vasileva, K. D., and Osinov, A. G., in: Evolution and Ecology of Unisexual Vertebrates, pp. 153–169. Eds R. M. Dawley and J. P. Bogart. New York State Museum Bulletin 466, Albany 1989.
24 Morris, M. A., Herpetologica *41* (1985) 267.
25 Morris, M. A., and Brandon, R. A., Copeia (1984) 324.
26 Kraus, F., Occ. Pap. Mus. Zool. Univ. Michigan *709* (1985) 1.
27 Billy, A. J., Can. J. Zool. *64* (1986) 2418.
28 Bogart, J. P., in: Polyploidy: Biological Relevance, pp. 341–378. Ed. W. H. Lewis. Plenum Press, New York 1980.
29 Dawley, R. M., in: Evolution and Ecology of Unisexual Vertebrates, pp. 1–18. Eds R. M. Dawley and J. P. Bogart. New York State Museum Bulletin 466, Albany 1989.

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Effects of retinoic acid on embryonic development of mice in culture

T. Watanabe[a] and R. M. Pratt[b] †

[a] *Department of Hygiene and Preventive Medicine, Yamagata University School of Medicine, Yamagata 990-23 (Japan), and* [b] *Laboratory of Reproductive and Developmental Toxicology, NIEHS, NIH, Research Triangle Park (North Carolina 27709, USA)*
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Summary. The effects of all-trans-retinoic acid (RA) (tretinoin) on the craniofacial development of mouse embryos were examined using whole embryo culture. In day 8 embryos cultured for 48 h, embryonic growth was inhibited concentration-dependently by all-trans-RA treatment. Most of the treated embryos exhibited hypoplasia of the primary palatal processes and a reduction in the development of the first visceral arches. In day 10 embryos cultured for 48 h, although embryonic growth was not inhibited at any concentrations of all-trans-RA, median cleft lip (93%), hypoplasia of the primary palatal processes (37%) and limb reduction deformities (48%) occurred commonly. Furthermore, RA treatment greatly reduced the size of the secondary palatal processes. The incorporation of ^{3}H-thymidine in the treated maxillary processes was decreased to 65% of the control value at 1.0×10^{-7} M all-trans-RA. These findings indicate that all-trans-RA is teratogenic in mouse whole embryo culture.
Key words. All-trans-retinoic acid; whole embryo culture; mouse embryos; craniofacial development; mesenchymal cells; palatal processes.

Teratogenic and embryolethal effects of retinoic acids (RA) have been observed in humans and experimental animals. In humans, there have been severe congenital malformations in newborns born to women who took 13-cis-RA [1,2]. The major malformations resulting from the embryonic exposure were cleft palate, micrognathia, and external ear and central nervous system abnormalities. In experimental animals in vivo and in vitro, developmental defects have been produced in various tissues including neural folds, the heart and limbs [3–5]. These are similar to those observed in human newborns.

In a series of our experiments, using whole embryo culture, 13-cis- and 4-oxo-13-cis-RA caused highly specific defects in mouse embryos. Day 8 embryos showed a marked reduction in the size of the visceral arches. This may be caused by 13-cis-RA inhibiting the migration of cranial neural crest cells [6,7]. A high percentage of day 10 embryos were affected with median cleft lip and severe limb reduction. In these embryos, the mesenchymal cells beneath the epithelium of the nasal and maxillary processes contained pyknotic nuclei (dead cells), and there was a dramatical reduction in the number of the mesenchymal cells and in ^{3}H-thymidine incorporation in the secondary palatal processes [8]. In culture, the treated palatal mesenchymal cells took a long time to proliferate [9]. We suggested that the cis-isomers of RA affect proliferation of cranial neural crest cells as well as oronasal mesenchymal cells. This appears to be related to the production of craniofacial malformations in rodents and humans.

Different hypotheses concerning the cellular mechanism by which retinoids produce craniofacial malformations in mammalian embryos have been proposed. Some of the malformations are considered to be due to an inhibition of the migration or proliferation of cranial neural crest cells and to programmed cell death of epithelium in facial processes [7,10,11]. Recent findings indicate that the inhibition of 13-cis-RA on palatal mesenchymal cell growth may contribute to facial clefting in mouse embryos [9,12,13]. It has also been demonstrated that the teratogenic effects of RA are attributable to excessive cell death [14,15].

In the present study, to determine the cellular mechanism of the development of craniofacial malformations in

retinoid-treated embryos, we examined the effects of all-trans-RA, an isomer of isotretinoin, on embryonic growth and development in whole embryo cultures. The results were compared with our previous research on the embryopathy of cis isomers of RA.

Materials and methods

Randomly bred CD mice were maintained in an animal room with artificial lighting on a 12-h light/dark cycle at about 23 °C. Nulliparous females were paired overnight with males of the same strain. Females were checked daily for the presence of copulation plugs and the day of a plug was designated as day 0 of gestation. Pregnant mice were killed by cervical dislocation on days 8 or 10 and embryos were collected from each uterus. Under sterile conditions, the conceptuses were transferred to Trowell's T8 medium (GIBCO Laboratories, Grand Island, NY) at room temperature. After removing maternal decidua and Reichert's membrane, day 8 embryos, ranging from 4 to 6 somite pairs, with visceral yolk sac, amnion, and ectoplacental cone intact, were prepared according to the technique of New[16]. Day 10 embryos were dissected free of accessory tissues, and were carefully pushed through a small tear in the functional yolk sac. The embryos were placed individually in 30-ml stoppered glass bottles containing, for day 8 embryos, 4.0 ml of culture medium composed of only heat-inactivated male rat serum and for day 10 embryos, 50% Waymouth's MB 752/1 medium (GIBCO) and 50% fetal bovine serum containing 2 mg/ml fetal bovine albumin. The procedures for the whole embryo culture technique have been described in detail in our previous papers[6, 8].

All-trans-RA (tretinoin) was dissolved in DMSO at 6 mg/ml (gassed with N_2, exposed only to yellow lights, and stored at −70 °C), and added at final medium concentrations of $10^{-7} - 10^{-5}$ M. Control cultures were given 4 µl of DMSO (final concentration of 0.1%). After 48 hours of culture, the embryos were harvested from the medium, and the overall growth and development of each embryo was assessed using a dissecting microscope. For the last 6 h of culture, some embryos were maintained in the presence of ^{3}H-thymidine (20 Ci/mmole) at 2.5 µCi/ml (New England Nuclear, Boston, MA). The embryos were extensively washed with phosphate-buffered saline (PBS, pH 7.2). For autoradiography, the embryo heads were carefully detached from the trunk and immediately fixed in Bouin's solution. After dehydration and embedding in paraffin, specimens were serially sectioned at 6 µm in the frontal plane and placed on slides. These slides were later deparaffinized in xylene, dried, and dipped in Kodak NTB-2 autoradiographic emulsion diluted 50% with distilled water at 45 °C. After drying, slides were stored at −70 °C for 2 weeks, developed, and finally stained with hematoxylin. Sections were evaluated for ^{3}H-TdR incorporation and cell morphology.

To measure DNA synthesis, individual embryonic parts (brain, maxillary process, mandible process and limb bud) were rinsed twice in PBS and immediately frozen. After thawing, each sample was sonicated in distilled water and sonicates (20 µl) of embryonic organs were spotted on glass microfiber filters. Dried filters were placed in 10% trichloroacetic acid (TCA) solution at 4 °C for 30 min, followed by three 5% TCA rinses (15 min), and then transferred to absolute ethanol. The dried filters were placed in 10 ml of scintillation fluid and counted in a Beckman scintillation counter (LS9800). The protein content in the embryonic parts was determined by the method of Lowry et al.[17] using bovine serum albumin as a standard, and the incorporation values were presented as disintegrations per minute (DPM) per µg of protein.

For statistical comparisons, the reproductive data, such as the number of embryos with malformations, were analyzed by chi-square test and Fisher's exact test. Student's t-test was used to analyze data concerning embryonic development and ^{3}H-thymidine incorporation[18].

Results

Table 1 shows the effects of the all-trans-RA on day 8 mouse embryos cultured for 48 h. At more than 2.5×10^{-7} M concentration, the crown-rump and head lengths in treated embryos were significantly shorter than in the DMSO controls. Embryonic growth was inhibited in a concentration-related manner. External examination of the craniofacial region showed a variable degree of hypoplasia of primary palatal processes, and first visceral arch defects were developed in a number of treated embryos (89%) (fig. 1). Only 5% of the DMSO control group showed anomalies.

In the embryonic growth and development of day 10 embryos treated with all-trans-RA (table 2), RA treatment at 1.0×10^{-6} M substantially increased embryolethality to 50% of the cultured embryos. However, there

Table 1. Effect of all-trans retinoic acid on day 8 mouse embryos in vitro

All-trans retinoic acid	Crown-rump length[a]	Head length[a]	Hyp. of primary palatal processes (%)	Visceral arch defects (%)
0 (DMSO)	4.0 ± 0.23	2.0 ± 0.14	0	0
1.0×10^{-8} M	3.9 ± 0.32 (98)	1.9 ± 0.20 (95)	0	0
1.0×10^{-7} M	4.0 ± 0.50 (100)	2.0 ± 0.22 (100)	16.7	16.7
2.5×10^{-7} M	3.4 ± 0.46* (85)	1.5 ± 0.26* (75)	100	100
1.0×10^{-6} M	3.1 ± 0.36* (78)	1.2 ± 0.35* (60)	100	66.7

[a] mean ± SD (percent of control), mm. * $p < 0.01$ (Student's t-test). Hyp.: hypoplasia.

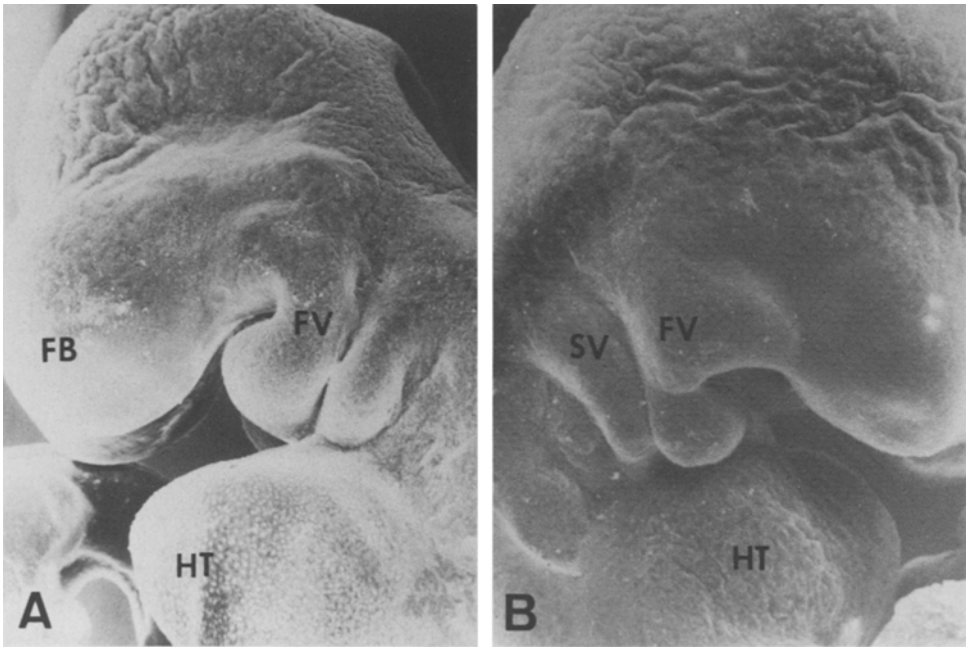

Figure 1. Scanning electron micrograph of the frontal facial region of day 8 mouse embryos cultured for 48 h. *A* control embryo. *B* all-trans-retinoic acid-treated embryo. The first visceral arch is severely reduced and abnormal. FB, forebrain; FV, first visceral arch; SV, second visceral arch; HT, heart. × 55.

Table 2. Effect of all-trans retinoic acid on day 10 mouse embryos in vitro

All-trans retinoic acid (M)	0 (DMSO)	1.0×10^{-7}	1.0×10^{-6}
No. of embryos used	33	34	54
No. of dead embryos (%)	7 (21.2)	9 (26.5)	27 (50.0)[b]
No. of live embryos examined	26	25	27
Embryo crown-rump length[a] (mm)	8.5 ± 0.32	8.3 ± 0.53	8.4 ± 0.51
Embryo head length[a] (mm)	4.5 ± 0.28	4.4 ± 0.41	4.3 ± 0.36
No. of embryos with malformations			
hypoplasia of primary palatal process	0	4*	10*
median cleft lip	0	11*	25*
lateral cleft lip	1	1	2
small mandible	0	4*	7*
short limb	0	2	13*
short tail	0	1	6*

[a] mean ± SD. [b] $p < 0.05$ (chi-square test). * $p < 0.05$ (Fisher's exact test).

was no difference in crown-rump and head lengths between the RA-treated and control groups, indicating that RA caused no embryonic growth retardation, including craniofacial development, even at the concentration of 1.0×10^{-6} M. The external malformations occurred primarily in the craniofacial regions: median and lateral cleft lip were prominent (92.6%), usually associated with hypoplasia of the primary palatal processes (37.0%), and small mandible (25.9%). Limbs and tails also developed defects at 1.0×10^{-6} M, significantly more than in the DMSO controls.

Autoradiographic and histological studies of the facial processes revealed some obvious differences between the RA-treated and control embryos. Treatment with RA dramatically reduced the size of the secondary palatal processes, in which labeled nuclei were reduced. Howev-

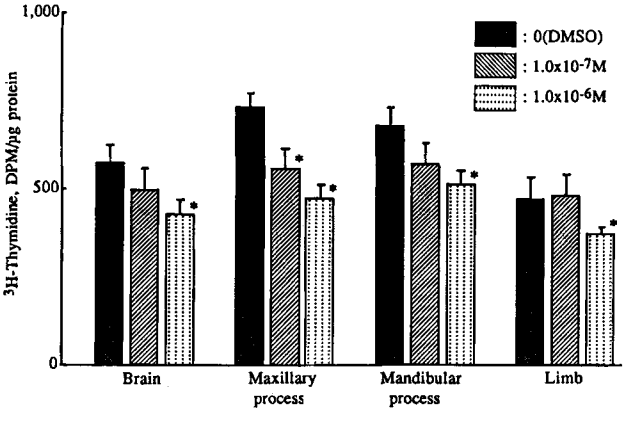

Figure 2. Effect of all-trans retinoic acid on ^{3}H-thymidine incorporation in day 10 mouse embryos in vitro. Statistical evaluation of the means by Student's t-test: * $p < 0.05$, compared with the respective controls.

er, no pyknotic nuclei were present in the nasal and maxillary mesenchymal cells adjacent to the epithelium in the all-trans-RA treated embryos.

The effect of RA on DNA synthesis was measured on day 10 by the incorporation of ^{3}H-TdR into TCA-insoluble macromolecules in the embryonic parts cultured for 48 h (fig. 2). Significant concentration-dependent differences between control and treated samples occurred in all parts of the embryos. At 1.0×10^{-7} M RA the ^{3}H-TdR incorporation in the treated maxillary process were decreased to 65% of the control value.

Discussion

The present results indicate the potential teratogenicity of all-trans-RA in mouse embryos in vitro. In day 8 embryos, embryonic growth was significantly inhibited concentration-dependently by all-trans-RA treatment, which is in accordance with the results of previous experiments with 13-cis- and 4-oxo-13-cis-RA[6]. The types of malformations resulting in all-trans-RA-treated embryos were similar to those observed with cis isomers. At 1×10^{-5} M of 13-cis-RA, approximately half of the embryos had very specific defects including a reduction in size of the first and second visceral arches. Similar results were obtained with the metabolite, 4-oxo-13-cis-RA (1×10^{-5} M). All-trans-RA produced developmental defects in the first visceral arches in all treated embryos even at a concentration of 2.5×10^{-7} M. These retinoids also produced a high percentage of craniofacial abnormalities in day 10 mouse embryos cultured with doses of retinoic acid at which embryos exhibited little, if any, growth retardation. All embryos treated with 13-cis-RA (2×10^{-5} M) had median cleft lips with or without lateral clefts, and most had severe limb reduction deformities. In addition, these orofacial and limb malformations appeared in most embryos given all-trans-RA at 1×10^{-6} M. The types and incidence of malformation were not different between the retinoids examined. However, treatment with all-trans-RA (1×10^{-6} M) resulted in a higher rate of embryolethality (50%) than occurs with the same dose of 13-cis- and 4-oxo-13-cis-RA. Thus, mouse embryos were relatively more sensitive to all-trans-RA and less sensitive to cis isomers of RA in vitro. It was also demonstrated in vivo that cis isomers were not as teratogenic as the trans isomer in mice[19-21]. The differences in teratogenicity of retinoids may be related to alterations in length and conformation of the conjugated side chain[22].

The cellular mechanisms by which retinoids exert embryopathy have not been clearly defined, but the direct effect of RA on the developing embryo may be confined to specific cell types. The forebrain and midbrain neural crest cells have been known to migrate from the neural folds into the frontonasal processes and first visceral arch[23,24]. In day 8 embryos exposed to 13-cis-RA, we observed defects in the first and second visceral arches, which suggests that these defects are due to the inhibition of cranial neural crest cell migration[6,7]. Similar changes may occur in the cranial crest cells from day 8 embryos treated with all-trans-RA.

On the other hand, the orofacial defects observed after RA treatment in day 10 embryos cannot be explained by an alteration in migration of cranial neural crest cells. Our autoradiographic examination showed that retinoic acid-induced inhibition of the proliferation of mesenchymal cells in secondary palatal processes was accompanied by a decrease in DNA synthesis[8,13]. Similar findings have been reported in the posterior region of secondary palatal shelves in rat embryos after retinoid treatment in vivo[25]. The addition of retinoids in the cell culture induced a longer cell proliferation time for embryonic palatal mesenchymal cells from humans and mice[9]. Abbott et al.[15] have recently demonstrated that mesenchymal cell death and reduced proliferation are not responsible for the development of palatal shelves in RA-treated mouse embryos in vivo. These findings show that RA may be a factor directly affecting the proliferation and viability of mesenchymal cells at the middle stage of organogenesis, thus inhibiting development of the secondary palatal processes. So, the induction of orofacial defects by RA can be attributed to different types of embryonic cells at the early and middle stages of embryonic development[12].

Previous experiments showed an accumulation of 13-cis- and all-trans-RA in the facial regions of mouse embryos in vivo and in vitro[6,26]. Also, specific proteins were found by two-dimensional gel electrophoresis to be altered in 13-cis-RA-treated embryos. Particularly in the craniofacial regions, such as the frontonasal and pharyngeal arch mesenchyme, retinoic acid receptor γ transcripts were observed during early mouse embryogenesis[27]. These findings indicate that retinoic acids or their major metabolites act at a nuclear level and induce changes in gene expression in these sensitive cells and tissues, which may be correlated with the onset of retinoid-induced abnormal development. Some of the effects of retinoic acids may be mediated by a specific cell binding protein. RA-binding protein and the nuclear receptors have been identified in various embryonic tissues in mouse and rat embryos[28,29]. However, the role of RA-binding protein in the mechanism of retinoic acid embryopathy remains to be elucidated. This study provides additional information about the potential teratogenicity of retinoic acids in mouse embryos in vitro. Our results have shown that the induction of orofacial malformations by retinoids can be attributed to defects in different types of embryonic cells, such as cranial neural crest cells and mesenchymal cells.

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† Deceased.

1 Ward, A., Brogden, R. N., Heel, R. C., Speight, T. M., and Avery, G. S., Drugs *28* (1984) 6.
2 Rosa, F. W., Wilk, A. L., and Kelsey, F. O., in: Teratogen Update: Environmentally Induced Birth Defect Risks, p. 61. Alan R. Liss, New York 1986.
3 Morriss, G. M., and Steele, C. E., Embryol. exp. Morph. *32* (1974) 505.
4 Davis, L. A., and Sadler, T. W., Teratology *24* (1981) 139.
5 Zimmermann, B., and Tsambaos, D., Arch. dermat. Res. *277* (1985) 89.
6 Goulding, E. H., and Pratt, R. M., J. craniofac. Genet. devl Biol. *6* (1986) 99.
7 Pratt, R. M., Abbott, B. D., Watanabe, T., and Goulding, E. H., in: Developmental Toxicology: Mechanisms and Risk, p. 227. Cold Spring Harbor Laboratory, New York 1987.
8 Watanabe, T., Goulding, E. H., and Pratt, R. M., J. craniofac. Genet. devl Biol. *8* (1988) 21.
9 Watanabe, T., Willis, W. D., and Pratt, R. M., J. nutr. Sci. Vitam. *36* (1990) 311.
10 Yasuda, Y., Itoh, K., Mizuno, N., Konishi, H., and Tanimura, T., Analyt. cell. Path. *2* (1989) 23.
11 Tamarin, A., Crawley, A., Lee, J., and Tickle, C., J. Embryol. exp. Morph. *84* (1984) 105.
12 Watanabe, T., Cong. Anom. *30* (1990) 79.
13 Yoshikawa, H., Kukita, T., Kurisu, K., and Tashiro, H., J. craniofac. Genet. devl Biol. *7* (1987) 45.
14 Sulik, K. K., Cook, C. S., and Webster, W. S., Development *103* (Suppl.) (1988) 213.
15 Abbott, B. A., Hill, L. G., and Birnbaum, L. S., Teratology *41* (1990) 299.
16 New, D. A. T., Biol. Rev. *53* (1978) 81.
17 Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. biol. Chem. *193* (1951) 265.
18 Sokal, R. R., and Rohlf, F. J., in: Biometry, 2nd edn. Freeman, San Francisco 1981.
19 Kochhar, D. M., Penner, J. D., and Tellone, C. I., Teratogen. Carcinogen. Mutagen. *4* (1984) 377.
20 Creech Kraft, J., Löfberg, B., Chahoud, I., Bochert, G., and Nau, H., Toxic. appl. Pharmac. *100* (1989) 162.
21 Creech Kraft, J., Kochhar, D. M., Scott, W. J., and Nau, H., Toxic. appl. Pharmac. *87* (1987) 474.
22 Willhite, C. C., Hill, R. M., and Irving, D. W., J. craniofac. Genet. devl Biol., Suppl. 2 (1986) 193.
23 Johnston, M. C., Anat. Rec. *156* (1986) 142.
24 Noden, D. M., Devl Biol. *42* (1975) 106.
25 Pick, J. B., and Evans, C. A., Experientia *37* (1981) 1189.
26 Ruberte, E., Dolle, P., Krust, A., Zelent, A., Morriss-Kay, G., and Chambon, P., Development *108* (1990) 213.
27 Dencker, L., in: Advances in the Study of Birth Defects, vol. 1, p. 1. MTP Press, Lancaster 1979.
28 Chytil, F., and Ong, D. E., in: Advances in Nutritional Research, vol. 5, p. 13. Plenum, New York 1983.
29 Kwarta, R. F., Kimmel, C. A., Kimmel, G. L., and Slikker, W., Teratology *32* (1985) 103.

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Development and hormonal control of thioredoxin and the thioredoxin-reductase system in the rat liver during the perinatal period

J. Demarquoy, A. Fairand, R. Vaillant and C. Gautier

Laboratoire d'Endocrinologie, URA CNRS 650, Groupe Régulations Cellulaires et Métaboliques, Faculté des Sciences, B.P. 118, F-76 134 Mont Saint Aignan cedex (France)
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Summary. The development and hormonal regulation of thioredoxin and of the thioredoxin-reductase system were investigated during the perinatal period in rat liver. An immunological procedure was developed in order to quantify thioredoxin in fetal and neonatal hepatocytes. Both immunoreactive thioredoxin and thioredoxin-reductase activity appeared on day 16.5 of pregnancy. The level of immunoreactive thioredoxin increased during the late fetal period, and its level was the same 24 h after birth. Moreover, its development was not subjected to hormonal regulation by corticosteroids and glucagon. In contrast, thioredoxin-reductase activity increased 3 times during the late fetal period and presented a marked increase 24 h after birth. In the absence of glucocorticoids there was no increase in the level of thioredoxin reductase, while administration of hydrocortisone acetate and glucagon to fetuses prematurely evoked its activity. This study suggests that if thioredoxin acts physiologically, this activity is related to the state of reduction of the molecule rather than to the total concentration in the liver.
Key words. Thioredoxin; thioredoxin reductase; perinatal period; liver; pancreatic hormones; corticosteroid.

The late fetal period is marked in the liver by the appearance and the development of metabolic pathways, such as urea formation in ureotelic mammals. The development of two urea-cycle enzymes, mitochondrial carbamyl phosphate synthetase-I (CPS-I; EC 6.3.4.16) and cytosolic argininosuccinate synthetase (ASS; EC 6.3.4.5), and their hormonal control, have been described previously: in vivo corticosteroids stimulate the accumulation of immunoreactive CPS-I in fetal mitochondria in an inactive form which is further activated by the administration of glucagon [1]. Recently, it was demonstrated in our laboratory that reduced thioredoxin is able to activate CPS-I and ASS in vitro [2-4]. The presence of thioredoxin and of the thioredoxin-reductase system in adult rat liver was previously reported [for review, see Holmgren [5]]. Thioredoxin is a small ubiquitous protein (12 kDa) presenting two redox-active half-cystine residues. Oxidized thioredoxin is reduced in vivo in a reaction catalyzed by thioredoxin reductase (EC 1.6.4.5) with NADPH as substrate. Reduced thioredoxin is involved in the reduction of