

## Studies on Hypoxia.

### VII. Effect of Prenatal Anoxia on Leucine-<sup>3</sup>H Incorporation of Neonatal Rat Pancreas and Submandibular Gland<sup>1</sup> (35224)

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It was previously reported [Morawa and Han (6)] that exposure of pregnant Sprague-Dawley rats to an atmosphere of purified nitrogen at different periods of gestation produced a rather serious lag in the differentiation of epidermal and connective tissue cells in offspring from the exposed mothers. This lag was in addition to the skeletal anomalies and retardation of dental development which were most pronounced in animals subjected to hypoxia during early days of fetal life [Murakami *et al.* (7); Ingalls and Curley (3); Degenhardt (1); Morawa and Han (6)].

Inasmuch as growth and differentiation depend directly upon the normal production of enzymes and proteins in general, it was suspected that the lack of differentiation of epithelial and connective tissue cells might be due to the prolonged effect of hypoxia on the capacity for protein biosynthesis by these cells. The present study was designed to evaluate whether or not a single hypoxic insult during the fetal life would show a prolonged suppression of labeled amino acid incorporation into the developing submandibular gland and pancreas during the neonatal period.

**Materials and Methods.** *Animals and anoxic conditions.* In each experiment, 4 pregnant rats were subjected to total anoxia for a period of 20 min in a manner described previously [Morawa and Han (6)]. Following exposure to anoxia, the rats were allowed to give natural birth until day 22 of gestation. If the mothers did not give birth by 22 days of gestation, the rats were anesthetized with ether and the fetuses were removed. These

offsprings were regarded as being equal to those neonates which were delivered naturally. Neonatal rats were used within hours after delivery. For each experiment, a total of 12 neonates were used for radioautographic preparation. These consisted of 3 litter mates from the control animal, 3 litter mates each from the 3 experimental groups representing animals insulted on days 12, 15, and 18 of fetal life. Experiments were repeated three times.

**Radioautographic preparation.** Each of the neonatal rats was injected intraperitoneally with leucine-<sup>3</sup>H in the amount of 10  $\mu$ Ci/g of body weight. The specific activity of the labeled amino acid was 3.90 Ci/m-mole. One animal from each of the 4 groups was sacrificed either at 15, 60, or 120 min following the injection of leucine-<sup>3</sup>H. The submandibular gland and the pancreas were rapidly excised, fixed for 24 hr in 10% neutral formalin, and embedded in parlodion and paraplast in the standard manner. Sections 6  $\mu$  thick were made and collected on slides coated with a subbing solution consisting of 0.5% pure gelatin and 0.05% chromium potassium sulfate in distilled water.

Five sets of slides were coated with Kodak-NTB 3 nuclear track emulsion, as described elsewhere [Smith and Han (9)], and then exposed for varying periods. An exposure period of 8 days was optimal for grain counts and, therefore, was used in the quantitation of average grain numbers. Because of the poor differentiation of duct elements during the neonatal period, the grain counts were made only over definite acinar cells. The results of the grain count were processed for Student's *t* tests using an IBM 7090 computer program.

**Results.** Since the quantitative data are

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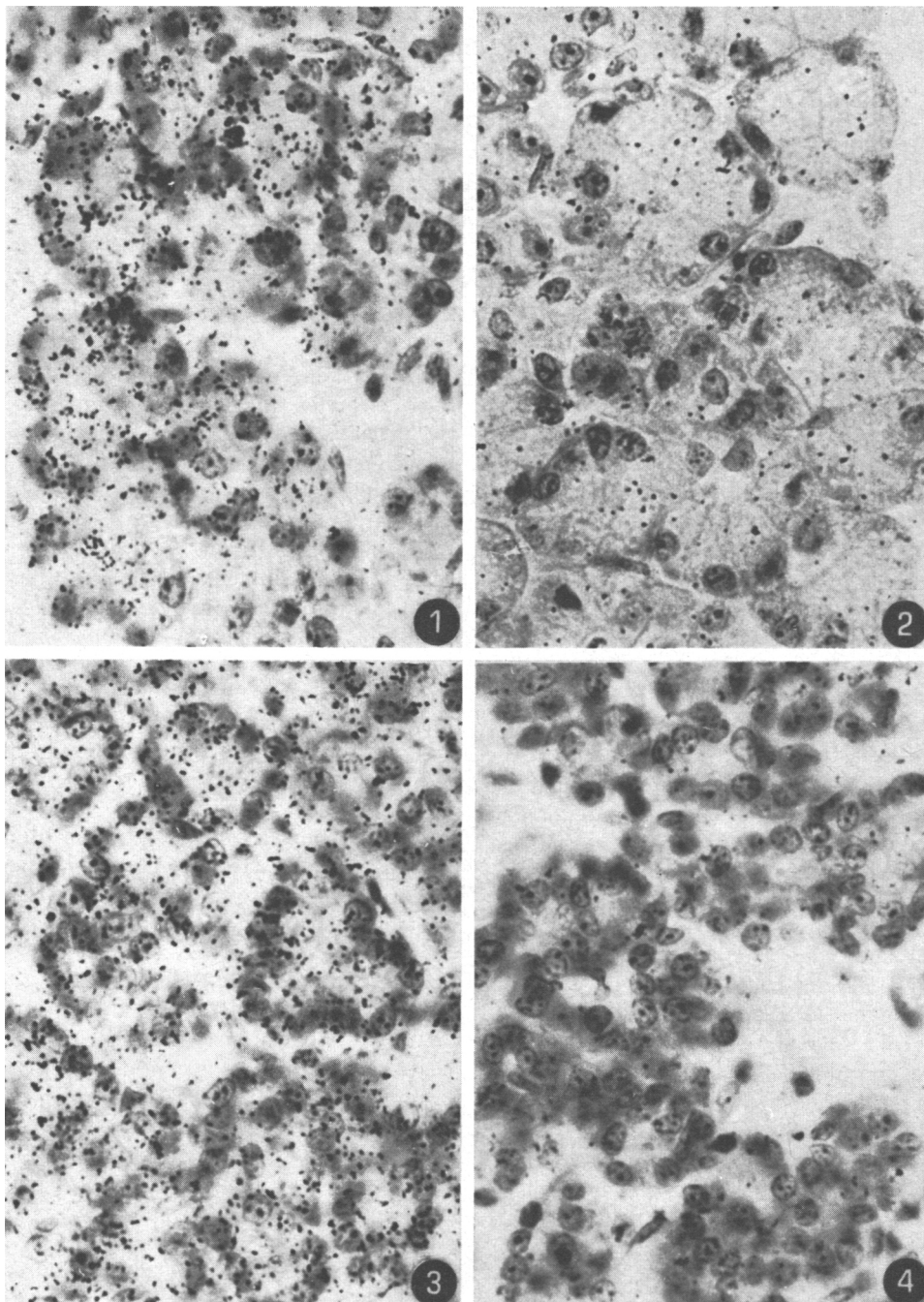


FIG. 1. Radioautographs of pancreas (Figs. 1 and 2) and submandibular gland (Figs. 3 and 4) of rat neonates. Pancreas of a control rat. Rats were sacrificed at 2 hr after the injection of leucine- $^3\text{H}$ . Pictures were focused at the levels of silver grains, and therefore the cells appear somewhat out of focus.  $\times 630$ .

FIG. 2. Pancreas of rat exposed to hypoxia on day 12 of gestation. The number of grains over individual cells is less than that of Fig. 1.

FIG. 3. Submandibular gland of a control rat.

FIG. 4. Submandibular gland of rat exposed to hypoxia on day 12 of gestation. A similar decrease in grain number is apparent.

TABLE I. Quantitative Radioautography of Leucine-<sup>3</sup>H Incorporation by Neonatal Rat Pancreas Following Anoxic Insult During Different Periods of Gestation.<sup>a</sup>

Time after <sup>3</sup> H injection (min)	Anoxic exposure (day)	Mean grain no. (SD)	% of control	Significance
15	Control	9.4 (± 2.0)	100	—
	12	6.2 (± 1.3)	66.4	<i>p</i> < 0.001
	15	4.9 (± 1.0)	52.5	<i>p</i> < 0.001
	18	7.2 (± 2.0)	76.8	<i>p</i> < 0.001
60	Control	11.6 (± 1.8)	100	—
	12	7.6 (± 1.6)	65.7	<i>p</i> < 0.001
	15	9.3 (± 1.9)	79.6	<i>p</i> < 0.001
	18	6.2 (± 1.1)	53.9	<i>p</i> < 0.001
120	Control	12.0 (± 1.3)	100	—
	12	6.1 (± 1.5)	50.7	<i>p</i> < 0.001
	15	8.0 (± 1.7)	66.6	<i>p</i> < 0.001
	18	7.3 (± 1.3)	60.9	<i>p</i> < 0.001

<sup>a</sup> Pregnant rats subjected to total anoxia for 20 min and immediately injected with 10 μCi/g of body wt of leucine-<sup>3</sup>H (sp act, 3.90 Ci/mmole), radioautographic exposure, 8 days.

recorded in Tables I and II, the illustrative figures of radioautographs are limited to the tissues of the neonates that had been anoxia-insulted on day 12 of gestation and were sacrificed 120 min after the injection of leucine-<sup>3</sup>H.

Figures 1 and 2 compare the pancreatic tissues of the neonates from control and experimental animals. It is clear that the number of grains in the experimental animals is notably less than that of the control pan-

creas. The submandibular gland shows a similar tendency (Figs. 3 and 4).

The results of grain counts from all of the experimental and control animals, given radioactive precursor at different times prior to sacrifice, are summarized in Tables I and II. Data for specific time periods are based on pooled counts obtained from all 3 experiments. Although a significant difference between the experimental and control animals is observed in rats injected with radioactive

TABLE II. Quantitative Radioautography of Leucine-<sup>3</sup>H Incorporation by Neonatal Rat Submandibular Gland Following Anoxic Insult During Different Periods of Gestation.<sup>a</sup>

Time after <sup>3</sup> H injection (min)	Anoxic exposure (day)	Mean grain no. (SD)	% of control	Significance
15	Control	4.3 (± 0.6)	100	—
	12	3.5 (± 0.8)	80.9	<i>p</i> < 0.02
	15	3.4 (± 0.7)	79.5	<i>p</i> < 0.01
	18	2.7 (± 0.5)	62.2	<i>p</i> < 0.001
60	Control	7.5 (± 0.8)	100	—
	12	2.5 (± 0.4)	33.0	<i>p</i> < 0.001
	15	4.6 (± 0.7)	61.4	<i>p</i> < 0.001
	18	6.4 (± 0.8)	71.7	<i>p</i> < 0.001
120	Control	8.9 (± 1.3)	100	—
	12	2.3 (± 0.5)	25.8	<i>p</i> < 0.001
	15	5.6 (± 0.6)	63.0	<i>p</i> < 0.001
	18	4.9 (± 1.0)	55.0	<i>p</i> < 0.001

<sup>a</sup> Pregnant rats subjected to total anoxia for 20 min and immediately injected with 10 μCi/g of body wt of leucine-<sup>3</sup>H (sp act, 3.90 Ci/mmole), radioautographic exposure, 8 days.

precursor 15 min before the sacrifice, the difference is most pronounced in animals sacrificed at 120 min. In animals of this group, the average grain number of the pancreas of experimental animals varies between 50% and 67% of the control value (Table I). In all cases the difference between the experimental and control pairs is significant at the level of  $p < 0.001$ .

Table II represents the quantitative data obtained by counting cells of the submandibular glands. The mean grain number in control animals is about 70% of that found for pancreatic acinar cells at 120 min when the mean grain number of the controls was the greatest. The results from the experimental animals are somewhat irregular in animals sacrificed at 15 min, although it is generally lower than the control group. In animals sacrificed at 60 and 120 min after the injection of the radioactive amino acid, the number of grains per cell is the lowest in fetuses insulted on day 12 of gestation, being 30% or less of the control value. Experimental animals insulted on days 15 and 18 of gestation have a mean grain number which is twice as high as those exposed to hypoxia on day 12 of gestation, although they were significantly lower when compared to the control. Excluding the animals that were sacrificed at 15 min, the level of significance between the control and the experimental animals is  $p < 0.001$ .

*Discussions.* The results described suggest the following three points: (i) As indicated by radioautographic quantitation following leucine- $^3\text{H}$  injection, the incorporation of labeled amino acid by the pancreas of normal rat neonates goes on at a greater rate than that of the submandibular gland. (ii) Of the two glandular tissues, the rate of incorporation by the submandibular gland is more profoundly affected despite the greater rate of leucine incorporation observed in the pancreas. (iii) In both organs there was a protracted and significant decrease of leucine- $^3\text{H}$  incorporation in all experimental animals insulted at different gestation periods.

Several previous workers have reported that, following exposure to anoxia, the synthesis of proteins was variously suppressed in adult organisms [Sanders *et al.* (8); Turner

and Turner (10)]. Earlier work from our laboratory has demonstrated that similar suppressive effects were found in neonatal animals following acute anoxic exposure during the first day of life [Smith and Han (9); Kim and Han (4, 5)]. Such effects were evident in glandular cells as well as among various types of connective tissue cells.

Insofar as the prenatal effects of anoxia are concerned, past studies have dealt primarily with teratological aspects observable in neonates born of hypoxia-treated mother [Degehenhardt (1); Murakami *et al.* (7); Ingalls and Curley (3); Morawa and Han (6)], or in chickens rendered hypoxic prior to hatching [Grabowski (2)]. These studies have clearly shown that a prenatal residence in a hypoxic environment could produce profound malformations in skeletal morphology. These findings have been suggested to be the result of the accumulation of lactic acid (2) and for possible derangement of sequential evolution of enzymes and other proteins at crucial points in differentiation [Smith and Han (9)]. These statements support the earlier observations of Murakami *et al.* (7) and Ingalls and Curley (3) who pointed out that the malformations were most serious in those animals who experienced hypoxia during differentiation of the body somites.

Except for the histological observations by Morawa and Han (6), which suggested the possibility of a sustained impairment of protein synthesis in epidermis, few have reported any continued effects of prenatal exposure to anoxia on protein synthesis. Whether the protracted suppression of leucine- $^3\text{H}$  incorporation observed in this study is due to a prolonged effect on the rate of transcription and translation of secretory products, or due simply to a retardation in the degree of differentiation cannot be determined as yet, and awaits for future experimentation.

*Summary.* A radioautographic study has been made of the effect of prenatal exposure of rats to anoxia on leucine- $^3\text{H}$  incorporation by the pancreas and submandibular gland. Pregnant rats were subjected to anoxia on day 12, 15, or 18 of gestation period. On delivery the offsprings were given an injection of leucine- $^3\text{H}$  and sacrificed at 15, 60, or 120 min later. Pieces of the pancreas and

submandibular gland were double-embedded, sectioned, and prepared for radioautography in a routine manner. Silver grains were counted over exocrine cells and compared with grain numbers obtained from appropriate control. Results indicate the following: (i) The incorporation of leucine-<sup>3</sup>H in control rats goes on at a greater rate in the pancreas than in the submandibular gland. (ii) The submandibular gland is the more profoundly affected of the two, despite the higher rate of leucine-<sup>3</sup>H incorporation in the pancreas. (iii) In both organs there is a protracted suppression of leucine-<sup>3</sup>H incorporation which supports previous histologic data.

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