Studies on Hypoxia. III. Effects on Leucine-^{3}H Incorporation by Submandibular Gland Cells of Rat Neonates* (33582)

JOON H. KIM AND SEONG S. HAN

Laboratory of Cell Biology, Dental Research Institute, and Department of Anatomy, The University of Michigan, Ann Arbor, Michigan 48104

Despite the numerous metabolic, morphological, and physiological studies that indicate serious effects of hypoxia on various developing organs, few have reported the acute effect of anoxia on rapidly developing glandular cells during the early neonatal period. However, it has been shown that cells of digestive glands in adult animals incorporate a reduced amount of radioactive amino acids following the exposure to hypoxia (1).

In a previous study we reported that the secretion of connective tissue matrices of hamster neonates was decreased after an acute hypoxic stress, as expressed by the incorporation of proline-^{3}H.(2). The present study was undertaken in order to test if the incorporation of a universal amino acid, leucine-^{3}H, was modified in cells of developing submandibular glands as the result of an acute anoxic insult.

Materials and Methods. Twelve new-born Sprague-Dawley rats from an inbred colony were subjected to "total anoxia" for a period of 20 min by placing them in a bell jar flushed with a continued flow of purified N_2. The oxygen content in the jar, judged by use of a Westinghouse oxygen analyzer, was 30 to 40 ppm. Immediately following the exposure to anoxia the rats were injected with 3 μC/g of body weight of leucine-^{3}H. The specific activity of this preparation was 5 Ci/mmole. The same number of control animals were handled in an identical manner, except that during the period within the bell jar, an adequate supply of fresh air was made available for normal respiration.

Animals were sacrificed in pairs at 15 min, and at 1, 4, 24, 48, and 72 hr after the injection. At the time of sacrifice the animals were decapitated, and following the removal of brain tissues from the calvarium, the head was sectioned through a midsagittal plane and fixed in Bouin's solution for 48 hr. The fixed tissues were double-embedded in parlodion and paraplast. The tissue blocks were oriented to allow the sections to go through the developing glands in parasagittal planes. Serial sections were made at 6 μ and mounted on 1 $\times$ 3-in. slides that had previously been cleaned and treated with a subbing solution, consisting of 0.5% pure gelatin containing 0.05% of chromium potassium sulfate in distilled water.

Slides having comparable histological areas were selected microscopically and coated with Kodak NTB-3 liquid emulsion in complete darkness. After drying, the slides were placed in small plastic slide boxes that contained packets of desiccant. The boxes were wrapped with two layers of lead sheet and an additional layer of light-tight paper. They were sealed in a plastic bag in which desiccant packets were placed, and were left in a refrigerator for a period of 5 or 8 weeks for exposure. At the end of the exposure period the slides were developed in complete darkness in 2, 4-diaminophenol dihydrochloride developer for 1 min. After fixation, the slides were stained in Harris hematoxylin and alcoholic eosin Y. From each slide, grain counts were made over an average of 70 or more cells and expressed in terms of the number of grains per cell for comparison. The t tests and analyses of variance were made with a computer program in IBM 7090.

Results and Observations. Figure 1 illustrates the data from quantitation of the radioautographic slides that were exposed for 5 weeks; it shows that in control animals there

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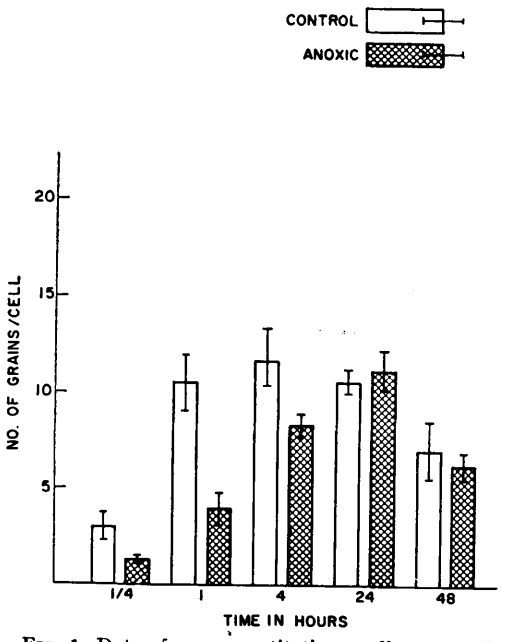

FIG. 1. Data from quantitative radioautography showing the early suppression of leucine-^{3}H incorporation by submandibular gland cells of rat neonates subjected to anoxia. The range indicates standard deviation; difference is significant ($p<0.001$) during the first 4 hr.

is a gradual increase in the average number of grains per acinar cell in the first 4 hr, whereas the average grain number is gradually reduced thereafter. Thus, the average grain number reached a peak of 11.62 at 4 hr from 2.66 and 10.46 at 15 min and 1 hr, respectively. In contrast, the average grain number of anoxia-treated animals in the early period was much less than that of the controls, showing only 1.35/cell at 15 min, 3.99 at 1 hr, and 8.26 at 4 hr. The level of significance at these 3 periods was $p<0.001$. The grain number in anoxia-treated animals increased to a level similar to that of the controls by 24 hr and thereafter. The slight difference observed during the late periods was insignificant.

While the geographic problems inherent in the light microscopic radioautography of paraffin sections do not allow an accurate histologic representation of the quantitative data gained through grain counts, photomicrographs in Fig. 2 illustrate clearly the differences observed between control and anoxia-treated animals during the first hour. It may be noted that at 15 min after the injection there are several grains per cell in the control (Fig. 2a) as opposed to the experimental gland in which none or only a few grains were localized in each cell (Fig. 2b). By 1 hr the amount of grains superimposing upon the cells in the control became so great that details of the cell structure were hard to resolve on photomicrographs (Fig. 2c). However, it might be noted that the grains were more concentrated in the apical portion of the cytoplasm. On the other hand, in anoxia-treated animals only a few to several grains were localized in a cell (Fig. 2d). A similar tendency was observed in sections from animals sacrificed 4 hr after the injection of the radioactive amino acid. Since there were no meaningful differences after 24 hr, as indicated in Fig. 1, no actual radioautographs are presented. Histologically, occasional mitotic figures were observed throughout the series, both in experimental and control animals.

Discussion. The incorporation of a universal amino acid, such as leucine-^{3}H, into the salivary glands of neonatal animals may reflect either or both of the following phenomena, namely, the synthesis of secretory proteins, and the elaboration of cytoplasmic proteins needed for growth and division. Thus, the changes in the average grain number of control animals during the first several hours might be regarded as an expression of the accumulation of newly synthesized cytoplasmic proteins for growth, or an accumulation of secretory proteins, or both since these neonatal animals have attained a minimum level of functioning of digestive glands. The gradual reduction of the grain number at later periods could reflect the reduction in the level of available precursors, the rapid attainment of secretory capabilities by the acinar cells, or the dilution of cytoplasmic grains by progressive divisions, or both.

On the other hand, the definite and rapidly occurring discrepancy of the average grain number between anoxia-treated and control rats during the early hours after the insult should indicate one or a combination of the

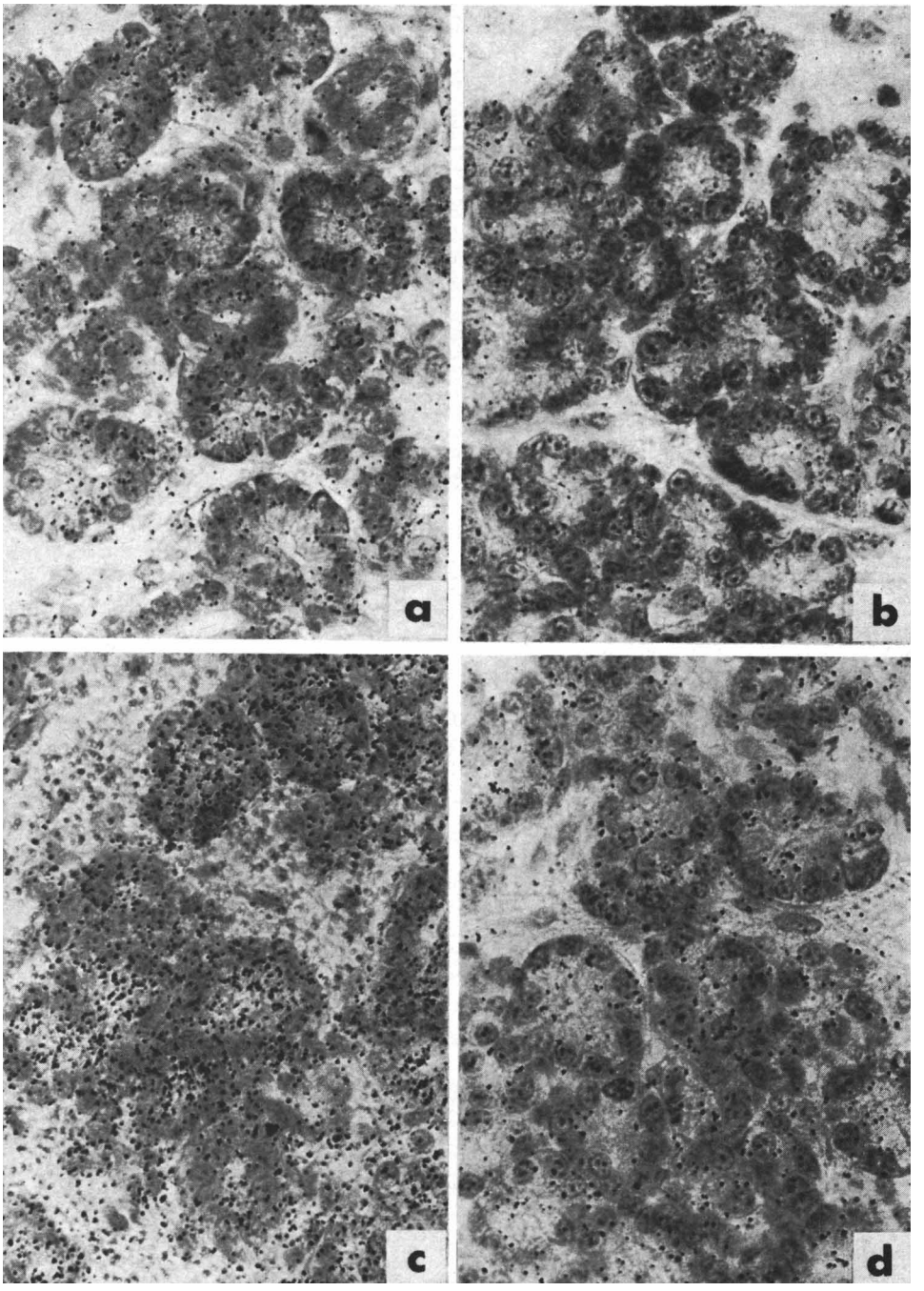

FIG. 2a and b. Radioautographs of submandibular glands from control (a) and anoxia-treated (b) rat neonates injected with leucine-^{3}H 5 min prior to sacrifice. A greater number of grains is observed over cells from the control gland. (c and d). Radioautographs of submandibular glands from control (c) and anoxia-treated (d) rat neonates injected with leucine-^{3}H 60 min prior to sacrifice. The discrepancy between the experimental and control animals is clear.

following possibilities: (i) The actual reduction of the synthesis of secretory or cytoplasmic proteins; (ii) the increase in the rate of secretion; or (iii) the increase in the rate of division leading to a speedy dilution of the radioautographic grains. Although there is no direct evidence to negate the last two possibilities, it is tempting to conclude tentatively that the early discrepancy in grain numbers might be the direct result of suppression of protein synthesis, either for secretory or cytoplasmic growth purposes, as there is no evidence that hypoxia serves to stimulate the secretory functions or divisions of cells.

In this connection we have observed that the synthesis of *secretory* proteins as expressed by the incorporation of proline-^{3}H into various types of connective tissue cells in neonatal animals is not suppressed during the first 6 hr after the insult, but was significantly suppressed by 24 hr after the insult (2). This late effect was thought to reflect the damage done to such cytoplasmic machinery needed for protein secretion as the endoplasmic reticulum, Golgi apparatus, etc., the lack of which would reduce the formation of secretory proteins even after the recovery from an immediate suppression of overall protein synthesis. If this is the case, the results from this study showing an earlier suppression of leucine-^{3}H incorporation might be related not so much to the secretory function of these developing glands, but rather to the suppression of overall protein syntheses. The lack of difference at 24 hr and thereafter suggests that the synthesis of proteins might have recovered within 24 hr.

Other studies of the effects of the hypoxic stress on protein synthesis have shown varying results. Based on radioautographic evidence Lubière and others (3) reported that different organs have varying sensitivity toward the hypoxic insult, while Sanders *et al.* (4), and Turner and Turner (1) both reported hypoxia-induced suppression of protein synthesis in the liver and pancreatic cells, respectively. Morphological studies of the immediate effect of hypoxia by use of the electron microscope have also revealed various types of detrimental changes, such as a swelling of mitochondria (5–7) or an increase in lysosomes (6, 8, 9). All of these authors have dealt with adult animals.

Whether the suppression of leucine-^{3}H incorporation observed in this study is due to a direct effect of anoxia on the cytoplasmic protein synthesis, or due to an indirect effect mediated through the suppression of ATP production, or both remains to be seen. The fact that the tissues in perinatal periods are in the process of changing from a relatively anaerobic state to a more complete aerobiosis tempts one to speculate on the possibility of a direct and relatively immediate effect on the protein assembly machines, i.e. polyribosomes, etc. Attempts are being made in our laboratory to answer this question through correlated biochemical and electron microscopic studies.

Summary. Effect of anoxia on leucine-^{3}H incorporation by submandibular gland cells of neonatal rats has been studied by means of quantitative radioautography. The results show that there is a marked suppression of leucine-^{3}H incorporation during the first 4 hr following the anoxia administration. There is an apparent recovery by 24 hr, and no difference between the experimental and control animals is observed after this time. It is concluded that the synthesis of overall cytoplasmic proteins is temporarily impaired after a brief exposure to anoxia in submandibular gland cells of neonatal rats.

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