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STUDIES ON HYPOXIA—I. GROSS AND HISTOLOGIC INFLUENCES OF MATERNAL ANOXIA UPON THE DEVELOPING RAT FOETUS

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Summary—As the first communication of a series to be reported, this article represents the result of a survey work which was designed to serve as the basis for further experimentation on the subject. Effects of maternal anoxia upon the developing rat foetus were studied by subjecting pregnant Sprague-Dawley rats to an atmosphere of 100 per cent nitrogen for a total of 18 min on the 10th, 14th, 17th or 20th day of gestation and their offspring were then evaluated morphologically, radiographically, and histologically. All defects of skeletal morphology were found in newborns that were treated on the 10th day of gestation. The animals treated on the 20th day of gestation exhibited a consistent retardation in eruption of the molars. Histologically, a serious lag in the differentiation of epidermal and connective tissue cells was observed in animals subjected to anoxia on the 10th day, while an engorgement of the blood vessels was apparent in animals which were subjected to anoxia on the 20th day. These changes are discussed in relation to possible biochemical and physiologic effects of anoxia on these cells.

INTRODUCTION

RECENT morphologic investigations of anoxia have dealt primarily with gross changes of morphology which have been induced during development (DEGENHARDT, 1960; and INGALLS and CURLEY, 1957) and with efforts to determine which of the primordial germ layers were most susceptible to anoxic insults (MURAKAMI, KAMEYAMA and KATO, 1956; and VIA, ELWOOD and BEBIN, 1959). Thus, INGALLS and CURLEY (1957) concluded that anoxia gives rise to many different manifestations, depending not only upon when it acts during pregnancy, but also upon where it acts on the foetus.

DEGENHARDT (1960) induced cranio-facial dysplasias in rabbits by using oxygen deficiency and reported extreme changes of hard tissues and the pulp of premolars when examined through polarized light. MURAKAMI *et al.* (1956) subjected mice to acute hypoxic conditions during pregnancy to determine which tissues were affected. Commonly manifested abnormalities were ectodermal in origin. Proliferation of the ependymal layers was observed in the abnormal tissues of the central nervous system. The dental tissues were retarded in development and hypoplastic dentitions were common. VIA *et al* (1959) investigated the effects of maternal anoxia upon the developing eyes and dentition and also reported a high percentage of ectodermal defects of dental and neural tissues.

This paper aims to record the results of a survey experiment on the effect of maternal anoxia upon the gross and microscopic appearance of the newborn rats, and thereby to serve as a basis for future communications which will demonstrate various effects of anoxia on cell structure and metabolism in different tissues subjected to similar conditions.

MATERIALS AND METHOD

A total of 28 Sprague-Dawley rats (*Rattus norvegicus*) were used throughout this study. All animals were maintained on Purina lab. chow and given tap water *ad libitum*.

Experimental

Anoxic environment. This was produced in a bell jar by replacing the air with a continuous flow of N_2 (High Purity Nitrogen, General Dynamics). The pregnant rats were exposed to the completely anoxic environment for a total of 18 min, in two periods of 12 min and 6 min respectively. The two insults were separated by a 30 min recovery period.

Experiment 1. *Radiography and microscopy.* Sixteen pregnant rats were divided into groups of 4 animals and received hypoxic insults as in Table 1. Three animals in

TABLE 1. DESIGN OF EXPERIMENT 1

Group	Time of insult	No. of rats insulted	No. of control rats
1	10th day of gestation	3	1
2	14th day of gestation	3	1
3	17th day of gestation	3	1
4	20th day of gestation	3	1

each group were used for experimental purposes and the fourth animal was used as a control. The control animals were pair-fed against the experimental animals, beginning on the day of anoxic treatment. This was achieved by giving the control animal the average of the food intake of the 3 experimental rats in its group. On the 21st day of gestation, the pregnant rats were anaesthetized with ether, sacrificed, and the foetuses removed. Only the foetuses which took an initial breath were included in this work. The foetuses were weighed and studied radiographically and histologically.

Radiography and histologic preparation. Twenty-four hours after fixation in 10 per cent formalin, each foetus was radiographed in both lateral and ventro-dorsal positions. The exposure was made at a source-to-film distance of 4 ft under a Picker Corporation X-ray machine. Kodak type M industrial X-ray film was used for all radiographs.

The entire foetus was fixed in 10 per cent formalin for 48 hr. The maxillae were separated from the skull, decalcified in 5·5 per cent Versene in 10 per cent formalin for

72 hr, washed in running water for 4 hr, dehydrated, and then double-embedded in parlodion and paraplast. After embedding, the maxillae were sectioned longitudinally

TABLE 2. DESIGN OF EXPERIMENT 2

Group	Time of insult	No. of pregnant rats
A	10th day of gestation	6
B	14th day of gestation	1
C	17th day of gestation	1
D	20th day of gestation	1
Control	—	3

at 7–8 μ, fixed to slides with albumin–glycerine adhesive, and alternate serial slides were stained with haematoxylin and eosin and Masson's trichrome.

Experiment 2. *Gross morphology and eruption of the dentition.* Twelve pregnant rats were divided into groups as indicated in Table 2. These animals were allowed to deliver normally. Three animals from each litter were sacrificed 18 days postnatally and 3 were sacrificed 25 days postnatally. The mandibles were separated from the skull, placed in 2 per cent KOH for 72 hr to remove soft tissues, washed in running water, dried for 48 hr, and photographed for study of the calcification and eruption of the dentition.

RESULTS

Gross radiographic and morphologic observations

The weight and mortality of foetuses from Experiment 1 is recorded in Table 3. Foetuses subjected to anoxia after the 10th day of gestation did not exhibit severe

TABLE 3. WEIGHT AND MORTALITY OF FOETUSES

Group	Total No. of foetuses	No. of stillborn foetuses	Percentage of stillborn foetuses	Average weight of live foetuses (range g)
1	52	10	19	(2·76–4·19)
2	39	9	23	(3·54–4·59)
3	48	20	42	(4·37–5·65)
4	33	3	9	(2·88–4·46)
Control	45	1	2	(3·88–4·34)

alterations of the skeletal morphology. In Group 2, insulted in the 14th day of gestation, only a cleft palate was found. No defects were visible in any of the other animals. All animals which received the anoxic insult on the 17th or the 20th days of gestation were grossly and radiographically comparable to the control animals. It might be

pointed out, however, that the percentage of stillborn foetuses was the highest in Group 3, insulted on day 17 of pregnancy.

The developing foetuses which received the insult on the 10th day of gestation (Group 1) displayed craniofacial dysplasias, spinal scoliosis, and a lack of or malformed calvarium (Fig. 1–4). Two palatal clefts also were observed. Thirty per cent of the animals in this group revealed some type of morphologic defect.

Macroscopic observation of the dentition

The molars of all animals appeared normal, regardless of the time of hypoxic treatment. However, there was an apparent retardation in eruption time of the molars when the state of eruption was studied on the 18th and 25th days after birth (Table 4).

TABLE 4. STATE OF ERUPTION OF MOLARS

Days postnatal	Group A	B	C	D	Control
18	+	+	—	++	—
25		—	—	+	—

— Normal.
\+ Mild retardation.
++ Moderate retardation.

Only one of the 43 foetuses in Group A, treated on the 10th day of gestation, survived 18 days postnatally. This animal displayed a mild retardation of eruption when compared with the control animals. The animals in Group B which received a hypoxic treatment on the 14th day of gestation also displayed a slightly retarded eruption 18 days postnatally. By the 25th day, there appeared to be a total recovery and the dentition was comparable to that of the control animals. The state of the dentition of the Group C animals did not differ from that of the control group.

The foetuses in Group D which were insulted on the 20th day of gestation appeared most sensitive to hypoxic insult (Fig. 5 and 6). The eruption of the molars was retarded in the experimental rats evaluated both 18 and 25 days postnatally.

Histologic observation

Histologically, the tissues which were treated during the early phases of their development displayed the most dramatic changes.

Integument. A comparison of longitudinal sections through the developing maxillary process, lateral to the incisors, revealed striking differences between the control (Fig. 7 and 11) and the Group 1 animals which were insulted on the 10th day of gestation. The epithelium of treated rats was very delicate, and had a thin layer of keratin, ill-defined granulosum and spinosum layers and a poorly organized basal layer (Fig. 8 and 12). The subcutaneous tissue contained numerous fibroblasts with hyperchromatic nuclei and poorly defined cytoplasm (Fig. 12). The experimental animals

in this group displayed a reduction in the number and size of hair shafts (Fig. 7 and 8) and a reduction in the number of vascular elements.

The integument of the animals in Group 2, insulted on the 14th day of gestation, exhibited the following subtle changes: the epithelium was slightly thinner and contained layers which were not distinct (Fig. 9 and 13); and nuclei of some of the underlying connective tissue cells were hyperchromatic.

The integument of the foetuses in Group 3 appeared similar to that of the control animals. On the other hand, hyperaemia of the subcutis consistently occurred in Group 4, subjected to anoxia on day 20 of gestation, and might have represented an early effect of O_2 deficiency (Fig. 10). However, the appearance of epithelial cells in this group was nearly normal, although many cells in the spinosum layer had somewhat swollen nuclei (Fig. 14).

Dental organs and incisors. The development of the dental organ appeared morphologically similar in the control and the animals which received the anoxia treatment on the 14th, 17th, and 20th days of gestation, hence no illustration is included here. The dental organ had four well developed layers, i.e. the outer dental epithelium, stellate reticulum, stratum intermedium and the inner dental epithelium. The basement membrane which separates the epithelial dental organ and the dental papilla (membrana preformativa) was very prominent. The only difference was a frequent hyperemia in the subodontoblastic region of the pulp as well as in the periodental connective tissues in animals subjected to anoxia on day 20 of prenatal life.

DISCUSSION

The histologic changes observed in the integument after the anoxic stress may be taken as significant evidence that serious biochemical alterations have occurred in the tissues. The primary biochemical lesion caused by anoxia may very well be related to the respiratory apparatus of cells, as suggested by previous studies (GRABOWSKI, 1964). However, the lack of differentiation of epidermis and the diminution of basophilia of fibroblasts indicate that the capacity for protein biosynthesis might be affected subsequently. Whether or not such an effect is entirely secondary to disturbances in cellular respiration is being tested by a series of short term experiments, as the point could not be clarified by a developmental study such as this.

Earlier experiments on the metabolic effects of anoxia have been concerned mostly with metabolism of glycogen, certain hormones and other metabolites (BONAVITA, GUARNER and SCARDI, 1964; CORNBLATH *et al.*, 1963; JACOB and BERNE, 1961; LOTSPEICH and WHEELER, 1962; MARKS, BHATTACHARYA and VERNIKOS-DANELLIS, 1965; PRITCHARD, HUSTON and MARTIN, 1963; ROUX, HAGERMAN and VILLEE, 1962; STAVE, 1965; THORN, 1961; TIISALA, 1962; and VILLEE *et al.*, 1958). With respect to the influence of hypoxia on protein synthesis, only a few reports are available. SANDERS, HALE and MILLER (1965) found a decrease in ATP concentration in the hypoxia-treated liver and brain accompanied by a decreased rate of incorporation of radioactive leucine into proteins of these tissues. On the other hand, TURNER and TURNER (1965), in

a cell free system of fractionated pancreatic proteins, reported a step-wise depression of amino acid incorporation into the protein fractions, when these fractions were taken from animals subjected to similar changes in environmental oxygen tensions. Our recent experiments, involving a radioautographic observation of H^3-proline incorporation by certain connective tissue cells subjected to anoxia, indicated that collagen synthesis is drastically suppressed following an anoxic stress (SMITH and HAN, 1968).

Insofar as the radiographic findings are concerned, the result of this study largely confirms those from previous studies (DEGENHARDT, 1960; INGALLS and CURLEY, 1957; and MURAKAMI *et al.*, 1956), except that no clear-cut dental anomalies in terms of morphology were found. This might be related to the fact that the primordia of dental organs of the rat molars are well-developed by the 10th day. Thus it is possible that the teratogenic effect of anoxia on dentition is minimal, and that the only consistent effect is a retardation of eruption.

It is of interest that a notable variation in skeletal morphology existed even among the litter-mates. This was particularly obvious in the animals treated on the 10th day of gestation; approximately 30 per cent of these animals displayed gross defects of a wide variety, while the rest appeared comparable to the control group. A combination of two factors may be considered to account for these differences. First, since only moderate developmental differences would occur among litter-mates at a given time during gestation, animals in specific periods of differentiation could be highly susceptible to insult, while those in closely related periods of differentiation might be relatively refractile to anoxia. Second, some developing animals may be inherently more resistant to reduced oxygen tension than others, although this is less likely to be the case.

The early engorgement of blood vessels observed in the subcutis and dental structures of animals insulted on day 20 of gestation suggests the degree of damage to circulatory functions in these regions, a point that has been indicated by previous works of physiologists (BRITTON and KLINE, 1945; ERNSTING, 1963; GORDON and KATZ, 1962; KAHLER, GOLDBLATT and BRAUNWALD, 1962; and MURRAY and YOUNG, 1963).

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Résumé—Le présent travail, première communication d'une série à être publiée, constitue un travail d'ensemble destiné à servir de base pour une expérimentation ultérieure. Les effets de l'anoxie maternelle sur le foetus de rat ont été étudiés en soumettant des rattes Sprague–Dawley à une atmosphère de 100 pour cent d'azote pendant 18 min, au 10ème, 14ème, 17ème, et 20ème jour de la gestation. Les diverses portées ont été étudiées morphologiquement, radiographiquement et histologiquement. Toutes les malformations squelettiques ont été observées chez les rats soumis à l'anoxie au 10ème jour de la gestation. Les animaux, traités au 20ème jour de la gestation, présentent des retards dans l'éruption des molaires. Un sérieux retard de différenciation des cellules épithéliales et conjonctives est noté histologiquement chez les rats, soumis à l'anoxie au 10ème jour, alors qu'une congestion vasculaire est visible chez les animaux soumis à l'anoxie

au 20ème jour. Les résultats obtenus sont discutés en fonction des effets biochimiques et physiologiques de l'anoxie sur ces cellules.

Zusammenfassung—Als erste Veröffentlichung einer Untersuchungsreihe soll dieser Artikel eine Grundlage für weitere Versuche bilden. Es wurden die Wirkungen der Anoxie auf den sich entwickelnden Rattenfoetus untersucht, indem Spraque–Dawley–Ratten am 10., 14., 17. oder 20. Tag der Trächtigkeit insgesamt 18 min lang einer Atmosphäre mit 100 Prozent Stickstoff ausgesetzt wurden. Die Jungtiere wurden danach morphologisch, radiographisch und histologisch untersucht. Bei Jungtieren, die am 10. Trächtigkeitstag behandelt worden waren, wurden alle Arten von Skelettdefekten gefunden. Die am 20. Trächtigkeitstag behandelten Tiere zeigten eine deutliche Verzögerung des Molarendurchbruchs. Histologisch wurde eine beträchtliche Verzögerung der Differenzierung von Epidermisund Bindegewebszellen bei den Tieren beobachtet, die am 10. Tag der Anoxie unterworfen worden waren. Bei den am 20. Tag durch Anoxie behandelten Tieren trat dagegen eine massive Füllung der Blutgefäße in den Vordergrund. Diese Veränderungen werden in Beziehung zu möglichen biochemischen und physiologischen Wirkungen der Anoxie auf diese Zellen diskutiert.

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PLATE 1

FIGS. 1 and 2. Radiographs of a control animal. The cranio-facial complex and the spine are well-developed.

FIGS. 3 and 4. Radiographs of an animal which was treated on the 10th day of gestation. This animal exhibits spinal scoliosis and a cranio-facial dysplasia.

FIG. 5. An enlargement of the dentition from the control rat which was sacrificed 25 days postnatally. The 1st and 2nd molars are fully erupted. Three cusps of the 3rd molar are visible. The scale shown in the background is in mm.

FIG. 6. The mandibular dentition of an animal which was insulted on the 20th day of gestation and sacrificed 25 days postnatally. Only two mesial cusps of the 3rd molar are barely visible. The scale shown in the background is in mm.

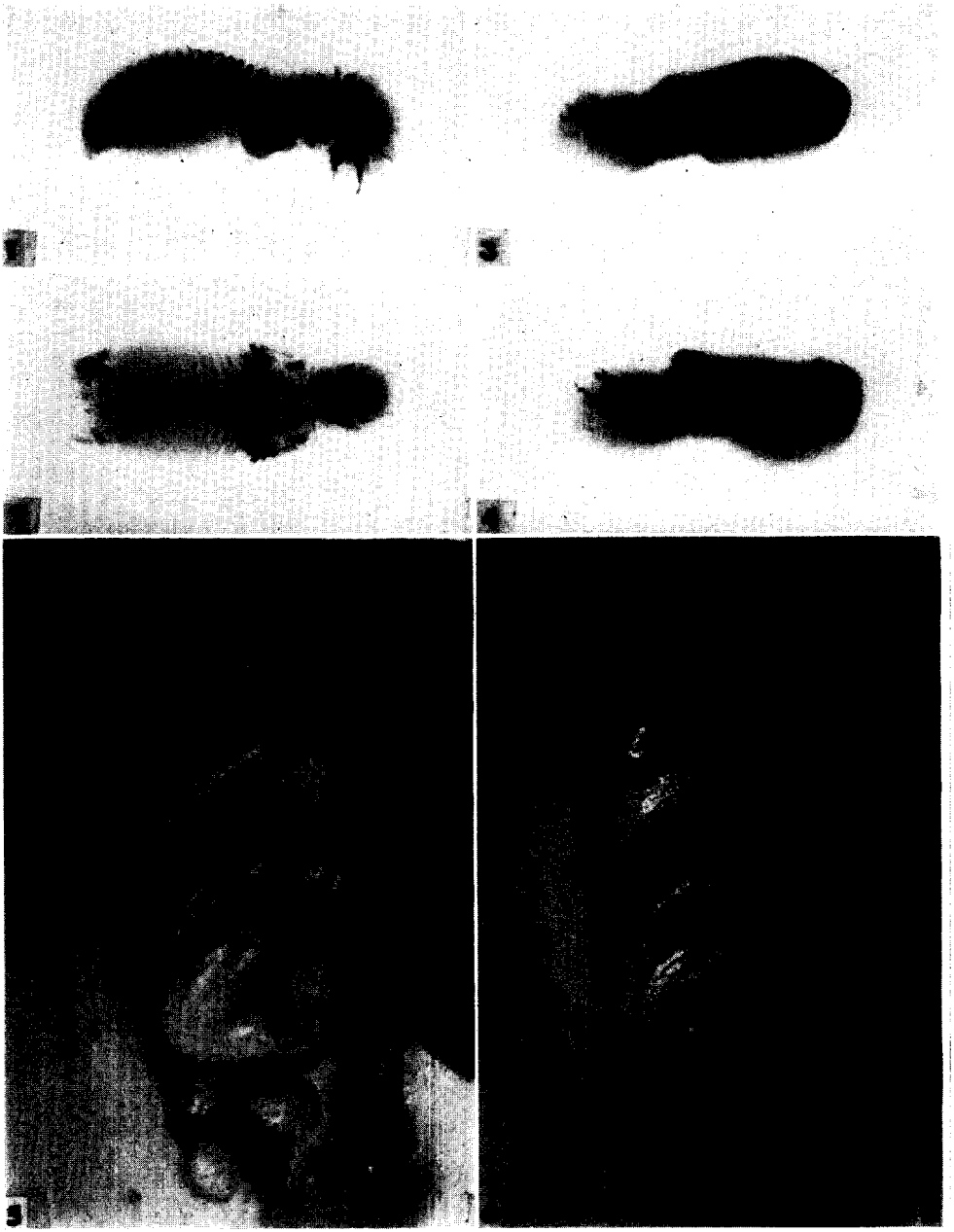

PLATE 1

A.O.B. f.p. 752

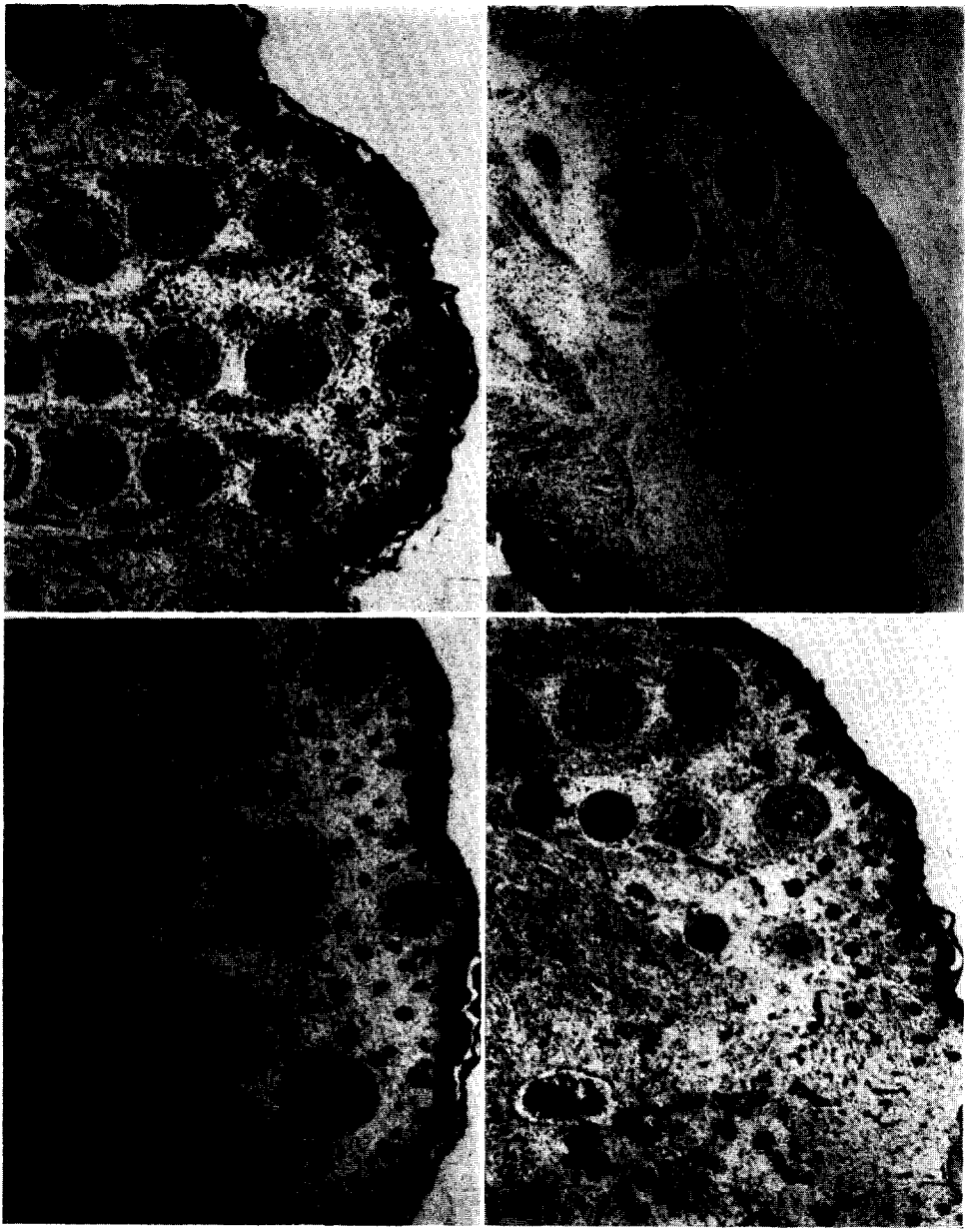

PLATE 2

PLATE 2

FIG. 7. A portion of the maxilla from a control animal. The hair shafts are numerous and well-developed. Masson's trichrome. × 40.

FIG. 8. A portion of the maxilla comparable to that appearing on Fig. 7, but taken from an animal which was insulted on the 10th day of gestation. The hair shafts are poorly differentiated and reduced in number. A distinct reduction in the vascular elements and muscle fibres is evident. Masson's trichrome. × 40.

FIG. 9. A portion of the maxilla comparable to that appearing on Fig. 7, but taken from an animal which was insulted on the 14th day of gestation. The hair shafts appear well-developed and are slightly larger than those of the control animals. Masson's trichrome. × 40.

FIG. 10. A portion of the maxilla from an animal which was insulted on the 20th day of gestation. This photograph was taken from a region slightly away from the snout which had well developed hair shafts and follicles. The extensive engorgement of blood vessels is visible in the open connective tissue space. Masson's trichrome. × 40.

PLATE 3

FIG. 11. The integument from a control animal. The epithelium has distinct layers with well-organized basal cells. Connective tissue elements are well-developed. Haematoxylin and eosin. × 630.

FIG. 12. The integument from an animal which was insulted on the 10th day of gestation. The epithelium has little keratin, poorly defined layers, and rounded cells in the spinosum layer. Nuclei of basal cells are irregular in shape and hypochromatic. Nuclei of the connective tissue cells are rounded and hyperchromatic. Very little cytoplasm is visible in these connective tissue cells. Haematoxylin and eosin. × 630.

FIG. 13. The integument from an animal which was insulted on the 14th day of gestation. The epithelium is relatively similar to the control animal, with moderate keratinization and a well-defined basal layer. Cells of the stratum spinosum have ill-defined shapes and the nuclei contain large aggregates of densely stained materials. Fibroblasts of the connective tissue are fairly well defined. Haematoxylin and eosin. × 630.

FIG. 14. The integument from an animal which was insulted on the 17th day of gestation. The epithelium and connective tissue cells are comparable to those of the control. Haematoxylin and eosin. × 630.

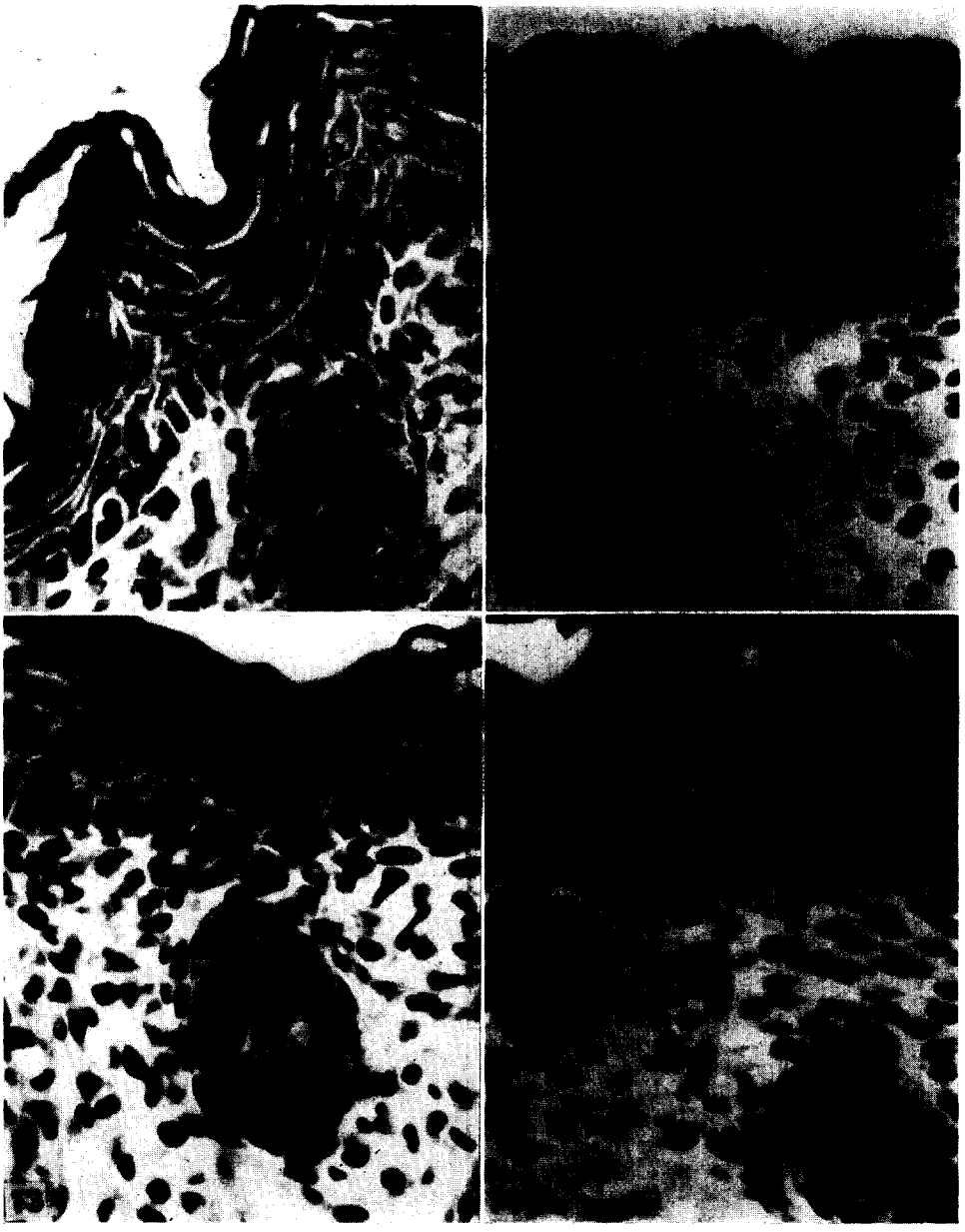

PLATE 3