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HISTOCHEMICAL DEMONSTRATIONS OF ACTINOMYCIN-INDUCED CHANGES OF CERTAIN OXIDATIVE AND HYDROLYTIC ENZYMES OF RAT INCISOR PULPS

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Summary—Levels of succinic and lactic dehydrogenases, cytochrome oxidase, alkaline and acid phosphatase activities of the rat incisor pulp were examined following a sublethal dose injection of actinomycin D. Control animals injected with physiological saline were pair-fed.

Succinic dehydrogenase and cytochrome oxidase activities were low in the pulp and presented insignificant alterations throughout the experiment. Lactic dehydrogenase and alkaline phosphatase activities of the dental pulp dropped rapidly during the first several days of the experiment and returned to the normal level by the end of the third week. Acid phosphatase activity showed rapid recovery after an initial drop, reached a peak on day 7, and returned to normal by the end of the second week.

The significant increase in acid phosphatase activity occurred at the time when cytoplasmic degenerations are most pronounced. While succinic dehydrogenase and cytochrome oxidase activities remained constant during the experiment, the normally higher lactic dehydrogenase activity showed a marked reduction in the experimental animals suggesting the dependence of pulp cells on anaerobic glycolysis, which is suppressed by actinomycin D. Variations in the level of enzyme activities observed in control animals are attributed to pair-feeding. Results of the present work give further supports to data obtained from previous ultrastructural studies.

INTRODUCTION

THE CYTOTOXIC action of the antibiotic, actinomycin D, has been tested in numerous *in vitro* and *in vivo* experiments. It was found that actinomycin D in high concentrations inhibits the activity of DNA-dependent RNA polymerase, (GIOVANNI and NOVELLI, 1963) thus blocking further synthesis of RNA (CASPERSSON *et al.*, 1963). Information in the literature is not without controversy as to the mechanism, but it is generally agreed that RNA and protein syntheses are abolished or drastically reduced by actinomycin D which specifically combines with the guanine portion of the DNA helix (GOLDSTEIN, SLOTNICK and JOURNEY, 1960; GOLDBERG, 1962; HARBERS and MÜLLER, 1962; PERRY, 1962; TAMAOKI and MUELLER, 1962; AMOS, 1963; CASPERSSON *et al.*, 1963; FRANKLIN, 1963; MERITS, 1963; HONIG and RABINOVITZ, 1965). Previous works made on the structural modifications in cells caused by this antibiotic appear to support the above description of the mechanism of action (JOURNEY and GOLDSTEIN, 1961; SCHOEFL, 1963; JONES and ELSDALE, 1964; JHEE and HAN, 1964; JHEE, HAN and AVERY, 1965; HAN, 1966).

The emphasis by enzyme histochemists in dentistry was placed in the past on the identification and localization of hydrolytic enzymes in the developing and adult

teeth (ENGEL and FURUTA, 1942; HOROWITZ, 1942; MORSE and GREEP, 1947; GREEP, FISCHER and MORSE, 1948; HARRIS, 1950; WISLOCKI and SOGNNAES, 1950; SYMONS, 1955; GLASSTONE, 1958; BURSTONE, 1960a,b; MORI *et al.*, 1960; MORI, TAKADA and OKAMOTO, 1961; MORI, TAKADA and OKAMOTO, 1962; TEN CATE, 1962; BALOGH, 1965). Oxidative enzymes, however, were investigated only recently (MORI, MIZUSHIMA and OSANAI, 1961; NUKI and BONTING, 1961; BALOGH, 1963; FULLMER, 1963; MIZUSHIMA, 1964).

The purpose of this study was to demonstrate effects of actinomycin D on certain respiratory and hydrolytic enzymes of the dental pulp by currently available histochemical techniques.

MATERIALS AND METHODS

Eighteen male Sprague-Dawley rats weighing 230-250 g each were used. Fourteen were paired by matching their body weights into an experimental and a control group. One day previous to the beginning of the experiment, the control animals were starved while the ones of the experimental group were given 50 g of rat chow. On the following day, the control animals were fed the same amount of food as consumed by their experimental partner during the 24-hr period. Pair feeding was subsequently maintained throughout the 21-day experiment. Water was given *ad libitum*. On day 0, the animals in the experimental group received an intraperitoneal injection of 0·125μg actinomycin D/g body weight dissolved in sterile physiological saline solution. Control animals were injected with an equivalent volume of saline.

After injection, rats of both groups were killed by ether anaesthesia on days 1, 3, 5, 7, 10, 14 and 21. Prior to dissection the animals were weighed, and the percentage of body weight change was plotted against time to correlate with the expected change in enzyme activities and body weight. Data from three additional experiments conducted under identical conditions were also utilized to construct the graph presented in Fig. 1.

After excision, the mandible was bisected at the symphysis and the incisor pulps were dissected in a manner described elsewhere (HAN and AVERY, 1963). Because of the small size of the tissue the following care was taken in the subsequent preparation. The dental pulp was removed with its odontoblastic layer and was placed on dry ice together with a piece of kidney cortex which served as a control for histochemical staining. The frozen tissues were then transferred onto a thin layer of ice in the bottom of a small aluminium foil cup which rested on dry ice. The cup was slowly filled with cold distilled water. The tissue embedded in ice was removed from the cup and without remelting was mounted for sectioning in a cryostat at $-25°C$. Sections 4μ thick were made, mounted on coverslips and placed in Columbia jars containing the proper substrate for different periods of time at the correct temperature as required by the respective techniques.

To check the aerobic and anaerobic modes of respiration three of the mitichondria-linked enzymes, namely, succinic dehydrogenase and cytochrome oxidase, and lactic dehydrogenase (LDH) were studied. Of hydrolytic enzymes, alkaline phosphatase was checked because of its involvement in phosphate metabolism related to

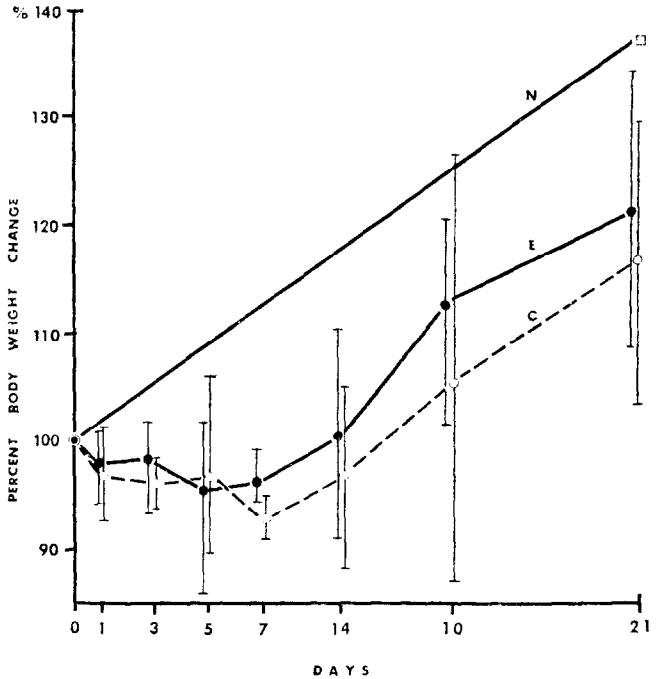

FIG. 1. The above graph shows the percentage body weight changes that occurred during the experimental period. N, normal body weight increase; E, body weight changes in the experimental group; C, body weight changes in the pair-fed control group.

mineralization and cellular activities, while the acid phosphatase was selected in order to determine possible lysosomal changes as previous studies showed an increase in lysosome-like bodies in protein-secreting cells after actinomycin (HAN, 1966). Succinic dehydrogenase was demonstrated according to the technique of Nachlas *et al.* as modified by BURSTONE (1962, p. 513). The activity of LDH was studied by the demonstration of DPN-linked lactic dehydrogenase activities (PEARSE, 1960, p. 911). In both techniques, nitro-BT was employed as the final electron acceptor. To avoid nonspecific staining of lipid, which attracts nitro-BT (BURSTONE, 1962, p. 495), sections were pretreated for 5 min in chilled acetone. Cytochrome oxidase was studied as described by ODA *et al.* (BURSTONE, 1962, p. 463). Gomori's lead nitrate method (PEARSE, 1960, p. 881) was applied for the demonstration of acid phosphatase, and the alkaline phosphatase was studied by using AS-MX phosphate as substrate with Red-Violet LB diazonium salt. Following the staining, tissues were examined and photomicrographs were taken with a Zeiss photomicroscope.

To determine the effect of initial starvation on enzyme levels in control animals resulting from the pair feeding, two rats were fed the average amount of food (e.g. 27 g) normally taken by the control animals while the two other rats were starved for a day. The pulp and kidney tissues of these rats were prepared and examined as described above.

RESULTS

Observations of pair-fed control animals

Figure 1 shows body weight changes of experimental and control animals as expressed by the percentage change of the initial value. After a continuous reduction the average low points are reached on the fifth and seventh days respectively for the experimental and control animals and then a steady recovery is noted. The control curve generally follows the fluctuation of the experimental group and, therefore, the changes in animal weights produced by the dose employed appear to reflect varying levels of food intake.

In order to facilitate a reliable comparison, histochemical observations to be reported were made from the midpulp region in all cases. Among the oxidative enzymes localized in the normal rat incisor pulp, LDH was found to be the most active, with succinic dehydrogenase showing moderate and cytochrome oxidase the least activity. All of these enzymes were visualized in the form of discrete cytoplasmic granules with some background staining in the case of LDH. On day 1 in the control rat, which essentially represented a 24-hr starvation effect, there was a minimal LDH activity (Fig. 2f), which showed a moderate increase by day 3 (Fig. 2g) and then dropped to a second low level on day 5 (Fig. 2h) when food intake was the lowest. By day 7 (Fig. 2i) a moderately intense reaction was observed, which did not change for the remainder of the experiment.

Topographically, the strongest reaction for LDH occurred in the cytoplasm of the subodontoblastic layer of cells. The intensity of staining in the odontoblasts was approximately half of that found in the cells of the kidney tubules. Fibroblasts of the dental pulp presented a divergent picture, showing a variation in the intensity of the reaction ranging from a minimal to moderate staining. Succinic dehydrogenase activities appeared to be restricted mainly to the odontoblastic layer, although there was a low enzyme activity in the pulp fibroblasts. The distribution of the cytochrome oxidase activity was similar to that of the succinic dehydrogenase.

Of the two hydrolytic enzymes, reaction products of acid phosphatase were observed in odontoblasts as densely-packed supranuclear granules (Fig. 3a, f). In the subodontoblastic layer of cells, granules were found in moderate numbers. Fewer granules were present in the cytoplasm of fibroblasts of the dental pulp. In contrast to LDH, a moderate acid phosphatase activity was present on day 1 (Fig. 3f). There was a significant decrease in acid phosphatase activity on day 3 (Fig. 3g) which recovered and increased beyond day 1 level by day 7 (Fig. 3i). In control sections of kidney cortex, acid phosphatase activity was seen to be located mostly in the paranuclear region of tubule cells.

Alkaline phosphatase activity was localized along apical plasma membranes of the pulp cells. The reaction products were somewhat diffusely distributed (Fig. 4j). Fibroblasts of the pulp contained variable amounts of the granular deposit perinuclearly and in their processes. In general, alkaline phosphatase activity appeared to decrease gradually from the apical portion of odontoblasts toward the centre of the pulp (Fig. 4j). In kidney tubules of control sections, alkaline phosphatase positivity was demonstrated in the apical region of the cells. Only slight variations were observed

in the intensity of staining in the control group during the experimental period (Fig. 4f–j). In addition a moderate enzyme activity was found in and around the walls of the pulpal arteries and arterioles for most of the enzymes examined.

Observations of experimental rats

Lactic dehydrogenase. The level of LDH activity on day 1 (Fig. 2a) was somewhat less than in the normal pulp which showed an intensity similar to that observed in 21-day animals (Fig. 2e, j). However, the level was considerably higher than in the day 1 control pair. The activity continued to decrease in the experimental group and reached a minimal activity on day 5 (Fig. 2c). From this point on, the enzyme activity returned to a moderate level by day 7 (Fig. 2d) and by day 21 the picture was indistinguishable from that of the normal (Fig. 2e).

Examination of the levels of activity of succinic dehydrogenase and cytochrome oxidase did not show any significant variation throughout the experimental period.

Acid phosphatase. The enzyme activity on day 1 (Fig. 3a) in the odontoblasts in the actinomycin-treated rat appeared to be unchanged and comparable to day 21 (Fig. 3e). However, as was true with LDH, the activity was somewhat greater than that of the paired control (Fig. 3f). By day 3 a marked drop was observed (Fig. 3b, g). From the 5th day on (Fig. 3c, h) a parallel increase of enzyme activity in both experimental and control groups was evident, except that experimental animals showed a much higher activity, reaching a peak on day 7 (Fig. 3d). By the end of the second week the staining intensity subsided to the level observed on the first day and became comparable to that of control animals (Fig. 3e).

The kidney cortex exhibited fluctuations in acid phosphatase activity similar to those of odontoblasts of the experimental and control groups.

Alkaline phosphatase. In general, changes observed in alkaline phosphatase activity were minimal. Only a slight increase in the enzyme activity was observed from day 0 to day 1 in the experimental animal (Fig. 4a). A limited but definite reduction was observed by day 5 (Fig. 4c) reaching a low point on day 7 (Fig. 4d). By the end of the second week the level of enzyme activity was raised to the day 0 level in both experimental and control tissues and remained unchanged for the rest of the experiment (Fig. 4e, j).

DISCUSSION

Normal enzyme histochemistry of the pulp

The fluctuation of enzyme activities presented by control animals is somewhat confusing. The initial drop appears to be related to the lowered food intake by control rats due to pair-feeding, since results from the starvation experiment show essentially the same picture. The effect of starvation is more obvious in enzymes with high levels of activity. Therefore, it might be indicated that, while pair-feeding is desirable for controlling the introduction of unwanted variables, the initial phase of pair-fed experiments should be interpreted with caution insofar as the enzyme histochemistry is concerned.

Levels of LDH activity in various pulp cells have been described by previous

workers in different ways. Thus, NUKI and BONTING (1961) examined the enzyme in the developing molar of the hamster by quantitative means and found it to be in the same order of magnitude in all cellular zones, with the zones more distant to sites of dentine and enamel formation representing the lower part of the range observed. BALOGH (1963) observed a marked activity in odontoblasts and the pulp of mouse incisors. FULLMER (1963), however, described LDH activity to be moderate in rat odontogenesis. MIZUSHIMA (1964) made observations similar to ours, in the rat, dog and hamster. He described LDH activity in the developing teeth as ranging from moderate to strong in the odontoblastic layer, while the dental pulp exhibited moderate activity.

That the activity of LDH is consistantly higher, in the rat incisor, than that of succinic dehydrogenase indicates the possible presence of a more active glycolytic pathway and a less active process of aerobic respiration. This hypothesis is also supported by the very weak cytochrome oxidase activity detected in these tissues, as observed by MIZUSHIMA (1964) and supported by our observations.

Phosphatase in the alkaline pH range in the odontoblast and pulp was first localized by HOROWITZ (1942) in foetal rats. ENGEL and FURUTA (1942) did not localize any phosphatase activity in odontoblasts and only some in the pulp of rats in the 1–4 day postnatal period. However, the presence of alkaline phosphatase activity was described in detail by HARRIS (1950) in the 4-hr old rat and by GREEP *et al.* (1948) in the 28-day-old rat in odontoblasts of the incisor pulp. The presence of this enzyme was substantiated in odontoblasts and dental pulp cells of man, monkey and rat by WISLOCKI and SOGNNAES (1950). Recent works concerned with the demonstration of alkaline phosphatase present a similar picture in terms of its localization in dental tissues (SYMONS, 1955; GLASSTONE, 1958; BURSTONE, 1960a, b; MORI *et al.*, 1960; MORI *et al.*, 1961; TEN GATE, 1962).

Our results are generally in agreement with the majority of the findings described in the literature. There is a spectrum of moderate to low enzyme activity proceeding from the odontoblastic layer toward the centre of the dental pulp.

Although acid phosphatase was described as absent from odontoblasts by BURSTONE (1960), MORI *et al.* (1961) demonstrated its presence in differentiating rodent teeth. BALOGH (1965) found the activity to be moderate in odontoblasts and weak in the pulp. Our observations show the presence of acid phosphatase in odontoblasts, where the activity of the enzyme is more intense than in fibroblasts of the pulp.

MORI *et al.*, (1961) and FULLMER (1963) found that succinic dehydrogenase activity increases with differentiation of odontoblasts and that the enzyme was more active in odontoblastic cells than in the cells of the pulp. Succinic dehydrogenase activity was low in our studies, but in general odontoblasts were more active than pulp fibroblasts. In contrast, BALOGH (1963) described strong succinic dehydrogenase activity in both the odontoblasts and the mesenchymal cells of the pulp in the adult mouse.

Enzyme activities in experimental animals

Similar initial reductions in the enzyme level of the alkaline phosphatase and LDH, along with the reduction in body weight, suggest a generalized impairment of

metabolic activities. The reduction in these enzyme activities is followed by a pronounced increase in acid phosphatase activity on the seventh day (Fig. 3d), after an initial drop on the third day (Fig. 3b) of the experiment. The decrease on the third day might possibly be the direct result of the inhibition in protein synthesis. What is significant is that, of the enzymes studied, only the acid phosphatase shows a very high level of activity during the time when cytoplasmic degenerations are most pronounced.

Succinic dehydrogenase and cytochrome oxidase activities remain at a relatively constant low level. On the other hand, the LDH activity, which is much higher than the preceeding two, shows a marked change in experimental animals, suggesting the dependence of pulp cells on anaerobic glycolysis, which is suppressed by actinomycin D (LASZLO *et al.*, 1966).

In view of the difficulty involved in quantitative estimation of histochemical results and starvation-induced changes in controls, it is not possible to assess the extent of the specific reduction caused by actinomycin D, although a definitely lower activity observed in the experimental pair on day 7 indicates that the effect persists through seventh day, which is followed by a recovery on day 14.

Cellular changes produced by actinomycin D have been observed with the light microscope in salivary glands (JHEE and HAN, 1964; JHEE *et al.*, 1965), and with the electron microscope in HeLa cells (JOURNEY *et al.*, 1961), in baboon kidney cells (SCHOEFL, 1963), in rat pancreas (JEZEQUEL and BERNARD, 1964), in differentiating amphibian cells (JONES and ELSDALE, 1964), and in dental pulp fibroblasts and parotid acinar cells (HAN, 1966). These observations list changes which include an enlargement of nuclei, fragmentation of nucleoli, cytoplasmic "blebbing", changes of the RER and Gogli complex, mitochondrial damage, increase in number of cytolysomes, and appearance of thread-like structures in nuclei. The above results suggest that, on histochemical examination of tissues from actinomycin-treated animals, a reduction in respiratory enzyme activity and an increase in lysosomal acid phosphatase activity might be expected. The reduction in mitichondria-related enzyme activities, such as LDH, might be correlated with structural changes in mitochondria (HAN *et al.*, 1966), which might succeed a reduced protein synthesis.

Although the cellular recovery would depend upon the amount of actinomycin administered, regenerative processes with the dose used in our experiments occur after the first 10 days, and are manifested by an increase of ribosomes and polysomes, restoration of the RER and mitochondrial structures (HAN *et al.*, 1966). Thus the return in lactic dehydrogenase activity might reflect the recovery of glycolytic activity to the normal level, while the increase in alkaline phosphatase activity may be related to regenerative changes and growth (STADTMAN, 1961, p. 57), as suggested by previous observations on developing and atrophic pulp tissues (FULLMER, 1964). The return of acid phosphatase activity to control levels suggests that structural "turnover" caused by the antibiotic has returned to its normal rate (SCHMIDT and LASKOWSKI, 1961).

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Résumé—Les concentrations des déshydrogénases succiniques et lactiques, en cytochrome oxydase, et en phosphatases alcaline et acide de la pulpe d'incisive de rat sont étudiées après injection d'actinomycine D. Des animaux témoins, injectés avec du sérum physiologique, sont soumis à la même alimentation.

Les activités en déshydrogénase succinique et en cytochrome oxydase sont peu élevées dans la pulpe et varient peu pendant la durée de l'expérience. Les activités en déshydrogénase lactique et en phosphatase alcaline de la pulpe dentaire diminuent rapidement au cours des premiers jours de l'expérience et reviennent à un niveau normal au bout de la troisième semaine. L'activité en phosphatase acide redevient normale après une chute initiale, augmente jusqu'au 7ème jour et reprend une valeur normale à la fin de la seconde semaine.

L'augmentation la plus importante de l'activité phosphatasique acide se produit au moment où les dégénérescences cytoplasmiques sont les plus prononcées. Alors que les activités en déshydrogénase succinique et cytochrome oxydase restent constantes pendant toute la durée de l'expérience, l'activité plus élevée de la déshydrogénase lactique est nettement réduite chez les animaux en expérience, indiquant toute l'importance de la glycolyse anaérobique inhibée par l'actinomycine D, pour les cellules pulpaires. Les variations de l'activité enzymatique, observées chez les témoins, sont attribuées au mode d'alimentation. Ces résultats confirment les conclusions d'une étude ultrastructurale antérieure.

Zusammenfassung—Nach Injektion einer sublethalen Dosis Aktinomycin D wurden die Aktivitäten der Bernsteinsäure- und Milchsäuredehydrogenase, der Cytochromoxydase sowie der alkalischen und sauren Phosphatasen in der Schneidezahnpulpa der Ratte untersucht. Kontrolltieren wurde physiologische Salzlösung injiziert; sie erhielten die gleiche Nahrung.

Die Aktivitäten der Bernsteinsäuredehydrogenase und der Cytochromoxydase waren in der Pulpa gering, sie boten während des gesamten Experimments nur unbedeutende Veränderungen. Die Aktivitäten der Milchsäuredehydrogenase und der alkalischen Phosphatase der Zahnpulpa fielen während der ersten Tage des Experiments schell ab und stiegen gegen Ende der dritten Woche wieder auf die normale Höhe an. Die Aktivität der sauren Phosphatase ließ nach anfänglichem Abfall eine schnelle Erholung erkennen, sie erreichte am siebenten Tage ihren Spitzenwert und kehrte gegen Ende der zweiten Woche zur Norm zurück.

Der signifikante Anstieg der Aktivität der sauren Phosphatase geschah zu der Zeit, wenn zytoplasmatische Degenerationen am stärksten ausgeprägt waren. Während die Aktivitäten der Bernsteinsäuredehydrogenase und der Cytochromoxydase im Verlauf des Experiments konstant blieben, zeigte die normalerweise höhere Milchsäuredehydrogenaseaktivität bei den Versuchstieren eine deutliche Verminderung, was für die Abhängigkeit der Pulpazellen von der anaeroben Glykolyse, die durch Aktinomycin D zurückgedrängt wird, spricht. Variationen in der Höhe der Enzymaktivitäten bei den Kontrolltieren werden der Ernährungsweise zugeschrieben. Die Ergebnisse der vorliegenden Arbeit ergänzen die aus früheren ultrastrukturellen Untersuchungen erhaltenen Angaben.

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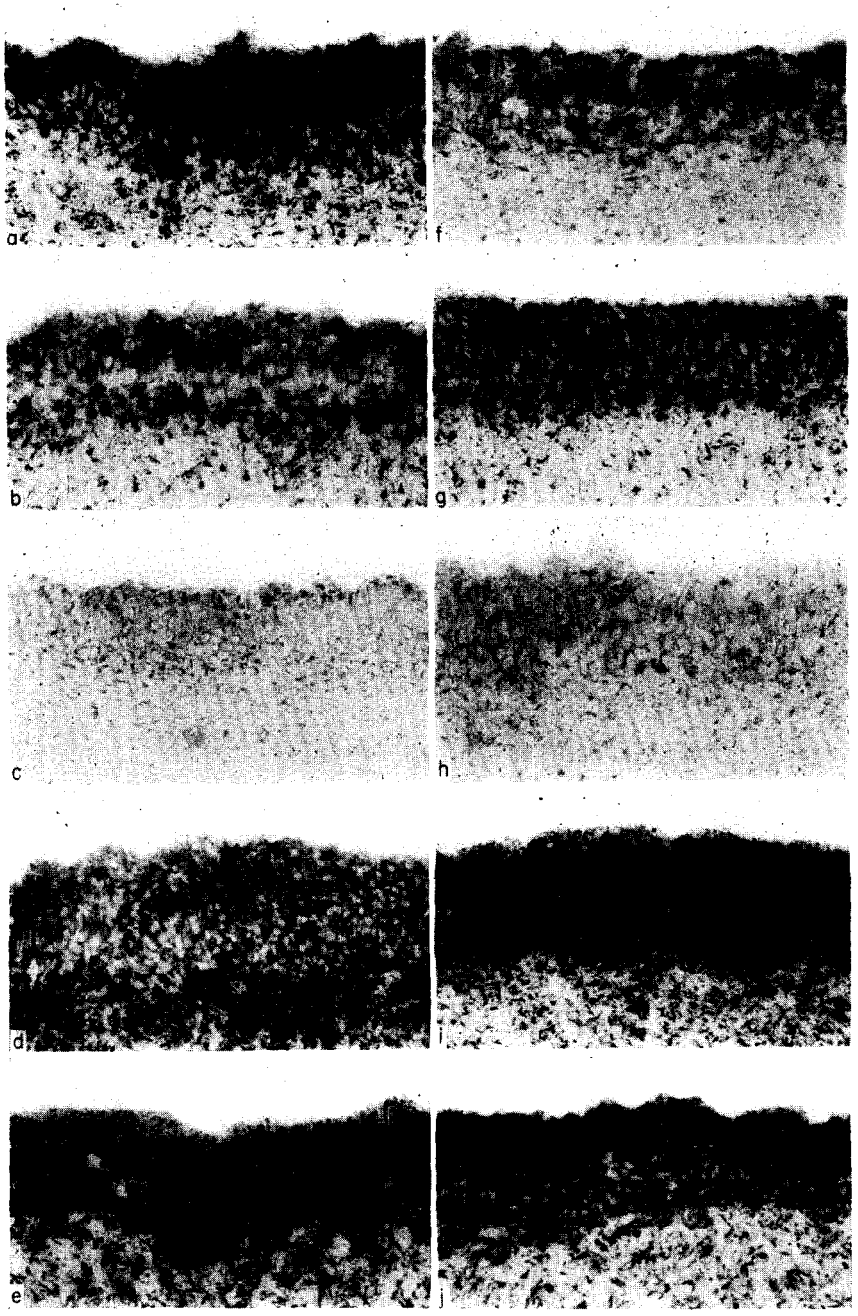

FIG. 2. Photomicrographs showing odontoblasts and adjacent pulp cells of rat incisors stained for LDH. (a–e = tissues from experimental animals killed on days 1, 3, 5, 7, and 21. f–j = tissues from pair-fed control animals killed on corresponding days.) × 272.

PLATE I
A.O.B. f.p. 274

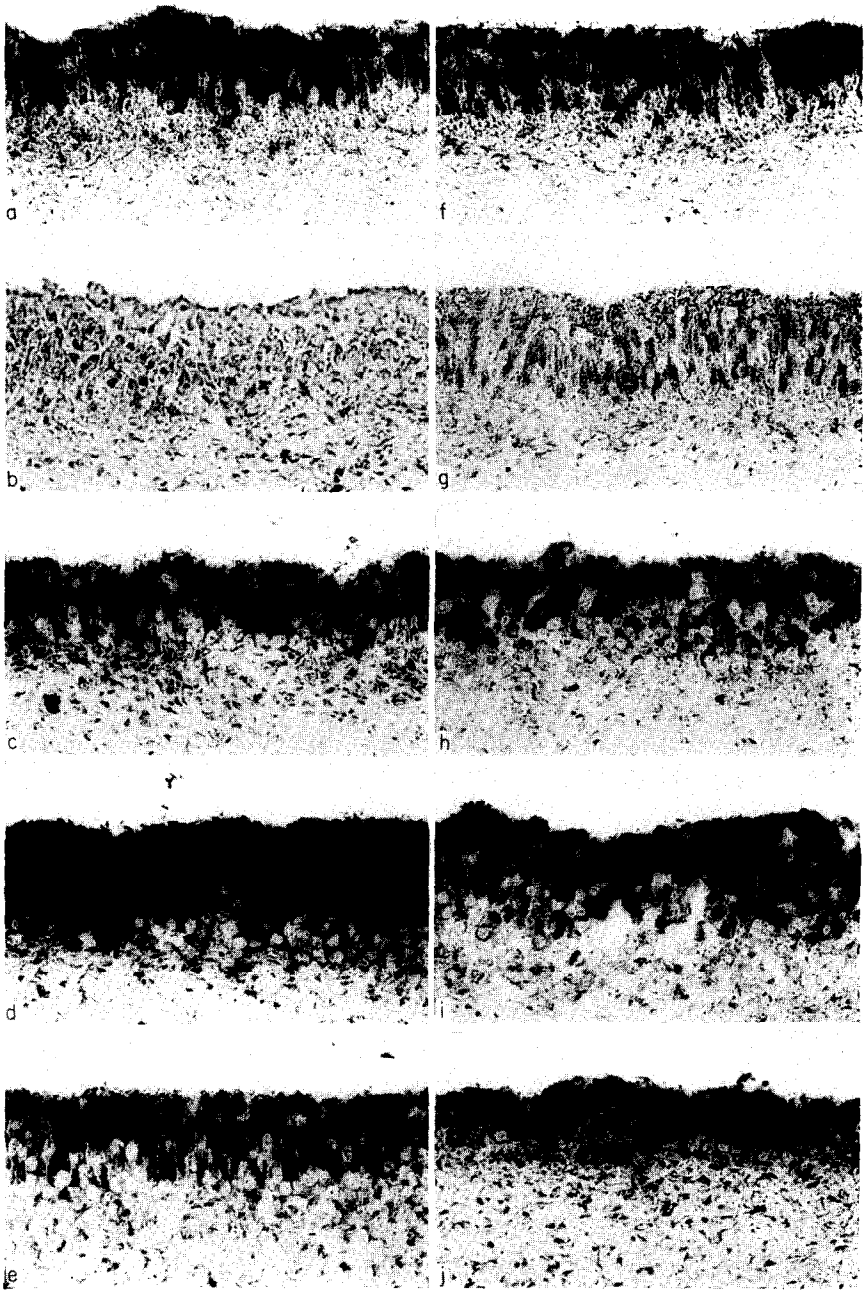

FIG. 3. Photomicrographs showing odontoblasts and adjacent pulp cells of rat incisors stained for acid phosphatase. (a–e = tissues from experimental animals killed on days 1, 3, 5, 7, and 14. f–j = tissues from pair-fed control animals killed on corresponding days.) ×272.

PLATE 2

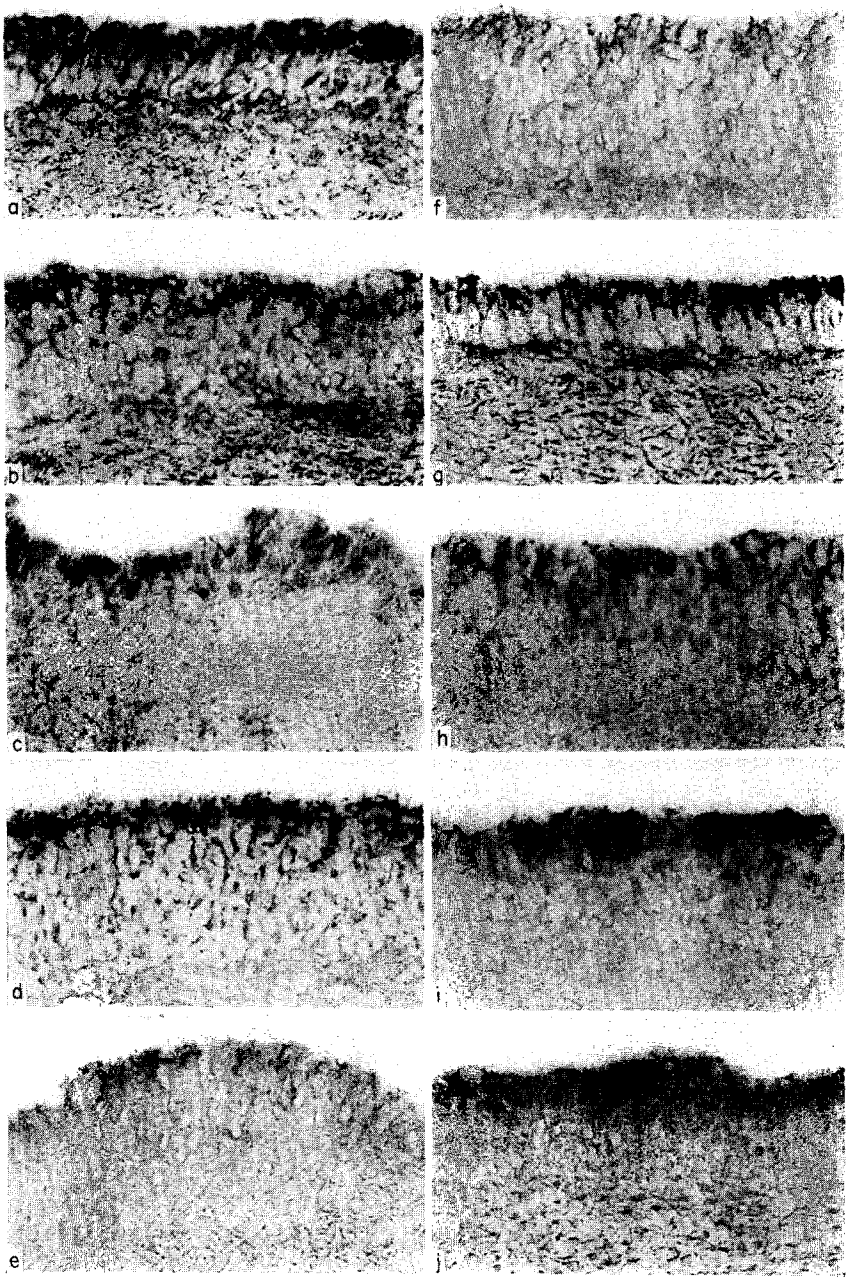

FIG. 4. Photomicrographs showing odontoblasts and adjacent pulp cells of rat incisors stained for alkaline phosphatase. (a–e = tissues from experimental animals killed on days 1, 3, 5, 7, and 21. f–j = tissues from control animals killed on corresponding days.) × 272.