SOME HISTOCHEMICAL VARIATIONS OF KERATOTIC LESIONS OF THE ORAL MUCOSA

by

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A THESIS

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INTRODUCTION

Preface and Semantics

The normal mucous membranes of the oral cavity may be covered by keratinized or non-keratinized epithelium, depending on the functions of the various regions. However, abnormal stimulation of any area may result in increased production of keratin in either a focal or diffuse distribution. As the keratin increases in thickness, it appears as a white area for which clinicians have evolved a variety of terms, e.g. hyperkeratosis, leukoplakia, keratosis, pachyderma oralis, leukokeratosis, etc.

Some lesions of the mucosa may appear to be the keratin-producing type, but after a careful history and clinical examination, may be found to be a necrotizing process (aspirin burn) or an overgrowth of organisms (monilia).

The basis of some keratin-producing lesions may also be elucidated by the history and clinical examination. Lupus erythematosus, white folded gingivostomatitis (white sponge nevus) and lichen planus are such entities. However, most of these lesions evade such definite categorization, even when a biopsy is performed, and will be routed into a variety of diagnostic categories according to the pathologist studying the case.

Obviously, clinical classification of white patches is confusing and perhaps more surprisingly, confusion concerning the microscopic

evaluation is also widespread (32,64). It would be simple to allow the problem to remain a subject for semantic arguments in academic circles, but when the matter of life or death hinges upon proper diagnosis and treatment, the potential malignancy of some of these white lesions must be properly reckoned with. Most professional men have now come to realize that clinical examination is often insufficient, that prolonged "watching" is dangerous, and that the white patch which fails to respond to conservative therapy is a definite candidate for biopsy. Even after the biopsy, however, which is so often considered definitive, the pathologist may be undecided about a particular lesion.

The following concepts will be used in this present discussion:

Keratosis: a clinical term referring to an undiagnosed white patch, which usually represents abnormal production of keratin, parakeratin, or prickle cells. Dyskeratosis may or may not be present.

Hyperkeratosis and parakeratosis: histologic terms referring to abnormal production of keratin which shows either no cell remnants (hyperkeratosis) or nuclear remnants (parakeratosis).

Leukoplakia: a histologic diagnosis referring to a keratotic condition in which there is microscopic evidence of dyskeratosis. It implies potential malignancy, and may vary from slight atypia to full-blown carcinoma-in-situ. Dyskeratosis is characterized by abnormal cell keratinization, hyperchromatism, variation in cell size and shape, increased and abnormal mitotic figures and loss of polarity. Not all of these criteria need to be present in a

single lesion for the evaluation of dyskeratosis to be made.

With the increasing use of histochemical procedures, it would seem reasonable that eventually the keratotic lesions of the mouth could be typified, and more information obtained concerning the physiology of these cells. If these techniques could offer more validity to diagnosis, prognosis or mode of treatment, their value would not be questioned. Histochemical procedures may also prove to be valuable in the study of oral pathoses other than the keratoses.

Intensive study of leukoplakia is especially important. If this condition is truly premalignant, is there some physiological sign that can be detected histochemically? Are the changes in these dyskeratotic cells of a specific or non-specific nature? My primary concern is to attempt to detect the critical difference(s) between normal tissues and/or cells, leukoplakia, and an irreversible malignant state.

A recent investigation has found a change in PAS staining characteristics in the basement membranes of white lesions of the mouth that are malignant. Some changes were also found in dyskeratosis which led the authors to suggest such a staining procedure may aid in the early detection of malignant changes in the epithelium (17).

The study of glycogen deposition in the epithelial cells of the mucosa offers a promising approach to the study of the keratinizing lesions. This substance has been studied quite intensely in recent years. Keratinization and the glycogen content are found to be inversely related in the oral mucosa, and glycogen content of normal buccal mucosa is five times greater than that found in "leukoplakia"

(keratosis*) (68,69). Inflammation tends to reduce keratinization but increases glycogen while both glycogen and keratinization increase with age. (62) Glycogen content has been related more to a state of hyperplasia and less to the type of keratosis; however, the parakeratotic lesions showed more glycogen than hyperkeratotic areas (63). These results, though, were obtained using leukoplakia only as a clinical entity, and no correlation was made with the histological state of the cells. Ayre (1) discusses the situation of a premalignant state of the cell, and concludes that glycogen is absent in both premalignant and malignant cells. On the other hand, Seibel (59) found large quantities of glycogen in the epidermis of the vulva with "leukoplakia", but it was inconsistent in its pattern. In full-blown carcinoma there was a great reduction of glycogen, but due to the spotty increase in "leukoplakia" (keratosis), Weibel states that one cannot predict the appearance of carcinoma on the basis of glycogen occurrence. He theorized that the glycogen provided anaerobic energy for keratin production due to local tissue anoxia. It also has a stimulatory effect upon the cells, resulting in excess keratin production and hyperplasia of the cells. The enzyme phosphorylase may be a finer measure of glycogen metabolism than staining for flycogen itself (7). This may offer an approach to clarification of the relation of glycogen to keratotic processes.

Many studies of "leukoplakia" or the keratoses have not made histologic descriptions of the clinical lesions. I feel that both macro and micro-scopic descriptions are necessary for histochemical correlations.

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* As defined on p. 6.

Enzyme Histochemistry

Enzyme histochemistry, as a young and vital branch of histochemistry, is currently receiving much attention from researchers. There are about fifty enzymes that can be demonstrated by histochemical techniques, not all of which are equally valid. Typification of tissues by the enzymes present and in the approximate amounts is a highly desirable goal. When cells enter into abnormal activities, there should be some enzyme systems affected since most of the metabolic activities are mediated through these vital components. Enzyme histochemistry then becomes of value when abnormal cell processes can be studied by these techniques. Improvement of techniques is continually progressing so that their validity is comparable to other histochemical procedures.

Prior to histochemical determinationa, enzyme activities were found by homogenization technics. Homogenized human frozen-dried skin was analyzed for the presence of acid phosphatase, aldolase, lactic dehydrogenase, malic dehydrogenase and glucose-6-phosphate dehydrogenase (33). These enzymes were found in consistent, measurable amounts even in the keratin layer of the sole of the foot, and were much more active in the epidermis than in the dermis. Eichel et al (20,21,24) determined some of the enzyme activities of various tissues from rabbits by homogenization techniques. Tissues from the oral region included tongue, gingiva, tooth pulp and submaxillary gland. Glucose-6-phosphate dehydrogenase, pyruvic reductase, succinic dehydrogenase,

DPN* cytochrome C reductase (diaphorase)**, cytochrome oxidase and catalase were demonstrated in consistent amounts. The liver was used as the 100 percent activity standard except for glucose-6-phosphate dehydrogenase for which the retina was the 100 percent activity standard. The oral tissues (tongue and gingiva) fell in the 10 - 30 percent activity range for all the enzymes. Glucose-6-phosphate dehydrogenase was more active in the gingiva than in the liver. Its very high activity in the retina was felt to reflect the need for this type of energy metabolism in an organ (eye) where the blood supply is not ubiquitous. In contrast, low activity in the liver may indicate the presence of adequate aerobic metabolic pathways.

Analysis of homogenized tissues helps lend credence to microscopic histochemistry, but does not elucidate the cell types contributing to the total activity found. Centrifugation of the homogenates to separate various cell fractions is a very useful technique also, but once again does not show the distribution of enzyme activity in specific cells.

Studies of the keratin-producing lesions by enzyme histochemical techniques are not prominent in the literature, and uaually the information is found in the descriptions of utilization of some particular technique upon a gamut of normal and abnormal tissues. Thus far, none of them have shown a consistent pattern of activity. In the past few years, however, there have been more studies of a histochemical nature

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*DPN: Diphosphopyridine nucleotide (coenzyme I). It serves as a link for specific dehydrogenases in cell respiration.

**Diaphorases: The flavoprotein enzymes that transfer hydrogen and electrons from the reduced coenzymes to the cytochrome system.

which are establishing base-line activities of some of the enzymes found in oral tissues (56,57,11,12,13,23,36).

The vaginal tract is another area of the body where the mucous membranes are reasonably accessible for study, and in addition, exfoliated cells can be easily studied with enzyme histochemical techniques. Smears of malignant and benign conditions were studied by both Papanicolaou stain and DPN-diaphorase activity (58). The malignant cells were found to retain high enzyme activity, but this was not conclusive enough to separate benign from malignant cells. Fishman _et al_ (26) feel that the ultimate elucidation of enzyme patterns of cells lies in the study of free cells. In utilizing enzyme histochemical techniques on tissue cultures of malignant epithelial cells, they found changes in enzyme activity during mitosis.

Enzyme histochemistry has not yet proved to be the panacea for those seeking answers to problems in oncology. Gomori (29) utilized enzyme histochemical techniques to study both normal and neoplastic tissues. His work can be considered some of the most original, classical efforts made in this field.

Acid phosphatase was found in metastatic carcinomas of the prostate and in some oral and gastric carcinomas (29). Others could not demonstrate such changes in oral carcinoma (60).

Phosphamidase is another enzyme found to be very active in carcinoma (30). This enzyme has no known role in biochemical schemes, but it may be related to the cells' physico-chemical work, processes of nonprotein synthesis and of adult histodifferentiation (38). Meyer and Weinmann improved upon the original technique and found phosphamidase in high concentrations in oral squamous cell carcinoma,

leukoplakia, papilloma and pleomorphic ("mixed") salivary gland tumor (37). Normal tissues were negative.

Beta-glucoronidase has been found mainly in the basal cells of epithelial tissues, especially those tissues that are directly influenced by estrogen. This enzyme is highly active in malignancies of these epithelial tissues, in contrast to the low activity found in connective tissue malignancies (39).

Aminopeptidase activity is high in the stroma about malignant apithelial tumors (14). Burstone feels that this enzyme may reflect the invasive potential of a neoplasm. Others do not agree, but feel that the enzyme reflects the non-specific proliferative response of connective tissue to such processes as invasive neoplasia, inflammation and repair (40).

Cytochrome oxidase activity is probably a good indicator of the general activity of the cytochrome system. Until recently, histochemical localization of this enzyme was not considered reliable but newer techniques (16) may prove more effective. Burstone (12) reports the localization of cytochrome oxidase in gingival epithelium and found that the enzyme was restricted primarily to crevicular epithelium, the epithelial attachment, and the basal cell layer of the free and attached gingiva. In tumors, cytochrome oxidase activity was generally lower than that found in normal cells of the same origin (31). Also, poorly-differentiated carcinomas were less active than well-differentiated carcinomas. However, some of the well-differentiated squamous cell carcinomas were highly active.

Dehydrogenases are one of the most important enzyme groups to be demonstrated histochemically. Since 1950 dehydrogenase histo-chemistry

has progressed rapidly with much interest in typifying normal and pathologic tissues. Pearse (52) is especially enthusiastic about this phase of enzyme histochemistry. Early investigators felt that certain dehydrogenase techniques would be useful in detecting malignant tissues in frozen surgical specimens (presuming the enzyme activity of malignant tissues is greater than the normal surrounding tissue)(6). The tetrazolium salt is reduced to a formazan to give a color reaction, thereby indicating dehydrogenase activity in tissues. (Methylene blue is another example of a substance which can be reduced by the activity of living systems).

Succinic dehydrogenase, lactic dehydrogenase, DPN diaphorase, and TPN*diaphorase have been studied in human tumors (41). The tumors varied greatly in enzyme activity, ranging from more to less than the activity of normal cells. No significant difference was found between mesenchymal or epithelial tumors, or between benign and malignant growths. Succinic dehydrogenase activity was low in the squamous cell carcinomas in which DPN diaphorase activity was high. TPN diaphorase was quite variable, and lactic dehydrogenase was about the same as in normal tissues. The authors suggested that these variations of enzyme activity in neoplasms may reflect the utilization of different metabolic pathways.

Using a gamut of the dehydrogenases, Wattenburg (66) studied the enzyme activities in a series of colonic lesions which included invasive carcinoma, malignant polyps, benign adenomatous polyps and hyper-

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*TPN: Triphosphopyridine nucleotide (coenzyme II). It acts as a link for specific dehydrogenases in cellular respiration.

plastic conditions. In the carcinomas there were changes in enzyme activity patterns not exhibited in the other lesions. DPN and TPN diaphorase activities were increased while succinic dehydrogenase, alpha-glycerophosphate and monoamine oxidase were reduced in activity. This study hints at a possible future in which such techniques may be of practical diagnostic value to the pathologist.

The Nature of the Problem

Histochemical enzyme techniques were employed in this investigation to study the activities of lactic dehydrogenase, DPN diaphorase, succinic dehydrogenase and glucose-6-phosphate dehydrogenase in keratotic lesions of the oral cavity. I wished to determine whether characteristic variations of these enzymes occur in the several keratin-producing lesions previously listed; and particularly to study enzyme activities of leukoplakia as compared to carcinoma and normal epithelium. The above four enzymes were chosen for several reasons:

1) The work of other investigators indicates that there may be demonstrable dehydrogenase activity changes in abnormal tissues.

2) Newer dehydrogenase techniques seem to localize enzyme activity quite well.

3) The enzymes chosen represent key points in the cells' metabolic pathways for obtaining energy. The sampling of various areas of this pathway may indicate if some portion is more or less active under abnormal conditions.

Figure 1 is a diagrammatic scheme of the cells' better known metabolic pathways for gaining energy, and the sites of enzyme activity to be sampled are indicated.

In the study of dehydrogenases, the enzyme to be investigated is utilized as it occurs naturally in the tissue. Exogenous substrate is

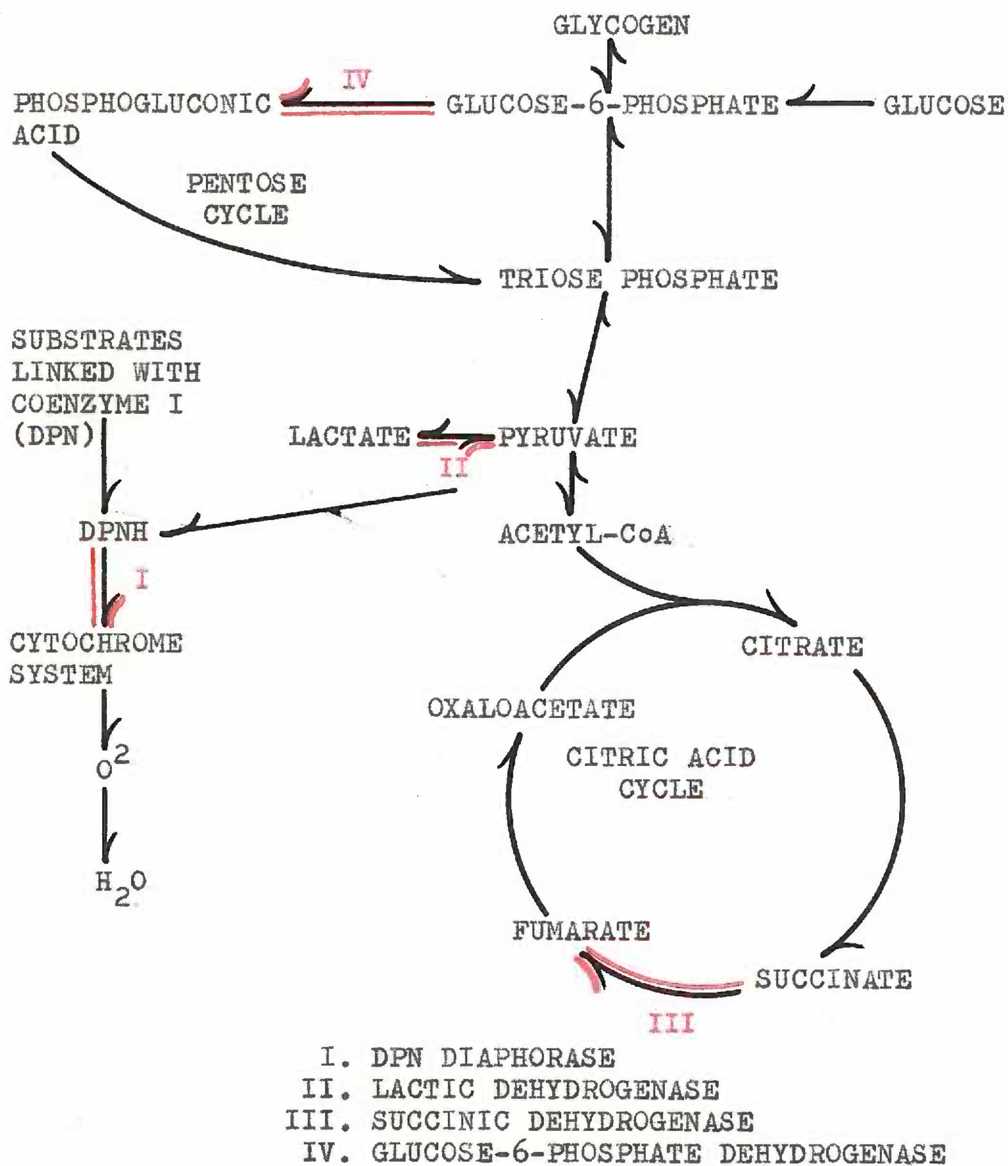


Figure 1. Partial scheme of oxidation and respiration within the cell. The reactions mediated by enzymes I, II, III, and IV are shown in red ink.

added, and its oxidation sets in motion the chain of events in which the hydrogen and electrons derived from the reaction are carried through the cytochrome system and finally to oxygen. In this instance, however, a tetrazolium salt is interposed which has a greater reduction potential than the next natural carrier in the system. The tetrazolium competes for the available hydrogen and is reduced to form an insoluble, colored dye (formazan). Figure 2 is a schematic diagram of the reaction sequences for the four enzymes.

The tetrazolium salt to be used in this study was recently developed by Tsou et al (61) and was given the name Nitro-BT ((2,2'-di (p-nitrophenyl)-5,5'-diphenyl-3,3'-dimethoxy-4,4'-biphenylene) ditetrazolium chloride)). Some of its favorable characteristics which the previous tetrazolium salts could not meet with equal proficiency are: (43)

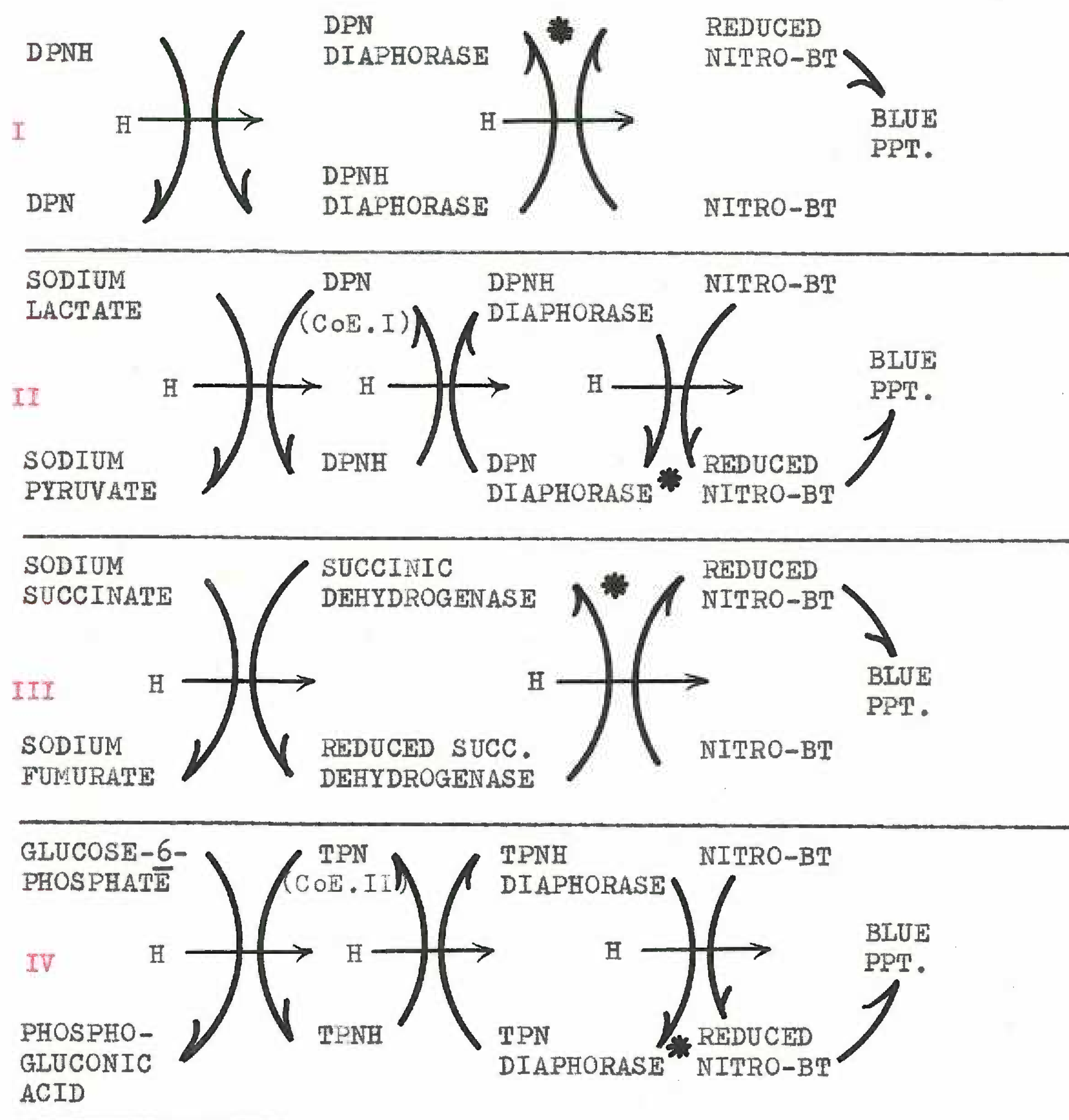
1) There is ready reduction to the formazan (blue color) and resistance to oxidation to the tetrazolium form.

2) It competes efficiently with the cytochrome system for the hydrogen, especially when inhibitors are used.

3) The formazan (blue color) produced is of a good quality in that it is an amorphous pigment which does not readily crystallize.

4) The formazan produced is not significantly soluble in the common organic solvents or in aqueous media, and has very good substantive properties which render it even more insoluble.

Use of the electron microscope shows that Nitro-BT can be used at this level of cell study (2). The formazan was found deposited in and on



✱ In the normal sequence, the cytochrome system is entered at this point.

Figure 2. Reaction sequence for demonstration of the four dehydrogenase systems. The formazan (blue precipitate) has high substantivity for protein.

the mitochondria and on the sarcoplasmic reticulin.

Pearse and Zimmerman (51) point out one factor which may lead to error in using these techniques. At levels above pH7 and becoming maximal at pH9, tetrazolium salts could be reduced by tissues in which the specific substrate was missing. Sulfhydryl inhibitors were used successfully, and therefore, they implicate the tissue-bound sulfhydryls in this phenomenon. This reaction has lead to the term of "nothing dehydrogenase".

These findings imply that a great deal of early dehydrogenase histochemistry is not as valid as previously thought, especially where a high pH was used in conjunction with the earlier tetrazolium salts. (27)

Some histochemists advocate using only fresh-frozen tissue for dehydrogenase study. In fact, Pearse (52, p.581) condemns the use of a fixative since the dehydrogenase systems have a hard enough time surviving as it is. On the other hand, Novikoff (46) feels that fixation by cold, formol-calcium does not destroy certain dehydrogenases. The present study utilized the fresh-frozen sections since I found in preliminary studies that glucose-6-phosphate dehydrogenase was lost during fixation with formalin.

Reactions should be run at a high velocity, keeping the incubation time to a minimum. Theoretically, a short incubation time helps prevent damage to the mitochondria and keeps alternate electron acceptors from taking up the supply of electrons. Cyanide is used in some reactions to act as a trapping agent and consequently removes reaction products. This results in more efficient enzyme action by allowing the reaction velocity to remain at a high level. Cyanide also blocks the

electron transport system near the end of the cytochrome complex and thereby prevents electron loss via this system. Other inhibitors used are azide and fluoride.

The effect of the incubating medium on the cell is somewhat minimized by buffering the reaction at pH7 and the use of a non-electrolytic hypertonic medium (polyvinyl pyrrolidone), which helps protect the mitochondria. Cobalt or magnesium may also be used to help protect the mitochondria.

DPN Diaphorase (I)*

DPN diaphorase acts as a link between reduced DPN (Coenzyme I) and the cytochrome system. This system reflects the activities of several DPN-linked dehydrogenases related to cell respiration (e.g. malate, glutamate, lactate, alcohol, befa-hydroxybutyrate, etc.). It is probably universally present in tissues and has been described as occurring in moderate amounts in oral tissues (23,24). Increased activities of this enzyme in carcinoma has been reported (41,66).

Lactic Dehydrogenase (II)*

Lactic dehydrogenase acts upon lactate to return it to pyruvate which may then proceed to the citric acid cycle or back to glycogen. Lactic acid represents the end product of anaerobic glycolysis and is present in large amounts in working muscle. Eichel found the oral tissues have a moderate amount of the enzyme (23). The serum lactic acid level of patients with carcinoma, lymphoma and leukemia has been found to be increased (5). This does not necessarily mean the

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*Roman numerals refer to designations of enzymes in Figs. 1 & 2 & Table I.

dehydrogenase activity is increased. Monis, et al (41) found high lactic dehydrogenase activity in squamous cell carcinoma of the oral cavity.

Succinic Dehydrogenase (III)*

The activity of succinic dehydrogenase in tissues has been of great interest for many years since its substrate, succinate, is an integral component of the tricarboxylic acid cycle which is considered to be the final aerobic phase of glycolysis. It is felt that the activity of the dehydrogenase will reflect the activity of the cycle. Succinic dehydrogenase activity in normal gingival tissues is fairly low. (23,24) A decrease of activity has been shown in human colonic carcinoma and ependymomas of mice, whereas it is slightly increased in benign adenomas.(66,49) Tumors induced in rat liver by a carcinogen exhibit high enzyme activity in glandular portions of the tumor, whereas areas of non-glandular tumor revealed no activity.(53) This type of enzyme distribution may explain why there may be a great variance in findings of those who assay for the enzyme without knowing the cellular morphology.

Glucose-6-Phosphate Dehydrogenase (IV)*

Glucose-6-phosphate is dehydrogenated by glucose-6-phosphate dehydrogenase to form phosphogluconic acid, providing an alternative pathway for glucose metabolism. Five carbon sugars are produced during the cycle which subsequently may contribute to nucleotide synthesis. In neoplasia, increased nucleotide metabolism may be indicated by changes in this system's activities.

The dehydrogenase is a TPN-linked enzyme and its localization in cells is somewhat obscure. Pearse (52) found high activity in the

growing edge of squamous carcinoma. The findings of Eichel in tissue homogenization studies have been mentioned previously. In the past year he has reported on its presence in the gingiva in low amounts (24).

METHODS AND MATERIALS

The specimens for the study were collected from several different sources: the Departments of Oral Surgery and Periodontology of the University of Oregon Dental School; surgeries of the University of Oregon Medical School Hospital, Multnomah County Hospital, Veterans Administration Hospital and St. Vincent Hospital; and offices of several private oral surgeons. Twenty-four of the specimens were placed directly on dry ice; 12 of them within three minutes, 8 within ten minutes, 2 within fifteen minutes and 2 within thirty minutes. The other 24 specimens were placed in a refrigerator freezer in an empty biopsy bottle within about three minutes. These latter specimens were from private oral surgeons, and dry ice was not immediately available. When the specimens were not to be processed immediately they were labeled, wrapped in "Saran" for prevention of dehydration, and stored on dry ice.

Sectioning of the tissue was accomplished by mounting the unfixed tissue on a metal chuck cooled by dry ice (-70°C) and cutting it on a microtome placed in a refrigerated box (Cryostat)(35). The temperature of the environment in the cryostat was maintained at about -18° C. The section was manipulated with a fine camel's hair brush as it was being cut to flatten the section and then picked up directly on a clean, dry cover slip. Each of the sections was cut at the six micron setting of the microtome. Serial sectiona were cut and in any given pair of adjacent sections, one was for control and the other for enzyme

demonstration. Every fifth section was stained with H & E for histological study. The mounted sections were stored in the cryostat environment until incubated.

The incubating media *were prepared as a (1) control solution in which only the specific substrate was omitted, and (2) a substrating solution in which all the components were present. Sections of rat kidney were also run with some of the test sections to sample the efficacy of the solutions. The solutions were made up in 10 ml. beakers which would conveniently hold two cover slips containing the sections. At first, each case was mounted separately on a cover slip for incubation, but later on, several cases were mounted on the same cover slip. The beakers were placed in a 37° C oven, and the sections allowed to incubate for given periods of time. For the lactic dehydrogenase and DPN diaphorase sections, it was found that 30 minutes was the optimal incubating period while for the glucose-6-phosphate dehydrogenase and succinic dehydrogenase, three hours was found to be the optimal period. Following incubation the sections were washed, counter-stained with nuclear fast red (kernechtrot), dehydrated through alcohol and xylol, and mounted on slides with "Clearex".

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*See Appendix I

FINDINGS

Forty-eight tissue specimens were examined, and Figure 3 shows the grouping of cases by routine histologic diagnoses. In some specimens there were several diagnostic findings, as for example, when a carcinoma is bounded by dyskeratotic non-invasive epithelium. These extra sites are not included in Figure 3, but are indicated in Table I which is a comprehensive compilation of the data.

Diagnosis		No. of Cases
Normal epithelium		17
Focal keratosis		4
Leukoplakia		13
Squamous cell carcinoma		11
Mucoepidermoid tumor		2
Other (follicular cyst)		1
	Total	48

Figure 3. Diagnostic categories of specimens studied by histochemical dehydrogenase techniques.

Cryostat sections stained with H & E tended to be of poor quality and made interpretation of fine changes in the epithelial cells difficult. Dyskeratotic changes were especially difficult to detect since this distinction is sometimes rather nebulous even in well-fixed sections.

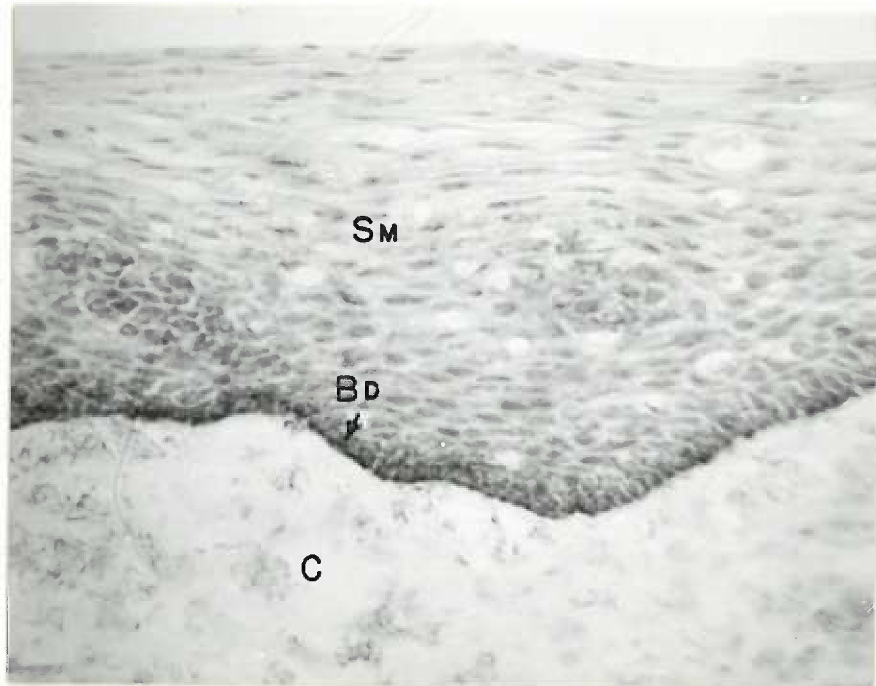

Figure 4. Normal epithelium from an irritation fibroma. Succinic dehydrogenase activity is seen mainly in the basal densities of the basal cells (Bd.) The activity of the enzyme quickly diminishes in the stratum spinosum although some perinuclear activity is seen in these cells (Sm). Some activity is seen in the connective tissue (C) which is largely composed of fibrous elements. The punched-out spaces in the epithelium are probably artifact (ice crystals). Activity evaluation is as follows: Overall staining -- 2+; Basal densities -- 4+; Basal cells -- 3+; Lower stratum spinosum -- 2+; Middle stratum spinosum -- 1+; Upper stratum spinosum -- 1+. (Mag. X 230)

There were other artifacts noted besides the problem of good H & E staining. Ice crystal formation was prominent in some specimens and the severity seemed to be a result of the manner in which the specimen was treated. Those specimens obtained from sources where freezing was initially by refrigerator showed the greatest ice crystal deformity, whereas those specimens quickly frozen on dry ice showed very little. Enzyme activity did not seem to be affected by the ice crystals.

Wrinkling, slight tearing and folding are present, but it is

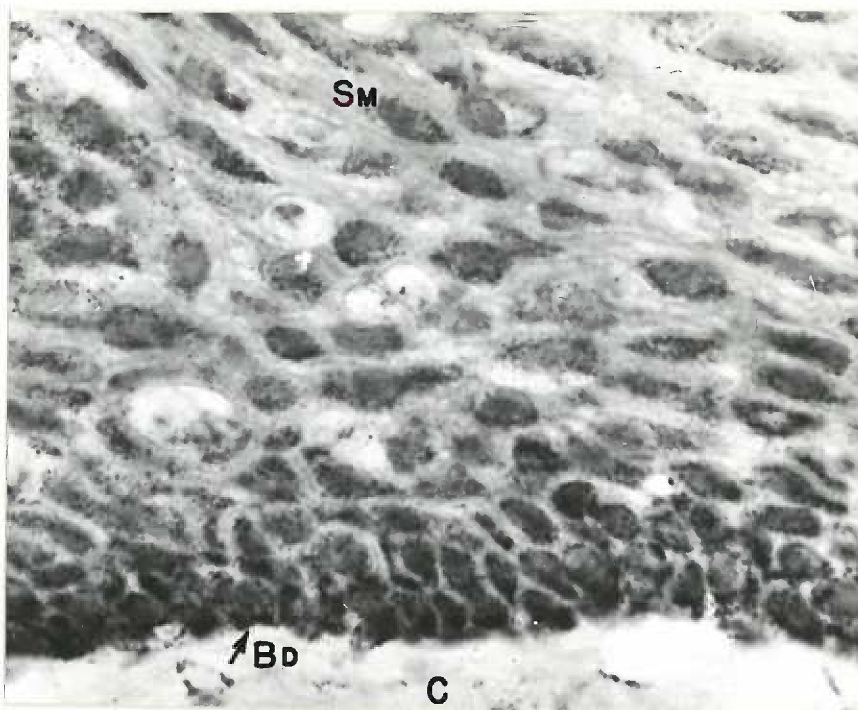

Figure 5. Normal epithelium from an irritation fibroma. The activity of succinic dehydrogenase is greatest in the cells of the stratum germinativum. The dots appear heaviest in the basal portion of the cytoplasm (Bd). Note the change in polarity in the middle cells of the spinosum (Sm). The formazan dots are found mainly in a perinuclear position while the nuclei are probably inactive. The connective tissue shows some activity (C). The vesiculated areas in the epithelium are probably ice crystal artifact. (Mag. X 450)

uncommon for them to seriously affect staining interpretation.

Each enzyme reaction was evaluated by the arbitrary assignment of a semi-quantitative numerical value to the staining intensity, ranging from 0 (negative) to 4+. The sections were categorized as to: (O) overall enzyme activity (including epithelium and connective tissues); (B) basal cell activity, (Sl) lower stratum spinosum activity, (Sm) middle stratum spinosum activity, (So) outer stratum spinosum activity, and (Bd) evaluation of the activity of basal densities. Basal densities is a term referring to rather high sites of activity in the

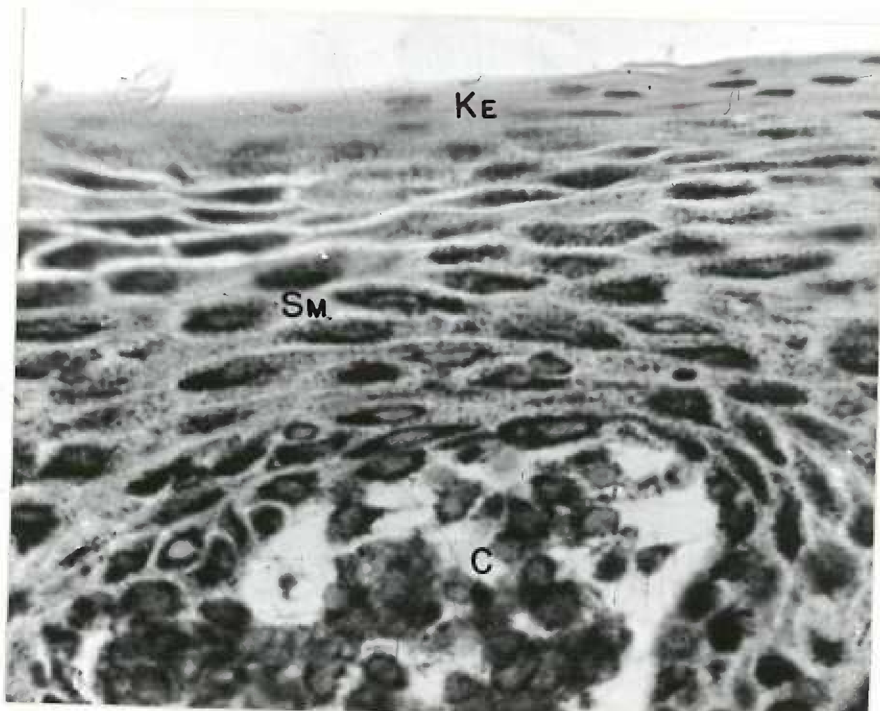

Figure 6. Squamous cell carcinoma of the lip. DPN diaphorase activity of carcinoma is shown in another area of the same tumor as in Fig. 12. Note that the tumor is well differentiated with very condensed perinuclear activity and rather large, discrete granules in other portions of the cytoplasm (Sm). There is some tendency for parakeratin formation (Ke). The connective tissue shows high enzyme activity (C). (Mag. X 450)

proximal cytoplasm of the basal cells (e.g. the portion of the basal cell in contact with the basement membrane). High activity may also be found in the proximal areas of other germinative cells. An example of evaluation is given in Figure 4. Although not evaluated in all cases, the activity of the enzyme in connective tissue was designated as (C).

The four dehydrogenases show consistent general levels of activity in the epithelium, and each enzyme has a fairly consistent pattern of activity in the various layers. There is some variation of activity of the enzyme from one area of the total section to another. The normal epithelial activity of succinic dehydrogenase and glucose-6-phosphate

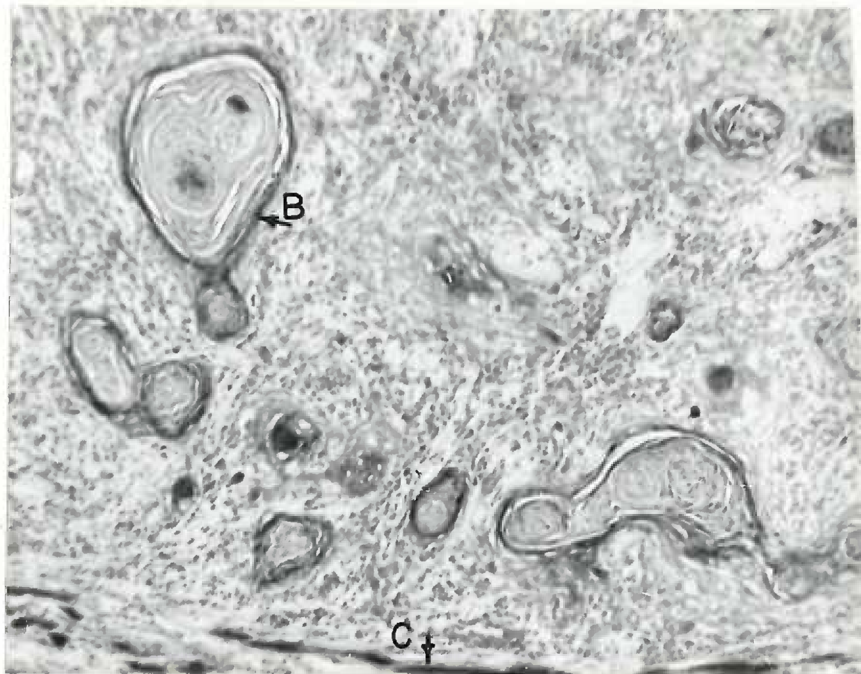

Figure 7. Squamous cell carcinoma of the lip. The glucose-6-phosphate dehydrogenase activity is seen in the basal cell around the epithelial pearls (B) and in muscle bundles (C). (Mag. X 120)

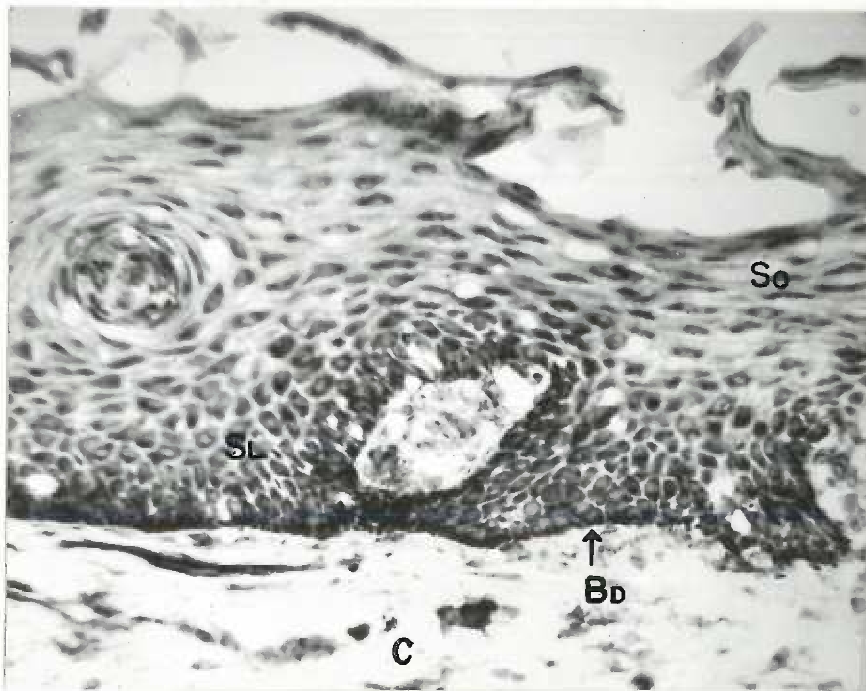

Figure 8. Normal epithelium from an irritation fibroma. DPN diaphorase activity. The lower stratum spinosum (Sl) and the basal densities are prominent (Bd). The upper stratum spinosum (So). The connective tissue (C). (Mag. X 230)

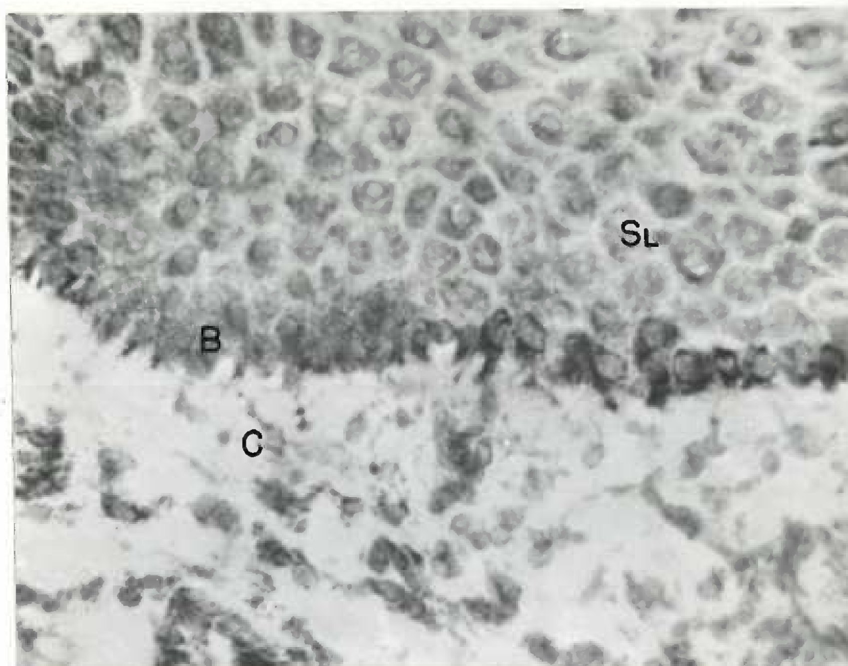

Figure 9. Leukoplakia of the gingiva. DPN diaphorase activity is generally reduced, especially in the basal cells (B). Lower stratum spinosum (Sl). Connective tissue (C). (Mag. X 300)

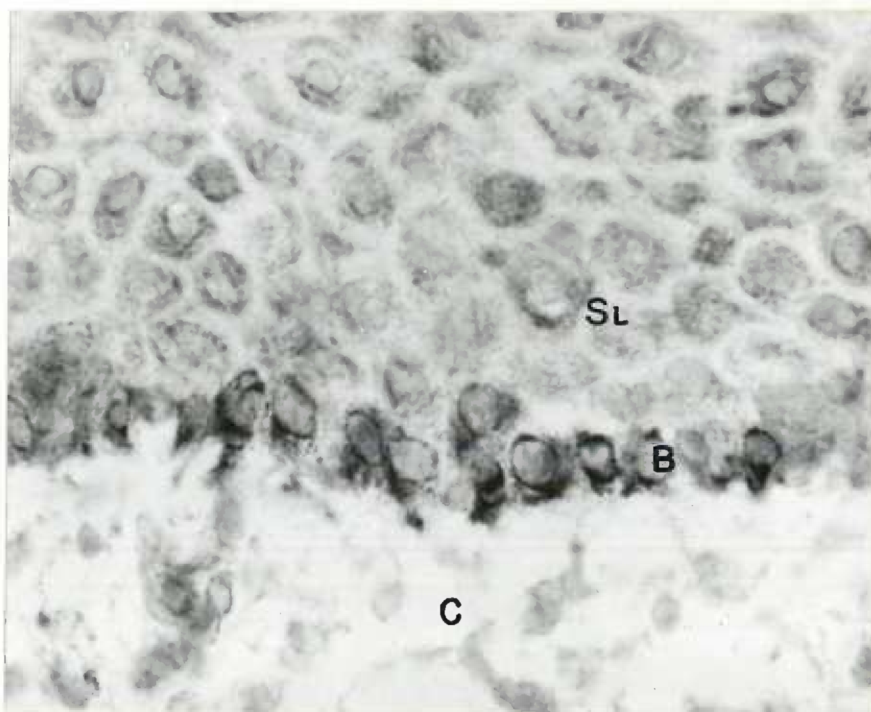

Figure 10. Higher magnification of Figure 9. Note the intense, condensed activity in some of the basal cells (B). Stratum spinosum (Sl). Connective tissue (C). (Mag. X 450)

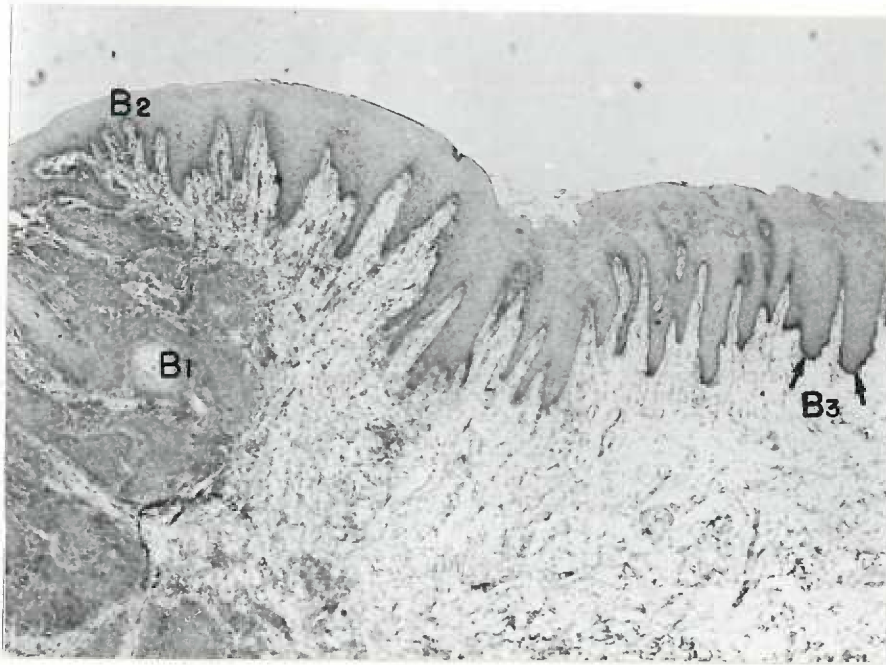

Figure 11. Squamous cell carcinoma of the lip. The carcinoma is invading the connective tissue (B1) and the junction of dyskeratotic epithelium with tumor is shown (B2). The basal cells of the epithelium in the area of (B3) show greater activity than cells closer to the carcinoma ("field effect" of activity loss). (DPN diaphorase) (Mag. x 20)

dehydrogenase was found to be low, while activity levels of lactic dehydrogenase and DPN diaphorase was fairly high as evaluated by the techniques used. Formazan reduction is probably directly proportional to enzyme activity, and therefore, the final intensity of the stain is controlled by the time of incubation.

In the epithelium, the quality of the formazan granules was often quite variable while not appreciably affecting the overall effect of enzyme activity. Increased granule size occurs haphazardly in both normal tissues and carcinoma. Some sections showed very little of this characteristic, while others exhibited a fairly high amount of it. Crystallization of the dye was also found to occur to a great extent in some sections and very little in others.

In areas of severe crystallization, evaluation of enzyme activity

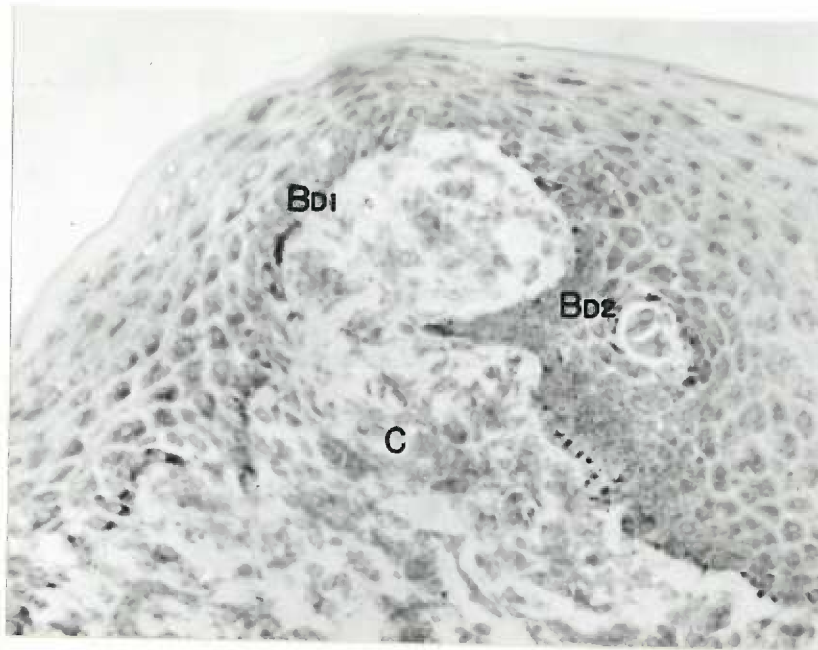

Figure 12. Squamous cell carcinoma of the lip. DPN diaphorase activity shown at the junction of dyskeratotic epithelium (Bd_2) with the portion of the carcinoma that shows invasive characteristics (Bd_1). Note the condensed areas of activity which stand out sharply from the light to medium activity of the other cells. Connective tissue (C). (Mag. X 230)

is somewhat compromised. The distribution of the formazan granules in the cytoplasm generally reflects the polarity of the cells. High power examination of the granules also reveals a linear distribution which also seems to reflect the polar relation of the cell. Cell nuclei and keratinized layers were found to lack enzyme activity in all four systems. Some of these characteristics are shown in Figure 5.

The evaluation of enzyme activity between various cell layers is difficult. In the basal cells, the stain is often fine and diffuse, giving a rather homogenous appearance. In cells of the spinosum, the granules were often more discrete and arranged in a perinuclear, polar fashion. Squamous cell carcinoma presents the same problem, depending on the differentiation of the cells. (Figure 6)

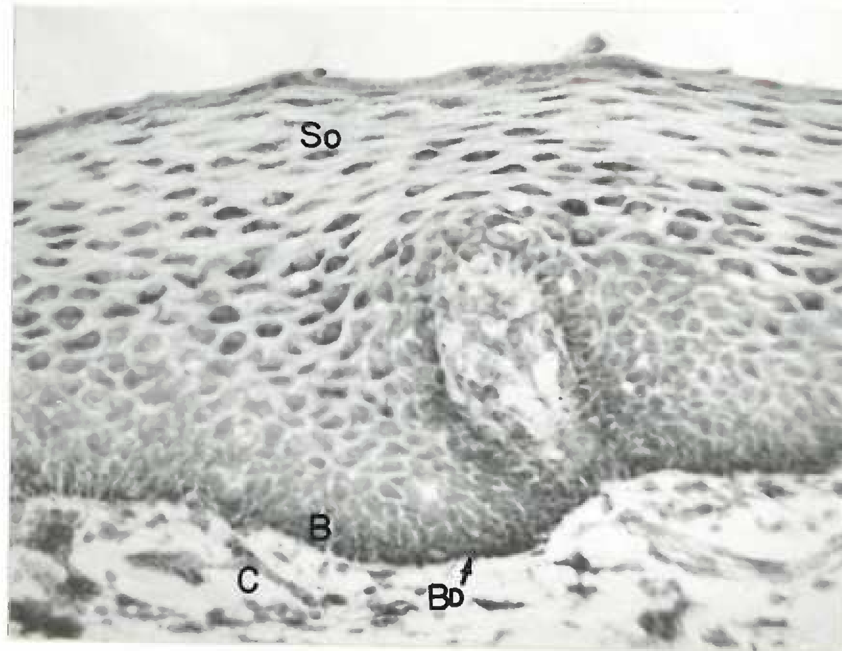

Figure 13. Normal epithelium from an irritation fibroma. Lactic dehydrogenase activity is seen throughout the epithelium, although in this particular case the cells in the lower stratum spinosum show reduced staining. The cells in the upper spinosum are quite active with the formazan dots arranged in a tapered, polar fashion about the nuclei (So). The basal cells (B) and basal densities (Bd). The connective tissue (C). (Mag. X 230)

Other components of the tissue sections showed moderate activity, especially striated muscle which was often very high in activity. The smooth muscle of blood vessels usually shows moderate activity. The cells making up the connective tissues showed very little to moderate activity. The areas of inflammation showed light to moderate activity. The connective tissue often serves to help evaluate the overall enzyme activity unless it is involved in an abnormal process (e.g. fibroma, inflammation, etc.). In the periphery of some carcinomas, the connective tissue activity may show increased activity, especially muscle fibers that are being encroached upon by the tumor. (See Figures 6 and 7)

DPN Diaphorase

The DPN-diaphorase procedure was found to be the technique showing

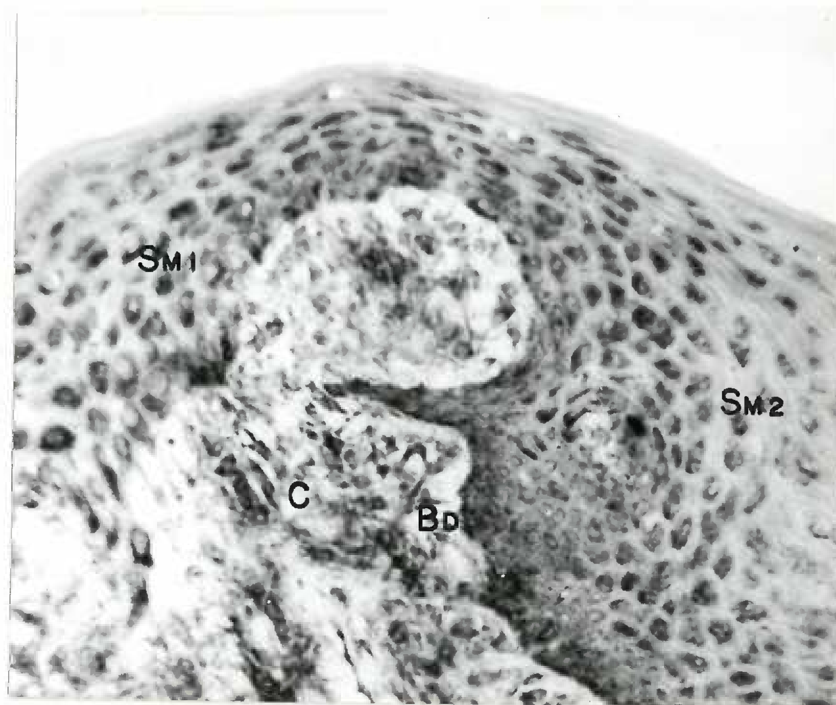

Figure 14. Serial section from specimen in Figure 12. Lactic dehydrogenase activity in the dyskeratotic epithelium (Sm2) and carcinoma (Sm1). There is some condensation of activity in the basal cells of area (Bd) and towards the carcinoma the cells of the spinosum show bizarre activity. Connective tissue (C). (Mag. X 230)

the most "finesse". In normal epithelium there was an orderly appearance to the enzyme activity although some variation occurred from area to area. The stratum germinativum showed the greatest activity and sometimes it was quite predominant in the basal layer. (See Figure 8) The activity within the cells tends to be rather homogenous with a perinuclear concentration. Basal densities are very common but not necessarily present, usually being absent at the bottom of the rete pegs. The basal densities often give the impression of very great enzyme activity in these portions of the basal cells. In the upper layers of cells, the enzyme activity tends to diminish, with a greater perinuclear concentration in a polar fashion. The granules also tend to become more discrete. The combination of change in granule size,

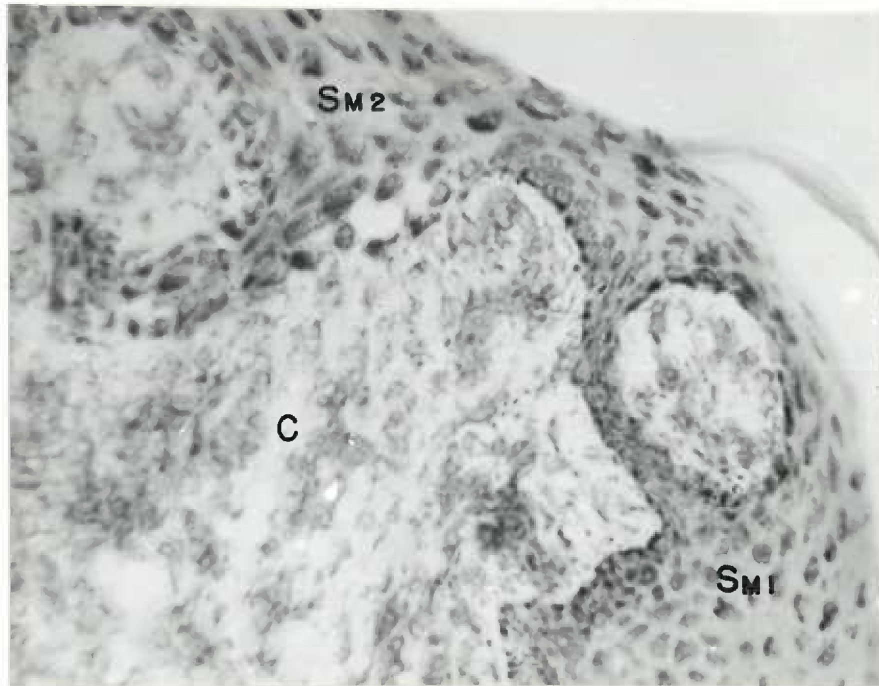

Figure 15. Serial section from specimen in Figure 12. Succinic dehydrogenase activity in the dyskeratotic epithelium (Sm_1) and carcinoma (Sm_2). Note the "pleo-enzymatic" "bursts" of activity in both areas of the epithelium, especially in all layers of the carcinoma. Connective tissue (C). (Mag. X 230)

increased perinuclear concentration and movement of epithelial cells farther apart, makes it difficult to compare the changes in activity through the various layers.

Focal keratosis displayed the same characteristics as the normal epithelium. There was some tendency for an increased variation in enzyme activity from cell to cell, but not noticeably different from variations found in some areas of normal epithelium.

In leukoplakia, the cells showed variability in enzyme activity from cell to cell, with the germinativum cells often losing some of the general, homogenous stain and individual cells exhibiting a rather intense condensation of activity. (See Figures 9 and 10) In some of the squamous cell carcinomas with adjacent epithelium present, there is

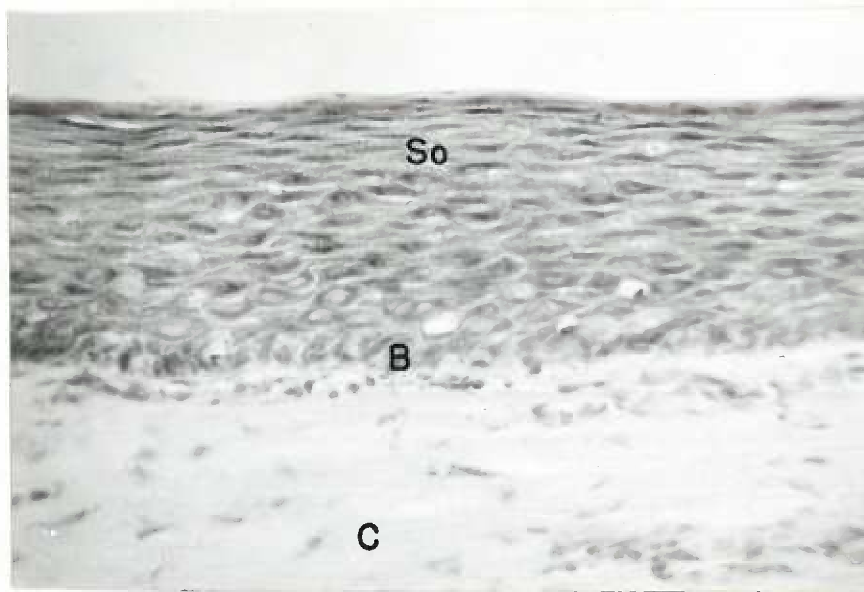

Figure 16. Normal epithelium from an irritation fibroma. Glucose-6-phosphate dehydrogenase activity. Outer stratum spinosum (So). The basal cells (B) are relatively inactive. (Mag. X 230)

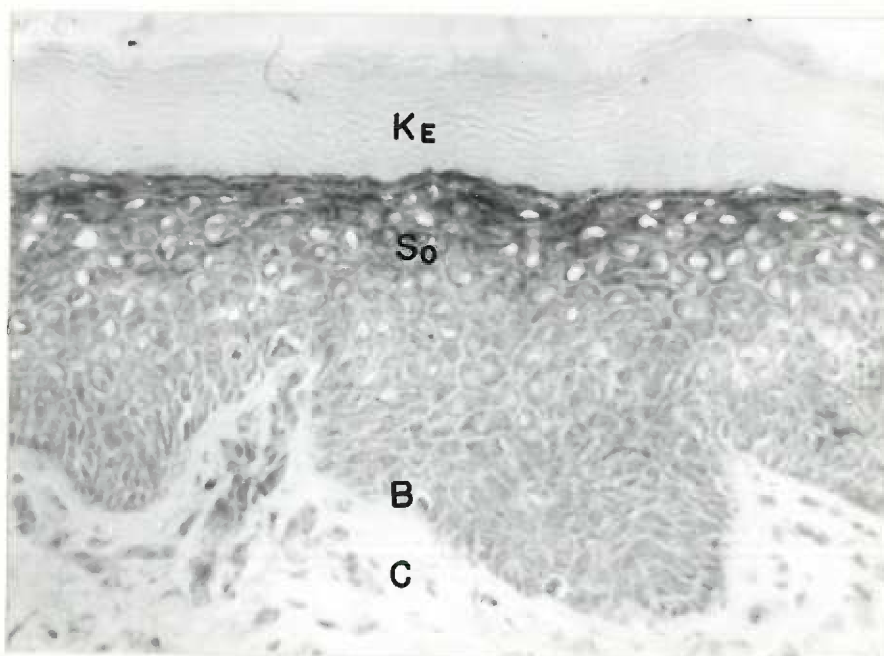

Figure 17. Dyskeratotic epithelium from the lip. Glucose-6-phosphate dehydrogenase activity. Outer stratum spinosum (including stratum granulosum) (So) fades in intensity towards the basal cells (B). Thick hyperkeratotic zone (Ke). Connective tissue (C). (Mag. X 230)

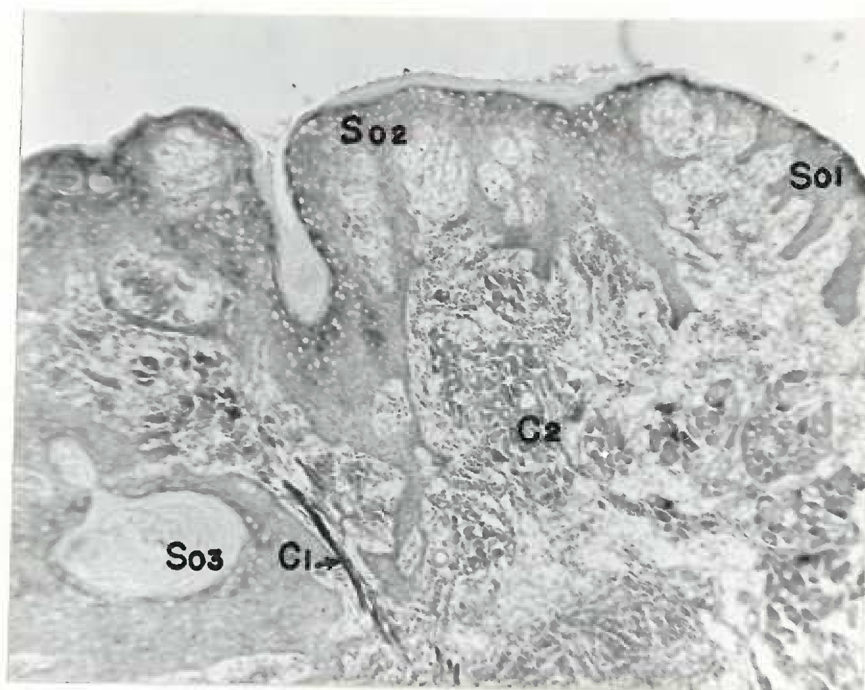

Figure 18. Squamous cell carcinoma of the lip. Glucose-6-phosphate dehydrogenase activity is seen in the dyskeratotic epithelium (So_1) in the surface area of the carcinoma (So_2) about an epithelial pearl (So_3) and in muscle fibers (C_1) and (C_2). The muscle fiber (C_1) shows very high activity while in the muscle bundles (C_2) there are occasional fibers showing increased activity. (Mag. X 45)

seen a "field effect" of activity loss but a few cells still display condensed, bizarre activity. (See Figure 11) There were ten areas showing these enzyme activity changes that were considered to be consistent with leukoplakia; eight areas were considered suggestive of leukoplakia; six areas were questionable. In the two former groups, the H & E sections had been diagnosed either as leukoplakia or questionable leukoplakia. In the group which was questionable on an enzyme basis, five cases were called leukoplakia or questionable leukoplakia on the H & E slides, and one case was primarily inflammatory. (See Table I)

The variability of enzyme activity was very great in carcinoma and the character of the distribution ranged from homogenous to heavily perinuclear. This distribution seemed to reflect the differentiation of the

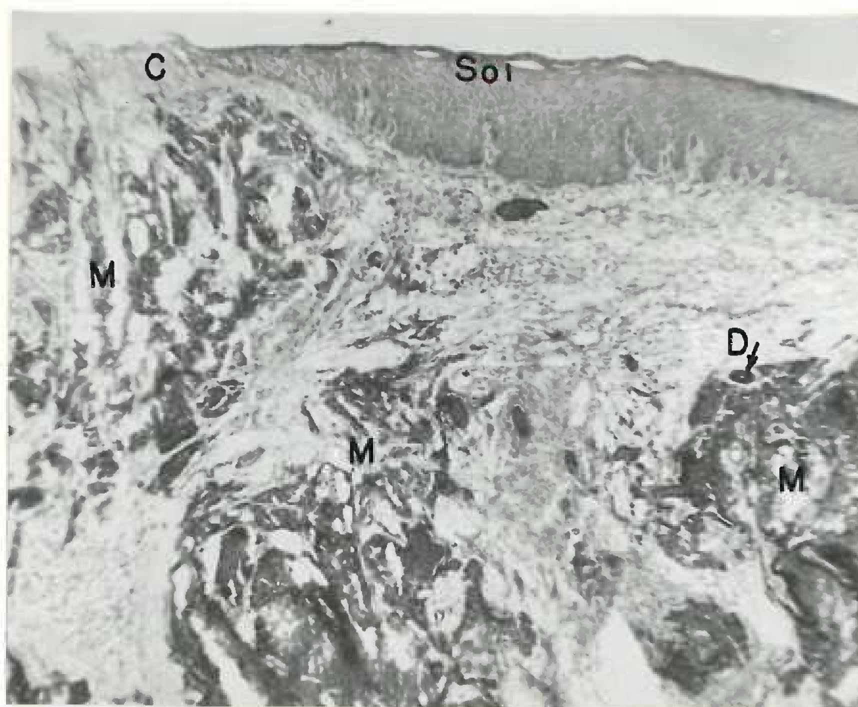

Figure 19. Mucoepidermoid tumor of the palate. Glucose-6-phosphate dehydrogenase activity is very striking in some areas of the tumor (M). Normal epithelium (Sol) shows moderate activity which is greatest in the outer spinosum. Ulceration of the epithelium is shown (C). A normal minor salivary gland duct shows very high enzyme activity (D). (Mag. X 45)

cells. The carcinomas tended to show enzyme activity equal to or greater than normal stratum germinativum. The great variance of haphazard activity in the individual cell often gave the impression of great "pleo-enzymism". (See Figure 12)

Lactate Dehydrogenase

Lactate dehydrogenase distribution was similar to that of DPN diaphorase. The reaction, though, resulted in a coarser appearance of the cells. There was a tendency for the enzyme to maintain good activity throughout the epithelial layers which was not as prominent in DPN diaphorase. Other features such as basal densities are present or absent as described for DPN diaphorase. (See Figure 13)

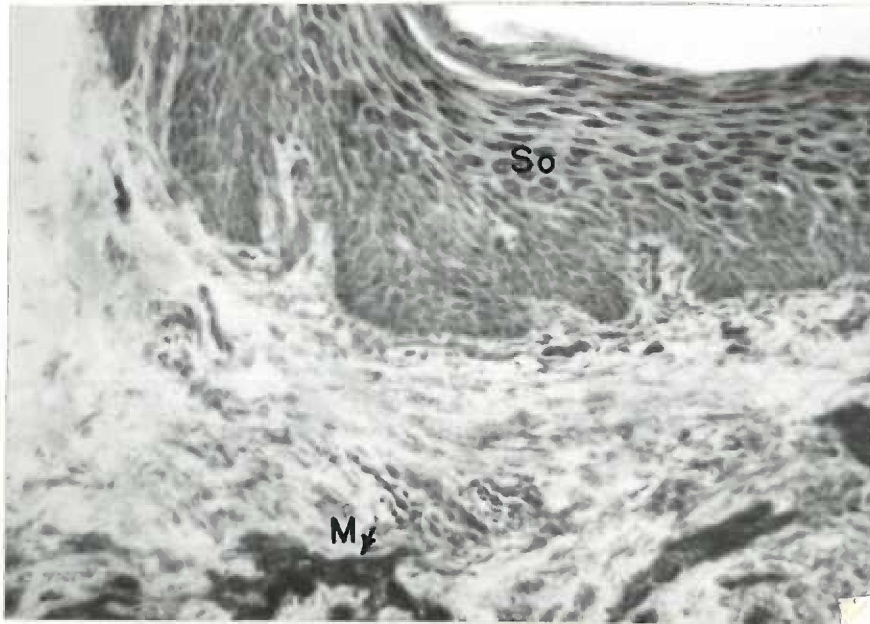

Figure 20. Another area of the specimen shown in Fig. 19. The intense activity of the tumor (M) is probably greater than that of the outer spinosum cells (So). (Mag. X 230)

Bizarre enzymatic changes in lactic dehydrogenase are seen in leukoplakia and carcinoma, but the distinction is less pronounced than in DPN sections. Enzyme activity in carcinoma is equal to or greater than in normal cells just as in DPN diaphorase sections. (See Figure 14)

Succinic Dehydrogenase

This enzyme shows its greatest activity in the basal cells, diminishing quite rapidly in the spinosum layers. (See Figure 4) There is a tendency for the greatest activity to be found in the basal densities.

In leukoplakia, the activity of the stratum germinativum may be haphazard and variable to the point of imparting a bizarre staining pattern. In questionable cases or questionable areas, this pattern may or may not be found, and therefore, the interpretation is equivocal.

The over-all activity pattern in carcinoma is sometimes one of less

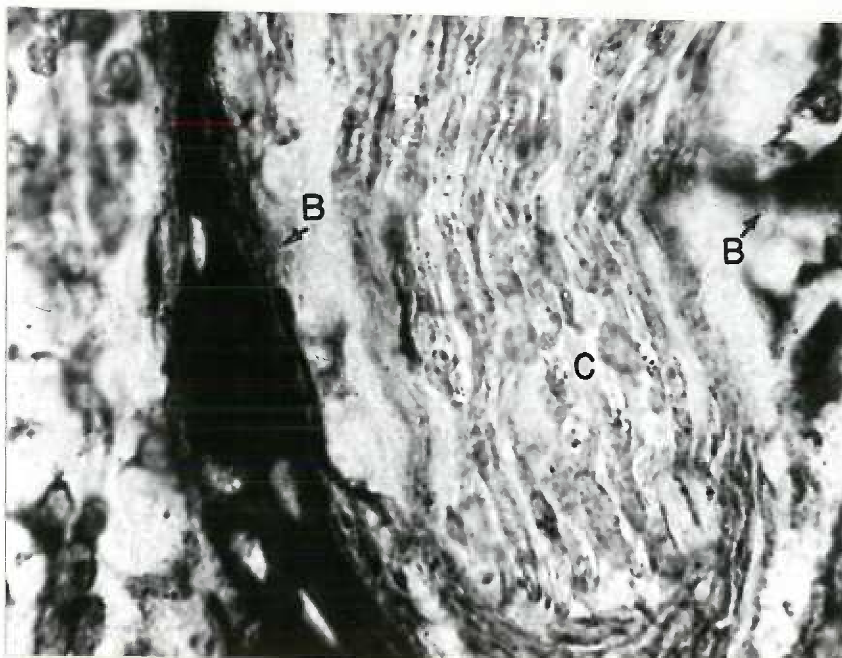

Figure 21. Squamous cell carcinoma of the tongue. High DPN diaphorase activity of the carcinoma cells (B) in perineural invasion (C). (Mag. X 450)

activity than in normal basal cells, however, the epithelium adjacent to a malignancy may show a haphazard burst of activity which lends a "pleoenzymatic" quality to the area (Figure 15). Variable areas in some carcinomas show increased activity.

<u>Glucose-6-Dehydrogenase</u>

Glucose-6-phosphate dehydrogenase is very scarce in the basal cells and increases in activity toward the surface of the epithelium. (See Figures 16 and 17) Its concentration is variable and usually areas of highest activity are found adjacent to a well-keratinized surface or a surface that shows a predilection for keratinization. In leukoplakia or squamous cell carcinoma, its activity again seems related to the degree of keratinization or differentiation of the cells. (Figures 17 and 18) Individual cells in some carcinomas show "bursts" of activity which may reflect early keratinization.

Striking enzyme activity was found in a case of mucoepidermoid

carcinoma (Figures 19 and 20). This activity was concentrated in diverse areas of the tumor and did not seem to be associated with invasiveness. Normal ducts of minor salivary glands showed high activity also. Glucose-6-phosphate dehydrogenase activity is occasionally found in the peripheral cells of squamous cell carcinoma, but it is not as striking as the intense activity of the mucoepidermoid tumor.

DISCUSSION

The validity of histochemical procedures is always open to question, and therefore, a discussion of some of the aspects of this problem is necessary.

Considering the results of this study, several generalizations can be made:

(1) For each of the four techniques used, general patterns of typical activity in the various epithelial strata were delineated which was fairly typical for each enzyme. If the activity was felt to be due to adsorption of the formazan at some distance from the enzyme site, one might expect more of a haphazard staining among the epithelial cells instead of what I consider to be a consistent pattern for each enzyme.

(2) The dye was quite substantive for the protein of the tissue and diffusion of formazan dots was not found beyond cell boundaries. When crystallization of the dye occurred it tended to transcend cell boundaries. The enzymatic inactivity of a thick stratum corneum was always in marked contrast to the underlying cells.

(3) Although a few control sections showed evidence of minimal endogenous reducing activity, the majority were negative in marked contrast to the test sections.

(4) The intensity of the formazan deposition was proportional to enzyme activity and the time of incubation. With good substantivity of the formazan, the localization to any given cell is thought to be fairly accurate.

Some tests performed by Nachlas _et al_ (43), are valuable in establishing the validity of using Nitro-BT as a dependable tetrazolium salt. It is worth summarizing a few facets of their study.

(1) Sections incubated without extrinsic DPN stained only slightly and when the sections were presoaked to remove the intrinsic DPN, they did not stain at all. Even upon addition of one of the specific dehydrogenases in the system, there was no reaction. Addition of DPN resulted in staining. This situation would indicate that it is the diaphorase that finally reduces the Nitro-BT, and not the specific dehydrogenase or DPN. This situation also lends credence to the overall process.

(2) Formulating a solution containing the substrate, its dehydrogenase and DPN yielded no color without the tissue. Also, if reduced DPN (DPN-H) is incubated with Nitro-BT, no color results.

(3) Incubation of tissue in a substrate plus exogenous dehydrogenase (and other necessary elements) results in color in the sections _only_ and not in the supernate. Such results are evidence for the diaphorase being tightly bound to the cell and reducing the substantive Nitro-BT.

Nachlas stresses the point that the diaphorase is the immediate substance responsible for the reduction of the tetrazolium salt, but

the specific dehydrogenase located in certain areas is responsible for activating the reaction, and therefore, the formazan deposition is specific for the localization of the dehydrogenase. DPN diaphorase would be expected to be a rather ubiquitous element to the cells, while the specific dehydrogenases are more specifically localized.

Very recent work by Novikoff et al (46) further supports the validity of Nitro-BT staining. He compared Nitro-BT with another tetrazolium salt and the degree to which they reflected intramitochondrial deposition. Using standard methods of staining mitochondria and electron microscopic studies, he demonstrated:

(1) Nitro-BT staining does indicate the intracellular localization of oxidative enzymes at the light-microscope level. By the electron microscope, it was found that the formazan tended to accumulate at the lipid-aqueous interface when lipid droplets are present.

(2) The Nitro-BT formazan dots reflect mitochondrial changes when they are purposely damaged.

(3) Boiled sections of tissue and the lipid fractions are not enzymatically active, showing that formation of the formazan is dependent upon an active enzyme system. The deposited formazan is not removed by extraction techniques which remove the lipids.

This study indicates that although there is an affect of intracellular lipids on the deposition site of the formazan, the stain is dependent upon enzyme activity and intracellular localization is valid at the light microscope level. It might be mentioned that this work casts doubt upon the concept of Pearse (52) that Nitro-BT is not

affected by lipids. Also, the "other" tetrazolium salt compared to Nitro-BT in this study was M.T.T.-Co^{++}(3-(4,5-dimethylthiazoly-2) -2,5-diphenyl tetrazolium bromide) which was found to be an unreliable indicator of mitochondrial activity. (This salt was developed in Dr. Pearse's laboratory.) Such findings would seem to indicate that the findings of every expert in the field of histochemistry is, as far as all the other experts are concerned, suspect.

Novikoff (47) does point out some factors that may lead to false localization. He feels that freezing and thawing is very damaging to the cells which cannot be adequately compensated by adding polyvinyl pyrrolidone to the substrate media. In this unfixed tissue, the soluble dehydrogenases may diffuse into the incubation media and lead to false localization. Another complication is that transhydrogenase may transfer electrons from TPNH to DPN and such activity can be abolished by use of fixative without loss of the other enzymes. In my study I noted no diffusion effect since each enzyme was generally consistent in its distribution pattern. The lack of staining of the control sections would seem to rule out any significant effect of transhydrogenase activity.

Pearse (50,p.545) feels that dehydrating and mounting the section in the organic media rendered the dye more susceptible to crystallization. In this study dye crystallization was variable and in some instances was severe enough to make interpretation of activity in groups of cells equivocal. This propensity for crystallization would vary considerable within the section itself. Another possible cause is the effect of freezing and thawing upon the cells, and it is my impression that the tissues frozen in the refrigerator freezers showed

more dye crystallization.

The size of the formazan dots was often variable. Groups of cells, both normal and abnormal, would sometimes show a striking granularity in contrast to the other cells. Monis _et al_ (41) attributed this finding in their study to some effect of the tumor. In this study, such an effect was not limited to the tumors, although succinic dehydrogenase sections of carcinomas seemed to be quite prone to exhibit it. I think that these changes may have been brought about by technical manipulations-i.e. early cell changes may affect the dye deposition if there is unmasking of fat.

There is a striking lack of nuclear staining and this has been attributed to the impermeability of the nuclear membrane. It would seem, though, that the sectioning process would cut through a few nuclei rendering the contents available for reaction. Under the microscope there are often formazan dots seen imposed upon the nucleus, but I feel these reactions lie above or below the nucleus. The explanation for the lack of nuclear staining is unknown and might be attributed to one or more of several processes:

(1) The enzymes studied do not exist in the nucleus.

(2) The nucleoplasm as a whole is impermeable to the tetrazolium salt.

(3) The enzymes are in the nucleus, but due to some physicochemical property, the formazan has no substantivity for the nucleoproteins and is deposited as perinuclear granules. The perinuclear accumulations of formazan dots has also been attributed to migration of mitochondria toward the nucleus (28).

The occurrence of basal densities (representing increased enzyme concentration) would seem to reflect the propensity for great metabolic activity at the junction of epithelium and connective tissue. The absence of these densities in variable areas of normal basal cells may indicate that groups of cells vary in their cycles of metabolic activity. The basal cells at the ends of the proliferative rete pegs seemed to show this activity loss most often, and there may be proliferative activity associated with such changes. It could be that in a proliferative phase, the cells undergo certain physico-chemical changes which mask the enzyme activity to this particular technique.

Increased activity of the connective tissue adjacent to squamous cell carcinoma was found at times and this was most prominent in residual muscle fibers of the involved connective tissue. Such increase in activity may reflect more of a degenerative or reactive change in the fibers rather than that due to specific influences of the tumor, since this characteristic is not found in all areas of invasive carcinoma. The increase in glucose-6-phosphate dehydrogenase activity of muscle bundles is especially prominent and may reflect the fibers' attempt to survive by utilization of this glycolytic pathway. (See figures 18,19) Increased activity of other connective tissue elements may be due to increased numbers of inflammatory and connective tissue cells. Moore et al (42) studied the connective tissue about several types of carcinoma and found that metachromatic staining was frequently altered. This alteration was histochemically comparable to that seen in repair of normal tissue. Metachromatic changes were attributed to the increased reproduction of mucopolysaccharides by young fibroblasts rather than by some depolymerizing action of the carcinoma cells. Also the

greatest alterations were found in the less malignant types (e.g. basal cell carcinoma). This may relate to the activity of aminopeptidase in the stroma of tumors. As had been mentioned, some feel that it reflects the generalized proliferative abilities of connective tissue rather than to specific action of the tumor (40,14). Cahn _et al_ (17) very recently reported changes in PAS stain reactions in the basement membrane of keratotic lesions which are dyskeratotic or frank carcinoma. They concluded that a loss of P.A.S. staining in this region may be an indication of malignant changes in a suspect lesion. I think these conclusions should be regarded with caution for several reasons:

(1) Not all of the lesions in their study exhibited these changes.

(2) They did not use inflamed tissue _per se_ (other than Lichen Planus) to observe its effect on the basement membrane of normal epithelium.

(3) They cite only _one_ bibliography reference which seems to indicate that correlation with the work of other investigators is lacking.

This study, though, is very interesting and the results may prove to be of value.

Some features of this study do not fit the findings or implications derived from other studies. Hershey _et al_ (33) found glucose-6-phosphate dehydrogenase activity in the keratin layer of the sole of the foot. His analyses were made by spectrophotometric techniques. In the present study the keratin layers were always negative; however, the sole of the foot was not included in the specimens. Another facet that does not agree on a histochemical basis is the demonstration by Burstone (12)

of cytochrome oxidase in only the basal-cells of free gingiva. In this study both DPN diaphorase and lactic dehydrogenase could be found in the spinous layers of the gingival epithelium. Since both of these enzymes are associated with the cytochrome system, it would seem there should be cytochrome oxidase in these cells. An alternative explanation may be that the hydrogen atoms evolved in these systems are utilized in other metabolic pathways. This explanation is not entirely satisfactory and the difference may well be due to variations in techniques.

In leukoplakia, DPN diaphorase seemed to exhibit the most definable change in enzyme pattern. Lactic dehydrogenase and succinic dehydrogenase also would often impart a bizarre appearance to the cells which had been evaluated as equivocally dyskeratotic on routin H & E sections. The concept of the precancerous cell is accepted by some, and Ayre (1) concludes that all cervical carcinomas pass through a premalignant state ranging from one to twenty years. I do not believe that the present study delineates an entity such as a precancerous <u>cell</u>, but perhaps it does indicate a state of leukoplakia (or premalignant state) on a <u>tissue</u> basis. Changes found in well-defined leukoplakia were very similar to that seen in dyskeratotic epithelium on the edge of a carcinoma, or even to cells in the tumor itself. But to designate any given cell in a field as either a precancerous or a normal cell cannot be done by this investigation at the present time. This study then, has not solved the original question as to how to determine when a cell or group of cells have embarked upon a truly malignant course. The findings do support the contention that the H & E interpretation of leukoplakia as a potentially malignant state is a valid concept.

The problem of interpreting the changes in enzyme activity pattern in the leukoplakic state still remains. Since mitochondrial stains were not performed on these sections, it is unknown if such intensive aggregation of activity is related to these cytoplasmic bodies. Description of such changes in mitochondrial distribution have not been found. Another possibility is that the cells are undergoing physicochemical transformations that are reflected to some degree by the condensation of activity. Bourne (8) describes how the mitochondria act as a "throttle" to the cell by virtue of their capability to change in size, to increase or decrease the number of cristae and to control the rate of substances passing into the reactive area. It is conceivable that any one or any combination of these "throttle" mechanisms may be responsible for the enzymatic changes in leukoplakia.

Consideration can also be given to recent thoughts on the mechanisms of carcinogenesis. Burnet (10) concludes there is no adequate theory for the process, and that it is very unlikely that they should all show the same biochemical lesion. He feels that a variety of mechanisms exists for chemicals, radiation, viruses and hormones to influence cellular and internal environmental processes so as to release clones of cells into abnormal and destructive proliferative activity.

Berenblum (3) and Walpole (65) present ideas on a two-stage mechanism of carcinogenesis. The first stage, initiation, is an immediate, irreversible change that is probably hereditary in nature. The second stage, promotion, is of a variable length of time and has an element of reversibility. The number of tumors eventually appearing is predetermined by the initiator, while the speed with which they appear is

dependent on the effectiveness of the promoting action. Some agents may act as both an initiator and promoter, or may only act at one stage. In promotion, there may be a delay in the maturation of the dormant tumor cell at the germinative stage bringing about a loss inequilibrium between division rate and death rate. This results in a buildup of a clone of these cells which become self-perpetuating after some critical level is attained. All promoters cause epithelial hyperplasia but if the initial change has not taken place, the process of hyperplasia is not promotion _per se_. Many agents can act as promoters, including certain normal physiological processes of the body (e.g. hormones).

This model can be carried further by incorporating some thoughts of Potter and Auerbach (55) who develop the concept of cell activities being regulated by enzyme systems which in turn are regulated by the presence of substances in the cell. The genes determine the presence of enzyme-forming systems (E.F.S.) which are thought to be the RNA constituents of cytoplasm. Therefore, the adaptive abilities of any cell is determined by the presence of the E.F.S., although in normal circumstances, not all the systems are utilized. The appearance and concentration of an enzyme is then dependent first upon genetic determination and secondly upon positive and negative feedback mechanisms acting upon the E.F.S. under environmental patterns governed by other genes. Carcinogenesis is a succession of somatic mutations which result in loss of negative feedback in the E.F.S. for those enzymes involved in cell division and eventually loss of systems needed for DNA synthesis. With deletion of DNA molecules specific for tissue surface proteins (antigens), metastasis and invasion may be more willingly permitted by the tissue. Successive mutations may be due to:

(1) inherent random errors in nucleic acid replication

(2) errors of replication induced by carcinogens

(3) loss of autonomous E.F.S. that fail to replicate and segregate fast enough during cell division.

Applying some of these thoughts to the present study, it can be theorized that the changes of enzyme patterns in leukoplakia reflect the changes in E.F.S. of these cells. The dyskeratotic epithelium on the border of carcinoma also shows these early stages of change. Actually, it might be well to change our concepts slightly and consider any lesion exhibiting leukoplakia as "malignant" and its further development into an invasive entity will depend upon the promoting agents within the environment. In actual practice, we do attempt to warn patients that white patches of the mouth may be premalignant and that they should avoid irritating and stimulating factors. It is also known that many of these lesions do not regress upon removal of an irritant, and it may be that carcinogenic initiation has taken place and promotion is in progress. I think that the proportional increase in cancer with age indicates that these early changes are actually tissues which have undergone initiation and which have an extended latent period of promotion.

The impressions gained in this study will have to be investigated further and an attempt made to derive conclusions that are more definitive and concise. The techniques will have to be improved so that the rather large number of variables present in this study will be reduced. The technique of fixation as suggested by Novikoff et al (46) will be explored, and if it is feasible, many of the technical artifacts can be reduced. Other enzymes which seem to reflect abnormal conditions will be utilized also. For example, I think both phosphamidase and

beta-glucuronidase may be of value, although in preliminary studies I was unable to obtain reliable, valid results.

Several types of keratotic lesions were not obtained during the period of investigation, and attempts will be made to secure them. As mentioned earlier, the histochemical typification of other oral lesions is a future goal. The high activity of glucose-6-phosphate dehydrogenase in the mucoepidermoid tumor was especially interesting and suggests further enzyme histochemical studies of salivary gland tumors.

Histochemical studies of oral smears may prove to be of value in conjunction with Papanicolaou stains. It has been mentioned that there are investigators who feel that enzyme studies of free cells will be of very great value (26). I feel this approach may be of great help, but it does not reflect the integrated and environmental effect that tissue cells in situ have on one another.

SUMMARY AND CONCLUSIONS

Forty-eight fresh surgical specimens of keratotic lesions from the human oral cavity were studies by histochemical techniques for the presence of four dehydrogenase systems. The fresh specimens were serially sectioned in a cryostat and incubated in appropriate substrate solutions to evaluate the activities of DPN diaphorase, lactic dehydrogenase, succinic dehydrogenase and glucose-6-phosphate dehydrogenase. Routine histologic study and shown the specimens to consist of normal epithelium, focal keratosis, leukoplakia*, and squamous cell carcinoma. Study of the enzyme activities of the specimens leads to several findings which must be considered impressions at the present time:

(1) The enzymes are present in the normal epithelium in generally consistent quantities, and in consistent patterns of activity in the various layers. For the techniques used, DPN diaphorase and lactic dehydrogenase showed relatively high activities while, those of succinic dehydrogenase and glucose-6-phosphate dehydrogenase were low. DPN diaphorase, lactic dehydrogenase and succinic dehydrogenase usually showed the most activity in the basal cells, often in the form of basal densities

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*In this report leukoplakia is used as a microscopic diagnosis of a keratotic lesion in which dyskeratosis is present.

(areas of high activity located in the proximal cytoplasm of the basal cell). These basal densities were prone to be present in groups of the basal cells, and absent in other groups, giving rise to the impression that they represented various stages of metabolic activity in the cells. The activity of succinic dehydrogenase often diminished greatly in the stratum spinosum while DPN diaphorase tended to diminish somewhat in the outer cells of the spinosum, and lactic dehydrogenase tended to have fairly uniform activity throughout the epithelium.

(2) In leukoplakia, the three enzymes (DPN, succinic and lactic dehydrogenase) demonstrated a tendency for the overall activity of the cells to appear diminished with a concomitant condensation of activity sites. In dyskeratotic epithelium adjacent to squamous cell carcinoma, there is a field effect of activity loss, while some cells show a condensation of activity, particularly the basal cells. DPN diaphorase shows these changes best, while the other two enzymes show a more haphazard activity which is sometimes equivocal. These changes may be reflecting the promotional effects of certain environmental agents upon cells which have already undergone the initial stages of carcinogenesis.

(3) The enzymes show very haphazard activity in carcinoma. My impression is that DPN diaphorase and lactic dehydrogenase activity is equal to or greater than that of the normal basal cell, while succinic dehydrogenase activity tends to be equal

to or less than that of the normal basal cells.

(4) Glucose-6-phosphate dehydrogenase shows a converse pattern of activity. Its activity is greatest in the outer stratum spinosum and diminishes to almost zero in the basal cells. In keratinized surfaces the activity is greatest in the outer cells of the stratum spinosum (or granulosum) but no activity is found in keratin and parakeratin. In non-keratinized surfaces, the activity is spread more evenly through the stratum spinosum. No specific change relating to squamous cell carcinoma or leukoplakia was noted. In some well-differentiated areas of squamous cell carcinoma, epithelial pearls were ringed by epithelial cells high in enzyme activity and there was some activity on the border of invading sheets of cells. In a mucoepidermoid tumor, very high activity was noted which was equal to or greater than that noted in normal salivary gland ducts (which also show high activity). The activity of this enzyme in the epithelium may reflect the shift of the cells from an aerobic from of glycolysis to an alternate, anaerobic pathway as the cells move away from the blood and oxygen supply present in the connective tissues.

(5) The enzymes studied did not show activity in the nuclei or in keratin. The intracellular pattern showed a perinuclear and polar concentration. Under high power of the microscope, the formazan dots tended to lie in a linear pattern in keeping with the cell polarity.

Future investigation will include work on better techniques of tissue preparation. Probably, great improvement can be attained in the H & E

stains of cryostat sections, as well as in the elimination of ice crystal artifact, dye crystallization and wrinkling of the sections. Duplication of these present findings by using fixation techniques (mentioned in the discussion section) is desirable. Even though the original proposal to histochemically determine the point at which a cell embarks upon a malignant course has not been achieved, I feel the study has made progress in this direction. The histochemistry of other keratin-producing lesions not obtained during this study still need to be investigated.

TABLE I(a)

COMPREHENSIVE PRESENTATION OF DATA

Key:

Oral Site:
A = alveolar mucosa
B = buccal mucosa
F = floor of mouth
G = gingiva
L = lip
P = palate
T = tongue

Microscopic diagnoses of the H & E fixed tissue and the cryostat prepared tissue.
n = normal epithelium
f = focal keratosis
l = leukoplakia
c = carcinoma
i = inflammation
q = questionable diagnosis
Letters in () denote additional diagnoses.

Grouped Cases: Cases processed simultaneously in the same solutions grouped by case number.

Case No.	Oral Site	H&E Diag.	Cryo. Diag.	Grouped Cases	Comments:
1	A	f	l	1	Enzymes I,II: changes consistent with leukoplakia (loss of basal activity with condensation of activity about nuclei).
2A+	T	c,(n)	c,(n)	2A	Enzymes I,II: strands of invasive carcinoma. Very active (4+).
2B+	T	c,(n)	c,(n)	2B	Enzymes I,II: show higher activity in carcinoma than in normal tissue. Some muscle and glandular elements show high activity. (3+) (Enzyme I)
3	A	l	lq	3	Enzymes I,II: changes consistent with leukoplakia.
4	Cyst	n	n	4	Follicular cyst shows a single to double cell layer (epithelial). No lumen is present.
5	L	l	l,(n)	5	Enzyme I: haphazard activity changes noted; questionable for leukoplakia.
6	B	n	n	6	Epithelium lost during sectioning.
7	G	f	f	7 (8A,8B, 9A,9B)	Enzyme I: poor section; other enzymes not unusual; some variable loss of basal densities.

+Specimens from the same patient.

TABLE I(a)

Enzyme:
- I = DPN diaphorase
- II = Lactic dehydrogenase
- III = Succinic dehydrogenase
- IV = Glucose-6-phosphate dehydrogenase

Area:
- O = overall activity
- Bd = basal densities
- B = basal cells
- Sl = lower stratum spinosum
- Sm = middle stratum spinosum
- So = outer stratum spinosum (or granulosum)

Activity:
- 0 = none
- t = trace
- 1 = slight
- 2 = low
- 3 = moderate
- 4 = high

	I						II						III						IV					
	O	Bd	B	Sl	Sm	So	O	Bd	B	Sl	Sm	So	O	Bd	B	Sl	Sm	So	O	Bd	B	Sl	Sm	So
	2	1	2	2	1	1	3	2	3	2	2	1	*	-	-	-	-	-	*	-	-	-	-	-
	2	3	2	2	1	1	3	3	3	2	2	1	*	-	-	-	-	-	*	-	-	-	-	-
	2	2	2	1	1	1	3	3	3	2	2	1	*	-	-	-	-	-	*	-	-	-	-	-
	2	2	1	1	1	1	2	2	1	1	1	1	*	-	-	-	-	-	*	-	-	-	-	-
	3	-	3	-	-	-	3	-	3	-	-	-	*	-	-	-	-	-	*	-	-	-	-	-
	3	2	3	3	2	2	3	-	-	-	-	**	*	-	-	-	-	-	*	-	-	-	-	-
	-	-	-	-	-	**	3	-	-	-	-	**	*	-	-	-	-	-	*	-	-	-	-	-
	2	1	2	2	1	1	3	3	2	2	3	2	3	4	3	3	2	1	2	0	t	t	1	2

*Incubation time insufficient to demonstrate enzyme.
**Technical interference with enzyme evaluation.

TABLE I(b)

Key :

Oral Site:
A = alveolar mucosa
B = buccal mucosa
F = floor of mouth
G = gingiva
L = lip
P = palate
T = tongue

Microscopic diagnoses of the H & E fixed tissue and the cryostat prepared tissue.
n = normal epithelium
f = focal keratosis
l = leukoplakia
c = carcinoma
i = inflammation
q = questionable diagnosis
Letters in () denote additional diagnoses.

Grouped Cases: Cases processed simultaneously in the same solutions grouped by case number.

Case No.	Oral Site	H&E Diag.	Cryo. Diag.	Grouped Cases	Comments:
8A+	T	c	c,(l)	8A (7)	Enzymes I,II,III: bizarre activity patterns consistent with leukoplakia. Little activity in basal cells and occasional condensed sites of activity. Enzyme IV found in occasional cells undergoing abnormal maturation.
8B+	G	-	n	8B	Enzyme I: notable lack of basal cell activity and basal densities (due to proliferation?). Heavy activity in middle stratum spinosum. Other enzymes of normal pattern.
9A++	T	-	c,(l)	9A	Enzymes I,II,III: bizarre activity patterns of epithelium with enzyme I especially showing change consistent with leukoplakia.
9B++	L. node	-	c	9B	Metastatic carcinoma cells in lymph node show activity equal to some basal cells of 9A (Enzymes I,II). Enzymes III & IV less active in carcinoma.
10	G	n	n	10	Normal activity characteristics. Enzyme II: basal densities very prominent.
11	G	n	n	11	Enzymes I,II,IV: shows fair activity in blood vessels. Enzyme II shows some haphazard activity in middle stratum spinosum while activity in basal cells is more homogenous and lacks basal densities.

+Specimens from different patients
++Specimens from the same patient

TABLE I(b)

Enzyme:
- I = DPN diaphorase
- II = Lactic dehydrogenase
- III = Succinic dehydrogenase
- IV = Glucose-6-phosphate dehydrogenase

Area:
- O = overall activity
- Bd = basal densities
- B = basal cells
- Sl = lower stratum spinosum
- Sm = middle stratum spinosum
- So = outer stratum spinosum (or granulosum)

Activity:
- 0 = none
- t = trace
- 1 = slight
- 2 = low
- 3 = moderate
- 4 = high

	I						II						III						IV					
	O	Bd	B	Sl	Sm	So	O	Bd	B	Sl	Sm	So	O	Bd	B	Sl	Sm	So	O	Bd	B	Sl	Sm	So
	2	t	1	1	1	1	2	1	1	1	2	2	4	4	2	2	1	1	1	0	t	t	t	1
	3	t	1	1	3	2	2	3	2	2	2	2	4	4	3	2	2	1	3	0	t	t	1	3
	1	t	2	1	1	t	3	1	3	3	2	2	3	4	3	3	2	1	1	0	t	t	t	1
			2						3						4						t			
	2	2	2	2	2	1	2	4	2	2	2	2	2	1	2	2	1	1	2	0	t	t	1	2
	3	1	2	3	3	3	3	t	1	1	3	3	1	t	1	t	t	t	3	0	1	1	2	3

TABLE I(c)

Key:

Oral Site:
- A = alveolar mucosa
- B = buccal mucosa
- F = floor of mouth
- G = gingiva
- L = lip
- P = palate
- T = tongue

Microscopic diagnoses of the H & E fixed tissue and the cryostat prepared tissue.
- n = normal epithelium
- f = focal keratosis
- l = leukoplakia
- c = carcinoma
- i = inflammation
- q = questionable diagnosis

Letters in () denote additional diagnoses.

Grouped Cases: Cases processed simultaneously in the same solutions grouped by case number.

Case No.	Oral Site	H&E Diag.	Cryo. Diag.	Grouped Cases	Comments:
12	L	l	l	12	Enzyme I shows change consistent with leukoplakia; enzymes II & III show some haphazard, bizarre staining patterns also.
14A+	L	-	l,(f)	14A	Enzymes I,II,III: show haphazard activity characteristics consistent with leukoplakia.
14B+	L	-	l,(f)	14B	Same changes noted as in 14A.
15	G	-	n,lq)	15	Enzymes I,II,III: show enough change in pattern so as to question the possibility of leukoplakia.
16	B	n	n	16	Ice crystal artifact prominent. Heavy granule formation in enzyme I,II & III. Some haphazard activity areas in enzyme III.
17	B	n	n	17	Enzymes I,II,III: very heavy activity in basal area. Normal appearance.
18	B	n	n,(fq)	18	Enzymes I,II,III: high activity in basal cells. Enzyme III shows some haphazard activity.
19	G	-	n,(lq)	19	Approximately the same characteristics as #18.
20	P	l	l	20 (23)	Enzymes I,II,III: haphazard, bizarre activity appearance with loss of activity in basal cells. Suggestive of leukoplakia.

+Specimens from the same patient

TABLE I(c)

Enzyme:
I = DPN diaphorase
II = Lactic dehydrogenase
III = Succinic dehydrogenase
IV = Glucose-6-phophate dehydrogenase

Area:
O = overall activity
Bd = basal densities
B = basal cells
Sl = lower stratum spinosum
Sm = middle stratum spinosum
So = outer stratum spinosum (or granulosum)

Activity:
0 = none
t = trace
1 = slight
2 = low
3 = moderate
4 = high

	I						II						III						IV					
	O	Bd	B	Sl	Sm	So	O	Bd	B	Sl	Sm	So	O	Bd	B	Sl	Sm	So	O	Bd	B	Sl	Sm	So
	2	2	2	2	2	2	3	3	3	3	2	2	3	2	3	2	2	t	4	0	t	t	2	4
	3	4	2	3	2	1	3	4	3	2	2	2	3	4	2	2	1	t	4	t	1	1	2	4
	4	4	3	2	2	2	4	4	4	3	2	2	2	2	1	1	t	t	3	0	t	t	2	3
	1	1	2	1	1	1	3	4	3	3	2	2	2	3	2	2	2	1	3	t	t	t	1	2
	2	3	3	2	1	1	3	4	3	3	2	2	3	3	3	2	1	1	2	0	1	2	2	2
	2	4	3	2	1	1	3	4	2	2	3	3	3	4	3	2	1	1	3	1	1	1	2	3
	2	3	3	2	1	2	3	3	2	2	1	1	3	4	3	2	1	t	2	0	t	1	2	2
	4	4	4	3	2	2	3	4	3	3	3	3	3	3	3	3	2	t	4	1	1	1	3	4
	1	1	1	t	t	t	2	1	2	2	1	1	1	1	1	t	t	t	2	0	t	t	1	3

TABLE I(d)

Key:

Oral Site:
A = alveolar mucosa
B = buccal mucosa
F = floor of mouth
G = gingiva
L = lip
P = palate
T = tongue

Microscopic diagnoses of the H & E fixed tissue and the cryostat prepared tissue.
n = normal epithelium
f = focal keratosis
l = leukoplakia
c = carcinoma
i = inflammation
q = questionable diagnosis
Letters in () denote additional diagnoses.

Grouped Cases: Cases processed simultaneously in the same solutions grouped by case number.

Case No.	Oral Site	H&E Diag.	Cryo. Diag.	Grouped Cases	Comments:
21	L	c	c(l)	21	Enzymes I,II,III: changes consistent with leukoplakia. Field effect of activity loss near the invading carcinoma. Very bizarre, haphazard activity of cells in carcinoma proper. Enzymes I,II,IV show greater activity in carcinoma while enzyme III activity about the equal to normal.
22	L	c	c(l)	22	Same characteristics as in #21. Enzyme IV activity high about some epithelial pearls of the carcinoma.
23	F	c	c(lq)	23 (20)	Enzymes I,II,III: some activity changes found in epithelium suggestive of leukoplakia. The activity of the carcinoma is greater than that of the normal (4+).
24	G	n	n	24	Good uniformity and consistency of activity.
25	B	f	f	25	Enzymes I,II: increased size of formazan dots giving an increased haphazard appearance; not considered to be leukoplakia.
26	B	l	l	26	Enzymes I,II,III: general lack of basal densities; increase in size of perinuclear granules; bizarre activity pattern suggestive of leukoplakia.
27	B	l	f(lq)	27	Enzymes I,II,III: lack of basal densities; some increase in granule size and bizarre appearance; questionable for leukoplakia.

TABLE I(d)

Enzyme:
I = DPN diaphorase
II = Lactic dehydrogenase
III = Succinic dehydrogenase
IV = Glucose-6-phosphate dehydrogenase

Area:
O = overall activity
Bd = basal densities
B = basal cells
Sl = lower stratum spinosum
Sm = middle stratum spinosum
So = outer stratum spinosum (or granulosum)

Activity:
0 = none
t = trace
1 = slight
2 = low
3 = moderate
4 = high

	I						II						III						IV					
	O	Bd	B	Sl	Sm	So	O	Bd	B	Sl	Sm	So	O	Bd	B	Sl	Sm	So	O	Bd	B	Sl	Sm	So
	1	2	1	1	t	t	3	3	3	3	2	1	4	3	3	3	2	1	2	0	t	t	1	3
	2	2	2	2	1	1	3	4	3	3	3	3	4	4	4	3	2	2	3	t	t	t	1	4
	1	2	1	t	t	t	3	4	3	2	2	2	3	4	3	3	2	1	1	1	t	t	1	1
	1	2	1	1	1	t	3	4	1	1	t	t	2	3	2	2	1	t	-	-	-	-	-	**
	1	1	1	2	1	1	2	1	1	1	t	t	1	1	1	t	t	t	4	t	t	t	2	4
	1	1	1	1	1	t	3	1	3	3	2	2	1	1	1	1	t	t	1	0	t	t	1	2
	1	1	1	1	1	1	3	1	3	3	3	2	1	2	1	1	1	t	1	0	t	t	1	1

**Technical interference with enzyme evaluation.

TABLE I(e)

Key:

Oral Site:
A = alveolar mucosa
B = buccal mucosa
F = floor of mouth
G = gingiva
L = lip
P = palate
T = tongue

Microscopic diagnoses of the H & E fixed tissue and the cryostat prepared tissue.
n = normal epithelium
f = focal keratosis
l = leukoplakia
c = carcinoma
i = inflammation
q = questionable diagnosis
Letters in () denote additional diagnoses.

Grouped Cases: Cases processed simultaneously in the same solutions grouped by case number.

Case No.	Oral Site	H&E Diag.	Cryo. Diag.	Grouped Cases	Comments:
28	L	f	f,(lq)	28	Enzymes I,II: increased granule size in some areas with slight haphazard pattern; questionable for leukoplakia.
29	P	n	n,(i)	29	Enzymes I,II,III: chronic inflammation does not seem to affect epithelial activity greatly; haphazard activity patterns questionable for leukoplakia. Enzyme IV: areas of higher activity appear related to degree of keratinization.
30	F	c	c,(l)	30	Enzymes I,II,III: epithelial changes consistent with leukoplakia; bizarre activity patterns in carcinoma: carcinoma shows activity equal to or greater than normal in all 4 enzymes.
31	G	n	n	31	High activity patterns in all enzymes; consistent and even pattern.
32	T	n	n,(lq)	32	Enzyme II: some haphazard activity patterns.
33	P	n*	n*	33	Enzyme II: some haphazard activity in epithelium. Tumor shows high activity for all enzymes and especially enzyme IV. Normal ducts also show high activity.
34	B	l	l	34	Enzymes I,II,III: changes consistent with leukoplakia.
35	L	n	n,(lq)	35 (36)	Enzyme I,II: slight changes in activity pattern; questionable for leukoplakia.

*Mucoepidermoid tumor with ulceration of mucosa.

TABLE I(e)

Enzyme:
I = DPN diaphorase
II = Lactic dehydrogenase
III = Succinic dehydrogenase
IV = Glucose-6-phosphate dehydrogenase

Area:
O = overall activity
Bd = basal densities
B = basal cells
Sl = lower stratum spinosum
Sm = middle stratum spinosum
So = outer stratum spinosum (or granulosum)

Activity:
0 = none
t = trace
1 = slight
2 = low
3 = moderate
4 = high

	I						II						III						IV					
	O	Bd	B	Sl	Sm	So	O	Bd	B	Sl	Sm	So	O	Bd	B	Sl	Sm	So	O	Bd	B	Sl	Sm	So
	3	4	3	2	1	1	3	3	2	2	1	t	1	2	1	t	0	0	4	0	t	t	1	4
	3	2	3	2	1	1	2	1	2	2	1	t	2	1	2	2	1	t	1	0	t	t	t	1
	1	1	2	t	t	t	3	4	3	3	2	2	3	4	3	3	2	t	t	0	t	t	t	t
	3	4	3	2	2	3	4	4	4	4	4	4	3	4	3	3	2	1	4	t	t	1	3	4
	2	2	2	2	1	1	3	2	3	3	2	3	3	4	3	2	1	t	4	t	t	1	2	4
	2	2	2	2	2	2	3	4	3	3	2	4	3	4	3	2	1	1	3	1	1	1	t	t
	2	2	2	1	1	2	3	3	2	2	2	3	2	3	2	2	1	t	2	0	t	t	1	2
	2	3	2	1	t	t	4	4	4	3	3	4	3	3	3	3	2	1	4	1	t	2	3	4

TABLE I(f)

Key:

Oral Site:
A = alveolar mucosa
B = buccal mucosa
F = floor of mouth
G = gingiva
L = lip
P = palate
T = tongue

Microscopic diagnoses of the H & E fixed tissue and the cryostat prepared tissue.
n = normal epithelium
f = focal keratosis
l = leukoplakia
c = carcinoma
i = inflammation
q = questionable diagnosis
Letters in () denote additional diagnoses.

Grouped Cases: Cases processed simultaneously in the same solutions grouped by case number.

Case No.	Oral Site	H&E Diag.	Cryo. Diag.	Grouped Cases	Comments:
36	L	l	f(lq)	36 (32,33 34,35)	Enzymes I,III: loss of basal densities and increased granularity of activity; suggestive of leukoplakia.
37	L	l	l	37	Enzymes I,II,III: loss of basal densities and some great increase of granularity; very suggestive of leukoplakia.
38	P	n*	n*	38	Slight haphazard activity patterns noted in enzymes I,II but not interpreted as leukoplakia. Tumor shows same activity as in Case #33.
39	L	l	f(lq)	39	Enzymes I,II,III: increased granularity and haphazard activity patterns are suggestive of leukoplakia.
40	G	-	n	40	Enzyme II: some increase in granularity and haphazard appearance.
41	G	n(i)	n(i)	41	Granulomatous areas (sarcoidosis) show good activity for all enzymes. Inflammation seems to affect epithelial activities but do not resemble changes of leukoplakia.
42	P	n	n	42 (43,44 45)	Enzyme II: slight haphazard activities. Enzyme IV: good activity in the connective tissues.

*mucoepidermoid tumor with ulceration of mucosa.

TABLE I(f)

Enzyme:
- I = DPN diaphorase
- II = Lactic dehydrogenase
- III = Succinic dehydrogenase
- IV = Glucose-6-phosphate dehydrogenase

Area:
- O = overall activity
- Bd = basal densities
- B = basal cells
- Sl = lower stratum spinosum
- Sm = middle stratum spinosum
- So = outer stratum spinosum (or granulosum)

Activity:
- 0 = none
- t = trace
- 1 = slight
- 2 = low
- 3 = moderate
- 4 = high

	I						II						III						IV					
	O	Bd	B	Sl	Sm	So	O	Bd	B	Sl	Sm	So	O	Bd	B	Sl	Sm	So	O	Bd	B	Sl	Sm	So
	2	2	1	2	2	2	3	2	2	2	3	2	2	4	2	1	t	t	2	0	t	t	1	2
	2	3	2	2	1	1	3	3	3	2	3	2	2	3	2	2	1	t	1	0	t	t	t	1
	2	1	2	2	2	2	3	3	2	2	3	2	3	2	3	3	1	t	2	0	t	t	t	2
	3	3	4	3	2	2	3	4	3	3	2	2	3	3	3	3	2	1	1	0	t	t	t	1
	1	1	1	1	t	t	2	3	2	2	2	2	3	4	3	2	2	1	2	0	t	t	1	2
	2	2	1	2	1	1	2	t	2	1	2	2	2	3	2	2	1	t	3	0	1	1	2	3
	2	2	2	2	1	1	2	2	2	2	3	2	2	3	2	2	1	t	4	1	1	2	3	4

TABLE I(g)

Key:

Oral Site:
A = alveolar mucosa
B = buccal mucosa
F = floor of mouth
G = gingiva
L = lip
P = palate
T = tongue

Microscopic diagnoses of the H & E fixed tissue and the cryostat prepared tissue.
n = normal epithelium
f = focal keratosis
l = leukoplakia
c = carcinoma
i = inflammation
q = questionable diagnosis
Letters in () denote additional diagnoses.

Grouped Cases: Cases processed simultaneously in the same solutions grouped by case number.

Case No.	Oral Site	H&E Diag.	Cryo. Diag.	Grouped Cases	Comments:
43	T	-	c	43 (42)	Enzymes I,II,III: homogenous activity in carcinoma with higher activity in the peripheral (basal) cells. Carcinoma activity equal to or greater than normal in I & II, and equal to normal in III & IV.
44	L	c	c,(l)	44	Enzymes I,II,III: field effect of activity loss in epithelium adjacent to carcinoma; consistent with leukoplakia. Bizarre activity in carcinoma. Enzyme I & II: activity in carcinoma equal to or greater than activity of normal. Enzymes III & IV: equal to or less than normal.
45	A	l	l	45	Enzymes I,II,IV: some haphazard activity patterns suggestive of leukoplakia.

TABLE I(g)

Enzyme:
I = DPN diaphorase
II = Lactic dehydrogenase
III = Succinic dehydrogenase
IV = Glucose-6-phosphate dehydrogenase

Area:
O = overall activity
Bd = basal densities
B = basal cells
S1 = lower stratum spinosum
Sm = middle stratum spinosum
So = outer stratum spinosum (or granulosum)

Activity:
0 = none
t = trace
1 = slight
2 = low
3 = moderate
4 = high

	I						II						III						IV					
	O	Bd	B	S1	Sm	So	O	Bd	B	S1	Sm	So	O	Bd	B	S1	Sm	So	O	Bd	B	S1	Sm	So
	2	2	2	2	2	-	2	2	2	2	2	-	1	2	1	1	1	-	3	0	2	1	3	-
	2	t	2	1	1	1	2	t	2	1	3	1	3	1	1	1	1	1	3	0	1	1	2	3
	2	t	2	2	1	1	2	t	2	2	2	1	2	2	1	1	1	t	2	0	t	1	1	3

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Appendix I

Substrate Solutions for Four Dehydrogenase Systems

DPN diaphorase: (as modified from the Histochemistry Manual of the University of Kansas School of Medicine (33), page 14)

0.2 M Tris. buffer pH7	1 ml.
Nitro-BT	5 mg.
Polyvinyl pyrrolidone	350 mg.
H_2O, distilled, q.s.	10 ml.

The solution is divided into two 5 ml. portions and 5 mg. DPNH (reduced DPN) is added to the one solution to make a final substrate preparation and the other solution is the control.

Succinic dehydrogenase: (as modified from the Histochemistry Manual of the University of Kansas School of Medicine (33), page 12)

0.2 M Tris buffer pH7	1 ml.
Nitro-BT	5 mg.
Sodium cyanide	2 mg.
Polyvinyl pyrrolidone	350 mg.
H_2O distilled, q.s.	10 ml.

The solution is divided into two 5 ml. portions and 200 mg. of sodium succinate is added to the one solution to make a final substrate, and the other solution is the control.

Glucose-6-phosphate dehydrogenase (as modified from Pearse's Text, Histochemistry, 2nd Ed., (49), page 911)

TPN	25 mg.
Sodium Azide	2 mg.
$Mg\ Cl_2$	1 mg.
0.2M Tris buffer pH7	1 ml.
NaF (0.01 M)	0.05 ml.
Nitro-BT	5 mg.
Polyvinyl pyrrolidone	350 mg.
H_2O, distilled, q.s.	10 ml.

The solution is divided into two portions and 75 mg. of Glucose-6-phosphate is added to one solution to make a final substrate, and the other solution is used for a control.

Lactic Dehydrogenase (as modified from Pearse's Text, Histochemistry, 2nd Ed., (49), page 911)

0.2 M Tris buffer pH7	1 ml.
DPN	25 mg.
Na cyanide	2 mg.
$Mg\ Cl_2$	1 mg.
Nitro-BT	5 mg.
Polyvinyl pyrrolidone	350 mg.
H_2O, distilled, q.s.	10 ml.

The solution is divided into two portions and 5 drops of sodium lactate (approx. 150 mg.) are added to one solution to make the final substrate, and the other solution is used for a control.