other 2 spots could not be identified with certainty from the standards used, one having an R_f-value (0.65) somewhat higher than adenine (R_f 0.61) and the other (R_f 0.43) rather higher than xanthine (R_f 0.40).

Low power microscopical examination of iridophore preparations demonstrated the presence of similar amounts of reflecting material in a^p/a^p and +/+ cells. The numbers of reflecting platelets increased as iridophore differentiation progressed. High power studies revealed striking differences in the organisation of +/+ and a^p/a^p reflecting platelet populations (figure 1). Day 1 +/+ iridophores provided populations of small, square to hexagonal platelets of uniform colour and luminosity. Platelets from later +/+ iridophores were of similar character but progressively larger in size. The increase in size and number of platelets during the maturation of +/+ iridophores can be correlated to the increasing powers of light reflection and the transition from silver to gold of iridophores in situ in +/+ larvae. Platelets obtained from a^p/a^p iridophores were irregular and angular in shape and heterogeneous in colour and luminosity. Although a^p/a^p platelet populations exhibited size ranges similar to those of corresponding +/+ populations, the size distribution of platelets within each range indicated a persistent preponderance of smaller platelets (figure 2). The less efficient reflecting powers and paucity of gold coloration of maturing a^p/a^p iridophores can be accounted for in terms of the observed atypical morphology and size distribution of a^p/a^p platelets.

The findings reported here suggest that a^p/a^p iridophores have some deficiency in the processes controlling the formation of reflecting platelets. Previous studies[3,4,6] have indicated that a^p/a^p oocytes lack premelanosomes and that melanosomes synthesized in a^p/a^p larval melanoblasts are abnormal in structure. These observations support the proposal that the a^p gene has a pleiotropic effect on organelle development in both melanoblasts and iridoblasts. Further investigation of this effect may aid in establishing the developmental and cellular relationships of these pigment cells.

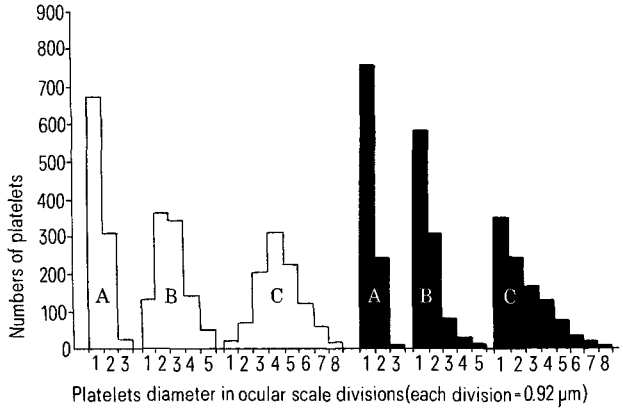

Fig. 2. Size distributions of +/+ and a^p/a^p (black hatched) reflecting platelets (1000 in each group) from day 1 (A), day 3 (B) and day 10 (C) iridophores. Note that while +/+ and a^p/a^p groups of platelets exhibit similar ranges of size, +/+ platelets tend towards maximum in the middle of each range whereas a^p/a^p platelets tend towards maximum in the lowest part of each range.

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Immunohistochemical demonstration of the loss of immunoreactive amylase from neoplastic human salivary gland[1]

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Summary. Antihuman parotid amylase antibodies raised in rabbits and horses were used as the primary antibodies in both the peroxidase-antiperoxidase and sandwich techniques for the localization of human amylase. Immunoreactive enzyme was demonstrated in the normal acinar cells of salivary glands and pancreas. Malignant transformation, which has occasionally resulted in ectopic production of amylase by various tissues, actually caused a loss of amylase synthesis by the transformed acinar cells of salivary glands and did not result in elaboration of amylase by transformed ductal cells.

Comparative peptide mapping studies indicate that human salivary and pancreatic amylases (Amy_1 and Amy_2) differ but slightly in amino acid sequence[3,4]. This should make it possible to use antibody raised against one or the other amylase for immunohistochemical localization of either. Ectopic production of amylase has been reported for certain tumors[5-9]. These tumor amylases closely resemble the salivary enzyme[4]. Several authors have used these properties of amylase and its antibody for immunofluorescent[11,12] and ultrastructural[12] localization of the enzyme. We report here results on localization of amylase in human salivary glands and pancreas, the loss of amylase expression by transformed acinar cells and lack of induction of amylase production in neoplastic ductal cells in human parotid gland tumors.

Materials and methods. Antihuman salivary amylase antibody was prepared in rabbits and horses. The IgG fraction of rabbit serum was isolated by ammonium sulfate precipi-

tation followed by DEAE-cellulose chromatography. The antibodies showed a single line of identity against pure parotid amylase, parotid saliva, whole saliva and extract of human pancreas in an Ouchterlony double diffusion system (figure 1). No precipitation was obtained with human spleen extract. Antibodies were easily absorbed with human pancreatic extract and not at all with human spleen extract.

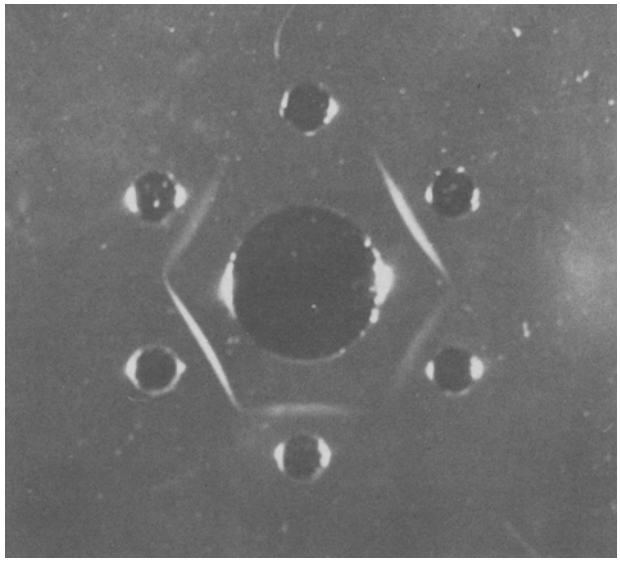

Fig. 1. Double immunodiffusion of rabbit antihuman parotid amylase IgG (center well) against human spleen extract (top well) and (clock-wise) parotid saliva, parotid amylase, human pancreas extract, whole saliva and parotid amylase.

Tissues were obtained at operation or post-mortem examination and processed in the manner customary for pathological specimens. 4-μm sections were placed on egg albumin coated microscope slides. Paraffin was removed from the embedded tissue sections and these were transferred through decreasing concentrations of aqueous alcohol into phosphate-buffered saline, pH 7.2–7.4. Pseudoperoxidase activity was blocked by a 30-min incubation in absolute methanol made 0.03% in H_2O_2[13]. The slides were stained for immunoreactive amylase with the immunoperoxidase sandwich technique as described by Kurstak[14] and the unlabeled antibody technique of Sternberger[15]. Primary antibody was used at 1:1000 dilution, at which concentration adequate specific staining was observed. Primary antibody incubation time was 24 h with the initial 3 h at 37 °C and the remainder at 4 °C. After reaction with 3,3′-diaminobenzidine and 0.001% H_2O_2, the slides were washed, dehydrated and mounted in Permount or Urysalon mounting medium and examined with the light microscope. Negative controls on consecutive 4-μm sections included a) no primary antibody, b) absorbed primary antibody c) no second antibody, and d) no peroxidase-antiperoxidase. Positive controls were sections of human submandibular gland.

Results. Immunoreactive amylase is localized in the acinar cells of the human parotid, submandibular (figure 2) and pancreatic glands. Duct cells, fat, vessels and connective tissue of the semilunar mucinous cells of the submandibular gland (figure 2), and the islet cells of the pancreas all are negative for immunoreactive material. When secretions are present in the ducts, they are invariably positive for amylase.

The neoplastic specimens examined were: 1. benign mixed tumors characterized by nests, cords and tubules of uniform appearing epithelial cells and demonstrating a myxoid chondroid or hyalinized stroma (5 specimens), 2. cystadenoma lymphomatosum (Warthin tumors) characterized by tubular structures and cystic spaces lined by columnar

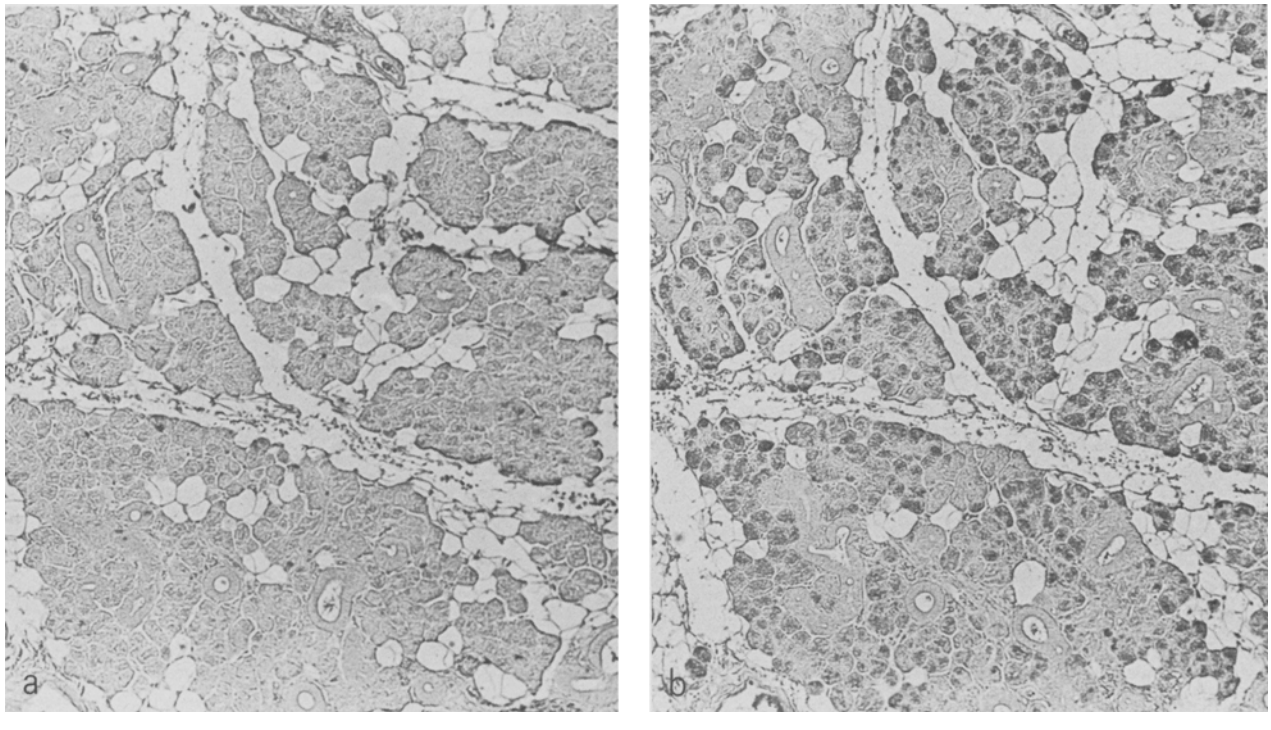

Fig. 2. Normal human submandibular gland, adjacent sections. *a* Control, treated with normal goat serum and *b* treated with rabbit antihuman parotid amylase antibody. Both sections were then treated with horseradish peroxidase conjugated goat antirabbit antibody and subjected to the peroxidase reaction.

acidophilic epithelium, supported by an abundant lymphoid stroma (3 specimens), and 3. mucoepidermoid carcinoma of high-grade malignancy with many epidermoid appearing cells and occasional mucous producing cell (1 specimen). These tumors are thought to arise from the ductal epithelium of the parotid gland. We also stained 4. acinic cell carcinoma (figure 3) which can be recognized by large polyhedral and rounded cells with small, dark eccentrically placed nuclei and abundant finely granular, somewhat basophilic cytoplasm (3 specimens). These tumors are thought to arise from acinar cells. No immunoreactive amylase was evident in any tumor cells observed.

Discussion. The most important component of immunolocalization techniques is the primary antibody. The antibodies used in this study were specific, sensitive and cross-reactive only with human amylase of different tissue origin (e.g., pancreas). We are able to show immunoreactivity on tissue sections with the primary antibody in dilutions as high as 1:5000 (results not presented). Specificity and cross-reactivity was shown by the single line of identity obtained with saliva or pancreatic extract and lack of precipitation with splenic extract in the Ouchterlony double diffusion system.

The immunoperoxidase technique gives exquisite localiza-

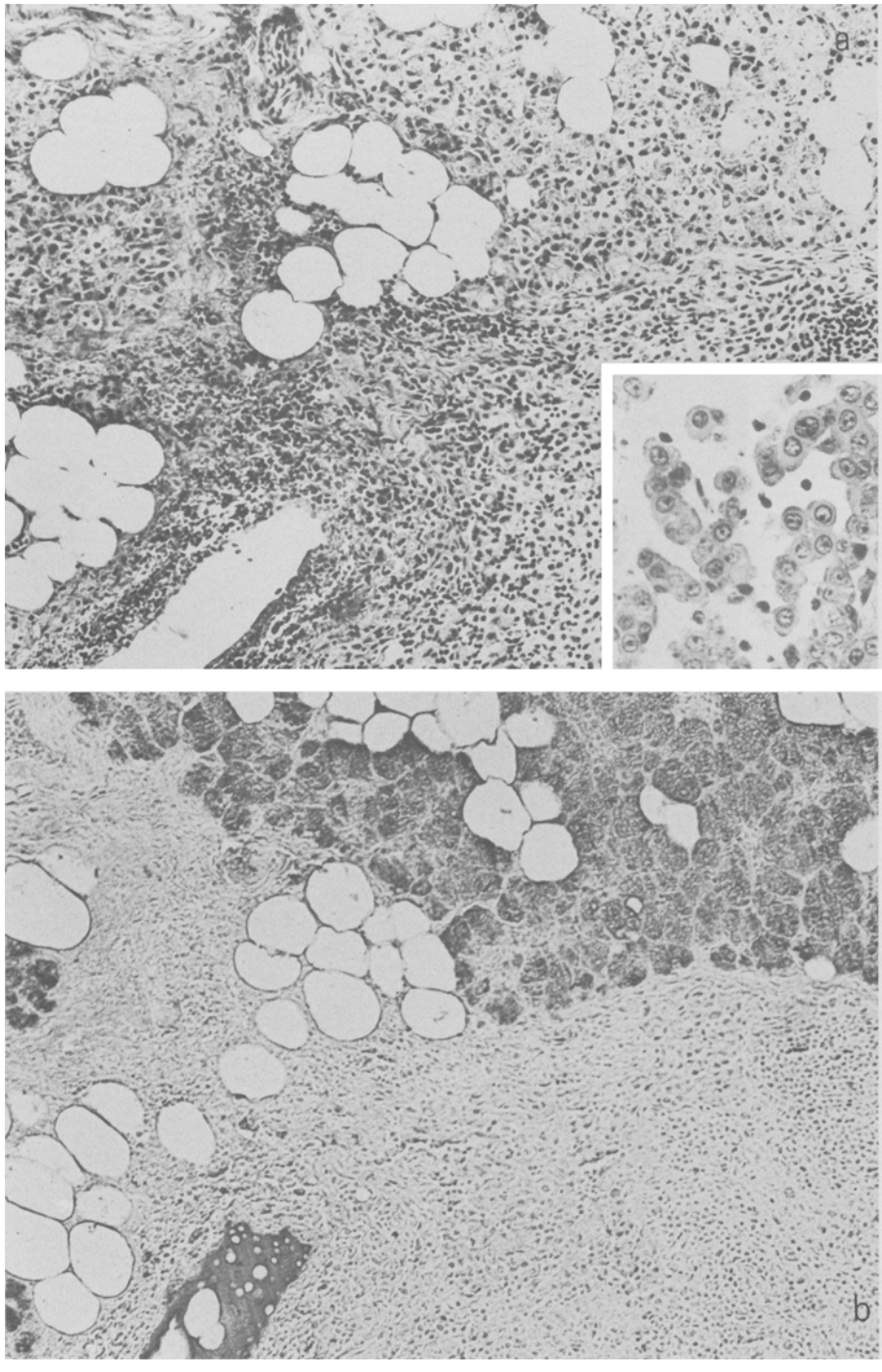

Fig. 3. Acinic cell carcinoma of human parotid gland, adjacent sections. a) Control and *b* anti-parotid amylase antibody treated. Both sections were then treated with conjugated second antibody as described in legend for figure 2, but only (a) was counterstained with hematoxylin and eosin. The inset in (a) is a high power view of the neoplastic cells. Note that only (b) shows positive stain and that is limited to the nonneoplastic (upper) portion of the section.

tion with the attendant advantages of light microscopic examination, ability to use fixed material previously embedded in paraffin, and permanent preparations. We have demonstrated that neoplasia may result in the loss of differentiated function. This is a variable but not uncommon finding in tumors[16]. We have also shown the lack of ectopic production of amylase in transformed ductal cells. Ductal cells are embryologically one step less differentiated than acinar cells. While some tissues that undergo malignant degeneration begin to elaborate amylase (lung, ovary) it is of interest that ductal cells of parotid gland do not.

Conclusion. We have demonstrated the ability to localize amylase in normal human tissues. We utilized this technique to study differentiation in parotid neoplasia. This technique can be used in various developmental studies and in the study of the evolution of neoplasia.

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Effects of the temperature of ice-seeding on survival of frozen-and-thawed mouse morulae

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Summary. Mouse morulae were frozen to −196 °C in the presence of 1.2 M ethylene glycol or 1 M glycerol. After ice-seeding in embryo samples at −10 °C or below, the survival rates in vitro were lower than those obtained by ice-seeding between −4° and −8 °C. The proportion morulae developing into live young in vivo after ice-seeding at −13 °C and freezing was lower than that of embryos seeded at −4 °C.

Since the first significant report by Whittingham et al.[1] on the survival of mouse embryos after freezing and thawing, inducing extracellular ice formation (ice-seeding) in embryo samples before slow cooling has always been done[2,3]. The temperature of ice-seeding affected the survival of 8-cell mouse embryos[2] and morulae and blastocysts of sheep[3,4] during freezing and thawing in the presence of dimethyl sulphoxide (DMSO). In the present paper we have examined the effects of the temperature of ice-seeding on the survival of mouse morulae after freezing and thawing in the presence of 1.2 M ethylene glycol or 1 M glycerol as the cryoprotectant.

Materials and methods. Female ICR mice were induced to superovulate, and mated[1]. Morula embryos were recovered from the reproductive tracts with a modified Dulbecco's phosphate-buffered salt solution (PBS)[1] at 80–84 h after the injection of HCG. The embryos were washed in several changes of PBS and 14–22 embryos were transferred to each test-tube containing 0.1 ml PBS. The tubes were cooled to 0 °C at 2 °C/min in a Dewar flask containing ethanol, and the cryoprotectant in PBS was added to samples at 0 °C in 2 increments of 0.05 ml at 10-min intervals. The final concentration of ethylene glycol and glycerol used as the cryoprotectant was 1.2 and 1 M, respectively. The samples were equilibrated at 0 °C for 10 min and cooled to the temperatures used for ice-seeding at 0.5–1 °C/min. They were seeded at between −4 and −13 °C by placing a cooled hypodermic needle in PBS. They were held at the ice-seeding temperature for 5 min and then cooled to −79 °C at 0.5−1 °C/min by adding dry ice to ethanol in a Dewar flask. The Dewar flask containing the frozen samples was placed in liquid nitrogen to be cooled from −79 °C to −120 °C at 1−2 °C/min, and then samples were transferred directly to liquid nitrogen. After being frozen at −196 °C for 1–24 h, the frozen samples were thawed at approximately 15 °C/min in air at room temperature.

After thawing, the embryos were cultured by the microdrop method in a modified Krebs-Ringer bicarbonate medium[1] under paraffin oil at 37 °C for 36 h in 5% CO_2 in air. The survival of frozen-and-thawed morula embryos was assessed by their ability to develop into expanded blastocysts during culture in vitro. Experiments were replicated 4–5 times and data were analyzed for statistical significance by the χ^2-test. The further potential for survival of frozen-and-thawed and unfrozen morulae was tested by transferring the blastocysts, which had expanded after 36 h in culture, to the uterine horns of females (5–10 blastocysts/horn) on day 3 of pseudopregnancy (day 1 is the day on which the copulating plug is found).

Results and discussion. As shown in table 1, the temperature of ice-seeding in embryo samples was important for the survival of frozen-and-thawed mouse morulae to the blastocyst stage in vitro, regardless of the cryoprotectant used. There were no apparent differences between the ice-seeding temperatures of −4 to −8 °C in the survival rate of mouse morulae after freezing and thawing in the presence of 1.2 M ethylene glycol or 1 M glycerol. After seeding at −10 °C or below, however, the survival rates of frozen-and-thawed embryos were lower ($p < 0.05$) than those obtained by ice-seeding at −4 to −8 °C.

As shown in table 2, the developmental rate for morulae to live young in vivo after ice-seeding at −13 °C and freezing to −196 °C was lower ($p < 0.01$) than that for morulae seeded at −4 °C.

The present results showed that the survival of mouse morulae frozen in the presence of ethylene glycol or glycerol was affected by the temperature of ice-seeding as