Reliance on Gomori's metal-salt method for acid phosphatase (1) for the ultrastructural identification of lysosomes has long been a weakness in the "lysosome concept". Many workers have indicated the necessity to extend the enzymatic localization of lysosomes to more of the hydrolytic enzymes which are characteristic of them. Unfortunately, the successful azo-dye methods for such enzymes as beta-glucuronidase (2) and non-specific esterase (3) have proved disappointing for ultrastructural observation. Insufficient density enhancement produced by the dyes, or extraction by the various solvents used in preparation of electron microscopy specimens have contributed to this lack of advancement.

The stimulus to formulate and develop acceptable methods for electron microscope cytochemistry has begun to yield a number of reliable procedures. Holt and Hick's pursue the development of the "azoindoxyl complex" principle with great success (4) and methods are now available for E600-resistant esterase (5) and aryl sulfatase (6, 7). It is to be expected that a considerable amount of new information concerning the hydrolytic enzymes of lysosomes may be forthcoming from these methods. In particular, the possibility of the detection of the differential distribution of the various hydrolytic enzymes in lysosomes may be forthcoming from these methods. In particular, the possibility of the detection of the differential distribution of the various hydrolytic enzymes in lysosomes in different tissues, should prove especially important. The possibility exists of further classification of the organelles that make up the "vacuolar apparatus" (see reviews 8, 9, 10).

Acid phosphatase-positive, membrane-limited granules have been demonstrated in keratinized epithelia of the mouse at the light microscope (11) and electron microscope levels (12). As part of an extensive investigation of lysosomes in murine keratinized tissues, the cytochemical localization of a number of hydrolytic enzymes now accepted as being of a lysosomal nature is in progress. Aryl sulfatase is the subject of this report.

METHODS

Samples of skin from the upper surface of the hind limbs plus esophageal epithelium and the cortex of the kidney of male Swiss albino mice were obtained rapidly after sacrifice by cervical luxation. Six mice were used in the cytochemical studies and a further six as controls. Tissues were minced into 1 mm³ pieces in cold (0—4°C) buffered 3% glutaraldehyde pH 7.2 (13). Fixation proceeded for 4 hours after which the blocks were washed for 24—48 hours in cacodylate-sucrose pH 7.2 at 0—4°C (14). Fifty micron sections were obtained from tissues by means of a freezing microtome.

The incubation procedure made use of p-nitrocatechol sulfate (2-hydroxy-5-nitrophenyl-sulfate) as substrate. The medium was prepared as suggested by Hugon & Borgers (15) utilizing barium chloride, as opposed to the lead nitrate capture reagent of Goldfischer (7). The barium sulfate reaction product is extremely insoluble and also produces sufficient enhancement of density in the electron microscope to require no conversion to the sulfide as is the case in light microscope technique utilizing lead nitrate (7). Barium has the added advantage of having a lower inhibitory effect on the enzyme than lead (16).

Subsequent to enzymatic incubation, the sections were osmicated in Zetterquist's formulation (17) for 1½ hours after a brief wash in buffered sucrose. Dehydration and embedding in Araldite were as previously published (18). No P.T.A. block staining was employed, to eliminate the possibility of misinterpretation of staining deposits.

Pale gold or silver sections were cut on a Porter-Blum MT2 ultramicrotome and mounted on Parlodion-coated copper grids. The sections were observed initially unstained, and for photographic representation, after lead citrate (19) and aqueous uranyl acetate staining (20).

Twenty micron sections were utilized in a correlated light microscopy study. Sections incubated in the solution containing barium chloride were observed by means of phase-contrast microscopy. Control sections were compared after incubation in the lead nitrate method of Goldfischer (7).
reaction product (lead sulfate) was converted to the sulfide with 1.0% ammonium sulfide solution. The enzymatic reaction product that was visualized in the light microscope was compared to the observations under phase-contrast conditions.

Control histochemical sections were incubated for 1 hour in substrate-free medium in which a high barium salt concentration was maintained.

RESULTS

The distribution of enzymatic reaction product in both light microscope and electron microscope sections was essentially similar to that previously observed for acid phosphatase (12). Lysosomes in the stratum basale of both the epidermis and the esophageal epithelium were of the same size range as those observed in the above study.

The distribution of barium sulfate precipitate varied slightly with the length of incubation. After short periods of incubation (15–20 mins.), the reaction product was localized over a percentage of the lysosomes in all of the tissues examined. Examples of lysosomes in the keratinized tissues after various incubation periods are shown in Fig. 1. The staining reaction in the kidney was utilized as a preliminary control and showed similar dis-

![Fig. 1. Lysosomes showing enzymatic reaction product (R.p.) of aryl sulfatase incubation. A:—from a basal keratinocyte of the epidermis after 45 minutes incubation, B-D:—from basal keratinocytes of the esophageal epithelium after the following incubation times: B-20 minutes, C-40 minutes, D-30 minutes. Lysosomal membrane (L.m.), A—× 135,000, B—× 45,000, C—× 97,000 D—× 92,000.](image-url)
tributution to that observed in the rat kidney (7, 16, 21). Figure 2 (A–C) shows the variation in enzymatic reaction product distribution with increasing incubation time.

After 45 minutes incubation, changes in localization of the reaction product were observed. There was a tendency for the mass of the electron-dense material to become localized around the periphery of the lysosomes adjacent to the limiting membrane (Fig. 2 C). This band of barium sulfate seems to be due to an artefactual redistribution of the enzyme or the reaction product caused by the lengthy incubation period. Observations of this nature stress the need to incubate sections for varying periods since incorrect deductions may be drawn from the sole examination of over-incubated sections. In this case it would appear that there was a central zone of enzyme inactivity in the lysosomes. Judging from sections incubated for shorter time intervals, there is no evidence to suggest that this is the case, and in fact the enzymatic reaction product was often seen to be concentrated in the central zones of lysosomes in such preparations (Fig. 2 A).

Excessive over-incubation of 1 hour or more produced increasing amounts of artefactual staining. Release of enzyme and/or reaction product from the lysosomes was evidently
Fig. 3. Mouse epidermis showing basal keratinocytes after 60 minutes incubation in aryl sulfatase medium. Enzymatic reaction product (R.p.) is observed overlying the lysosomes (L) as well as dispersed in the immediate vicinity of the organelle. Basement membrane (B.m.), intercellular space (I.s.), mitochondrion (M), plasma membrane (P.m.), × 75,000.

produced by the prolonged incubation in acid medium at 37°C (Fig. 3). Variable quantities of reaction product were visualized in the cytoplasm adjacent to the ruptured granules.

In agreement with the observations on acid phosphatase staining in lysosomes of mouse skin (12) in this study, longer periods of incubation for the keratinized tissues were required to produce a reaction product over lysosomes, which was as concentrated as that seen in kidney. This may indicate more than a quantitative difference in enzyme localization since it may reflect a decreased accessibility of substrate to the lysosomes in keratinized tissues or may represent decreased permeability of the limiting membranes.

Large numbers of organelles of varying sizes, corresponding to the various enzymatically-
positive components of the "vacuolar apparatus" (9), were stained in the kidney but some granules with the morphological appearance of lysosomes contained no observable enzymatic reaction product. Serial sections were examined to ensure that the reaction product, if present, was not merely in other parts of the organelles that were not included in a particular section.

In the epidermis and esophageal epithelium the majority of the positive organelles were confined to the stratum basale and the layer immediately above. Very few positive granules were observed in the stratum granulosum, and none at all in the stratum corneum.

No staining reaction was observed over Odland bodies (Membrane-coating granules). In the case of these organelles, however, a degree of caution must be exercised before definitely pronouncing the absence of aryl sulfatase activity. The granules have a high degree of electron density and, as such, this renders observation of any overlying enzymatic
FIG. 5. Mouse epidermis showing parts of a Langerhans cell and a basal keratinocyte. Aryl sulfatase incubation 40 minutes. Irregularly shaped organelle with electron-dense content and apparent aryl sulfatase activity as indicated by the enzymatic reaction product. Lysosome (L), mitochondrion (M), nucleus (N), plasma membrane (P.m.), tonofilaments (T), × 52,500.

Reaction product extremely hazardous. It is to be hoped that application of suitable autoradiographic methods in conjunction with labelled substrates may resolve this problem. Conflicting reports have been made concerning the presence of other hydrolases such as acid phosphatase in Odland bodies. A negative report for acid phosphatase in such organelles in murine epidermis and esophageal epithelium (12) is contrasted with positive results in the intermediate zones of normal and psoriatic human epidermis (22) as well as in human buccal mucosa (23) and in guinea pig epidermis (24). At the present time it seems unlikely that Odland bodies contain aryl sulfatase since incubations of 1 hour failed to produce any appreciable quantities of diffuse reaction product in their vicinity. If they contain
aryl sulfatase activity, it is to be expected that over-incubation would release at least a fraction of the enzyme. The alternative exists, however, that the enzyme is present but is either very strongly bound and thus inaccessible or is only present in minute quantities and thus undetectable by such inefficient methods.

Positively-stained lysosomes were regularly observed in Langerhans cells. Organelles with the morphological appearance of lysosomes have previously been observed in Langerhans cells (25) and shown to have a positive acid phosphatase reaction (12).

An example of an aryl sulfatase-positive granule in a Langerhans cell is shown in Figure 4. As has been noted previously, the specific granules which are characteristic of Langerhans cells are not always present in abundance. No specific granules are shown in the cyto-
plasmic zone included in Fig. 4, but they were present elsewhere in the cell, in limited numbers. Positive reactions were often obtained in Langerhans cells in association with organelles which did not have the compactness or obvious membrane delimitation of the "storage granule" form of lysosome (8). Examples are illustrated in a Langerhans cell (Fig. 5) and also in a basal keratinocyte (Fig. 6). In such organelles there are often sheaths of dense osmiophilic material of a similar appearance to the "myelin figures" observed in residual bodies in the kidney and many other tissues (10). It may be suggested that the organelles observed in this investigation are autolysosomes or telolysosomes (9) and represent the end products of digestion of cellular components since, in general, lysosomes seem to be deficient in rapidly-acting lipolytic enzymes (10). It is to be expected that material segregated into auto-lysosomes will not be digested quickly if lipid material is present to any great extent in its constitution. There are more of these irregular bodies present in Langerhans cells in this investigation than in adjacent keratinocytes. If they represent telolysosomes this would indicate that the segregation of cellular organelles for digestion or autophagy is more frequent in Langerhans cells. The synthetic activities of the Langerhans cell (26) may require such a constant turnover of the subcellular components to a far greater extent than do the keratinocytes.

The lysosomes in keratinocytes were most often observed in the perinuclear cytoplasmic zone, which is largely free from fibrous elements. Occasionally in this zone, some signs of a rudimentary Golgi complex were seen. As a rule the Golgi complex is not well developed in cell types that do not "export" substances that are manufactured in the cell (27). Since keratinocytes produce intracellular fibrous elements, there would appear to be little or no reason for them to possess extensive Golgi complexes. It is important, however, to remember that the synthesis of the various hydrolytic enzymes of lysosomes has long been a suspected activity of the Golgi complex and acid phosphatase has recently been demonstrated in staining patterns that are suggestive of Golgi vesicles in cells of the albino rat, cheek epithelium (28). The rudimentary Golgi complex of keratinocytes may therefore be involved to a major degree with the processing of the lysosomal hydrolases.

A small amount of diffuse reaction product, not clearly associated with any particular structural element, was observed in the stratum granulosum and stratum corneum especially after prolonged periods of incubation. This may represent a true site of unsequestered aryl sulfatase or may prove to be an artefactual redistribution of previously bound enzyme or reaction product.

In all cases the control sections showed no enzymatic reaction product. The ultrastructural preservation deteriorated noticeably in the sections that were incubated for longer than 1 hour and these could not be used to gain any information concerning the correctness of localization of enzymatic reaction product.

**DISCUSSION**

Aryl sulfatase has been demonstrated at the ultrastructural level in a number of different tissues from various animals (6, 7, 15, 16, 21, 29) and plant sources (30). In general, these results confirm the hypothesis that aryl sulfatase is associated with membrane-limited organelles conforming to the definition of lysosomes. It seems that the two lysosomal aryl sulfatases, types A and B, which have different pH optima in the acid range (31), are present to some extent in different cell types (7). Since the incubation medium used in the present investigation was buffered at pH 5.2, the aryl sulfatase identified in both murine keratinized tissues and kidney is probably type B. This appears to be more widely demonstrable in mammalian tissues than type A, which has a pH optimum of 4.2. As regards the slight amount of cytoplasmic staining, little can be proposed at present. This may have resulted from diffusion from lysosomes damaged during incubation or may reflect a true localization in insoluble microsomal aryl sulfatase type C (Roy—quoted in 7). Further investigations are necessary with varying pH of the incubation medium before any comment may be made on the possible sites of localization of aryl sulfatase types A and C in keratinized tissues.

The identification of acid phosphatase (12),
and in this investigation aryl sulfatase activity, as well as the observed presence of a single limiting membrane, indicates lysosomes are probably present in keratinized epithelia. These observations satisfy at least some of the criteria necessary for complete identification of lysosomes (32).

The natural substrates on which aryl sulfatase type B acts are not known with any certainty, and thus any suggestion concerning the normal physiological role of the enzyme must be tentative. It may be that aryl sulfatase B is inactive as such in vivo or that its properties in the intact cell may prove to be rather different from those characterized in the case of the isolated enzyme. The present state of ignorance regarding the physiological roles of the enzyme is regrettable since its widespread distribution in various tissues and species suggest that it may have an important part to play in the cellular economy. A clearer understanding of the normal physiological roles of other lysosomal hydrolases such as phosphatases and $\beta$-glucuronidases may prove to be the key to solving the enigma of the functions of the aryl sulfatases.

Aryl sulfatase staining of cytosomes in rat proximal convoluted tubule cells is often non-uniform (21). In this investigation (21) the incubation medium utilized a lead salt as the capture reagent and the staining product (lead sulfate) had a marked tendency to accumulate near the inner surface of the cytosome membranes. This peripheral, "ring-like" localization of reaction product when lead was used as a capture reagent has been noted previously (16). Correlated studies using a barium salt as the capture reagent (16) produced a uniform distribution of enzymatic reaction product similar to that observed in the present investigation.

The origins of the hydrolytic enzymes in the granular layers of keratinized epithelia remain obscure and little may be proposed at present. Unfortunately, the numerous studies at the ultrastructural level (see 12) using cytochemical techniques have failed to resolve the question of the apparently diffuse nature of acid phosphatase and other lysosomal hydrolases in the granular layer cells. It may be suggested that the release of enzymes from lysosomes is a natural event occurring at the level of the granular layer—cornified layer transition. The enzymes would seem to have an essential functional involvement with the extensive cellular remodelling that occurs. If this is the case, convincing evidence of disrupted and partially disrupted lysosomes should prove demonstrable in electron microscope preparations. The absence of confirmative results may indicate that the enzymes present in the lysosomes in this zone may be readily solubilized and thus released from their limiting membrane by the techniques of fixation and subsequent processing. Refined techniques are required for the biochemical separation of lysosomal fractions from the epidermis (33).

The proposed fragility of the lysosomal membranes may be a function of the physiological state of the cells in the granular layer. The conditions of pH and other factors may be such at this stage of the cell cycle as to render the lysosomes excessively fragile, while lysosomes in the basal layers are much more resistant. Further research concerning alternative methods of fixation and tissue preparation may provide the solutions to these important problems. Recently, Tabachnik and Perlish (34) have demonstrated lysosomes in the stratum granulosum of guinea pigs. They demonstrated a particulate localization as well as the commonly produced diffuse reaction for acid phosphatase using an azo-dye method. Latency in the granules was demonstrated by means of Triton X-100 pre-incubation, which abolished the punctate staining.

The demonstration of lysosomes at the ultrastructural level, in keratinizing epithelia, by cytochemical techniques, has raised a number of interesting points for speculation. It is not unreasonable to suppose, that if the un-sequestered hydrolytic enzymes present in granular layer cells are the product of disrupted lysosomes, either passing through the epithelium with keratinocytes or injected by a "cytocrine-transfer" mechanism (35), then convincing evidence should be available of sufficiently large numbers of intact lysosomes, either in basal or spinous layer keratinocytes or in Langerhans cells. To accommodate the processes of cellular autolysis occurring constantly at the transition between granular and cornified layers the quantities of hydrolases required are probably quite high since the
metamorphosis is so abrupt. The relative paucity of cytochemically demonstrable lysosomes, even in basal layer keratinocytes and Langerhans cells, when compared to other tissues such as the kidney, may lead research workers to make a re-evaluation of the extent of the contribution of lysosomes to the events occurring in the granular layer.

Electron microscope autoradiographic studies of amino-acid incorporation into murine keratinized tissues (see 18) have demonstrated the high degree of metabolic activity present in granular layer cells. The possibility exists that at least a portion of the synthetic activity demonstrated in granular layer cells may be directed towards production of hydrolytic enzymes freely into the cytoplasm. If this were the case, the keratinocytes may have the ability to synthesize hydrolytic enzymes in inactive or inhibited states and thus, only in the cytoplasm of upper layers of the epithelia are conditions of pH, oxygen tension, activators, etc. such as to facilitate the full lytic capabilities of the enzymes. The presence of unsequestered hydrodrolases in granular layer cells would in no way invalidate the previously proposed involvement of lysosomes with intracellular digestive processes and mitosis in the basal layer (12).

SUMMARY

1. The sites of aryl sulfatase activity were examined in the epidermis and esophageal epithelium of the mouse, using combined cytochemistry and electron microscopy.

2. The staining reaction in proximal tubules from the cortex of mouse kidney was utilized as a preliminary control.

3. Discrete, punctate staining was observed in basal layer cells of both keratinized epithelia at the light microscope level.

4. Enzymatic reaction product (barium sulfate) was observed in association with organelles morphologically identifiable as lysosomes.

5. Lysosomes were observed in relatively small numbers in the cytoplasm of basal keratinocytes and Langerhans cells.

6. Very few positively stained lysosomes were observed in the granular layers of either epithelium.

7. Diffuse reaction product was observed in the granular and cornified layers.

8. Positive enzymatic reactions were obtained over organelles containing aggregates of osmiophilic material. It was suggested that these bodies represent auto-lysosomes or telolysosomes.

9. The involvement of acid hydrolases with the events occurring in the granular—cornified layer transition was discussed and some speculation concerning the extent of the participation of lysosomes included.

REFERENCES


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