FINE-STRUCTURAL LOCALIZATION OF
ADENOSINE TRIPHOSPHATASE IN THE
RECTUM OF CALLIPHORA

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SUMMARY
Adenosine triphosphatase (ATPase) activity in the rectal papillae of Calliphora has been studied by biochemical and histochemical techniques. The microsomal fraction contained a Mg$^{2+}$-activated ATPase with a pH optimum of 8-0. The enzyme was not stimulated by the addition of Na$^+$ plus K$^+$ and was insensitive to ouabain. Histochemical studies using modifications of the Wachstein-Meisel method showed that at pH 7-2 this Mg$^{2+}$-activated ATPase was specifically localized on the intracellular surface of the lateral plasma membranes. A similar though less intense reaction was obtained with adenosine diphosphate and inosine triphosphate, but not with guanosine triphosphate, uridine triphosphate or β-glycerophosphate as substrates. At an acid pH (6-6-8-8), very little reaction occurred on the lateral plasma membrane but some reaction product was present in mitochondria and nuclei. Very little enzyme activity was found in the flattened rectal epithelium. These results are discussed in relation to the available data on transport ATPases and on the structural basis of fluid transport by rectal papillae. It is proposed that the ATPase localized on the stacks of lateral plasma membrane may be involved with ion secretion into the intercellular spaces to create the osmotic gradient necessary to extract water from the lumen.

INTRODUCTION
Reabsorption of ions and water by the rectum of insects is an active process which can occur against considerable ionic and osmotic gradients (Phillips, 1964a, b; Ramsay, 1955a, 1964). In the rectal papillae of the blowfly, Calliphora erythrocephala, it has been postulated that fluid is directed from the lumen into the haemolymph via an extensive system of intercellular spaces (Gupta & Berridge, 1966a; Berridge & Gupta, 1967). It was argued that in this tissue an active secretion of solute into the intercellular spaces creates favourable conditions for an osmotic influx of water from the lumen (Berridge & Gupta, 1967). From the fine-structural evidence alone, it appeared that the most likely sites of such ion secretion were the lateral plasma membranes, which were folded into stacks, and were closely associated with mitochondria. Furthermore, because potassium predominates in the urine secreted by the Malpighian tubules (Ramsay, 1953, 1955b; Berridge, in preparation) and is reabsorbed from the rectum of Schistocerca ten times faster than sodium (Phillips, 1964b), it was assumed that potassium is the major cation transported at such sites.

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It is now generally believed that adenosine triphosphate (ATP) is the immediate source of energy for the active transport of sodium in cells (Caldwell, Hodgkin, Keynes & Shaw, 1960; Whittam, 1958; Baker, 1966); it has been proposed that this energy is released by a specific ATPase (Judah & Ahmed, 1964; Skou, 1965; Post & Sen, 1965; Baker, 1966). Indeed, a Mg$^{2+}$-activated ATPase has been demonstrated biochemically in a wide variety of tissues engaged in active transport (see Skou, 1965; Glynn, 1964). However, only that part of the enzyme activity which is associated with the cell membrane and which, in homogenized fractions used for biochemical studies, simulates the properties of the corresponding transport mechanism in intact tissues is to be regarded as a ‘transport ATPase’ (Skou, 1957; Kinsolving, Post & Beaver, 1963; Glynn, 1964). Prominent among these properties of ‘transport ATPases’ is a stimulation of enzyme activity by Na$^+$ plus K$^+$ ions and inhibition by certain cardiac glycosides (Glynn, 1964).

Since biochemical studies of homogenized fractions cannot localize the actual sites of ion transport in a multicellular system, histochemical methods for the localization of ATPase in tissue sections have been employed to identify such sites (Torack & Barnett, 1963; Goldfischer, Essner & Novikoff, 1964; Bartoszewicz & Barnett, 1964; Wachstein & Besen, 1964; Kaye & Pappas, 1965; Kaye & Tice, 1966; Farquhar & Palade, 1966; Kaye, Wheeler, Whitlock & Lane, 1966; Rostgaard & Behnke, 1966; Shiose & Sears, 1966). Although a magnesium and, to a certain extent, a substrate specificity for the enzyme have been demonstrated in certain tissues investigated by histochemical methods, specificity for transported cations and inhibition by cardiac glycosides have not (Farquhar & Palade, 1966; Rostgaard & Behnke, 1966; Tice & Engel, 1966). This has led certain authors (for example Tormey, 1966) to doubt the validity of the available histochemical methods for demonstrating ‘transport ATPases’. There are, however, reasons to believe that a specific transport ATPase may not necessarily be a different enzyme from the Mg$^{2+}$-activated ATPase demonstrated histochemically, and the two may in fact be interconvertible (Farquhar & Palade, 1966).

The biochemical and histochemical aspects of fluid-transporting systems in insect tissues have received little attention. Although alkaline phosphatase has been demonstrated in the secretory and reabsorptive epithelia of many insects (Bradfield, 1946; Day, 1949; Berkaloff, 1959; Guraya, 1960; Mazzi & Baccetti, 1963; Baccetti, Mazzi & Massimello, 1963; Sohal & Copeland, 1966), the role of this enzyme in ion transport is by no means clear. The possible involvement of an ATPase in ion reabsorption by the rectal papillae of Dacus oleae has been suggested by Baccetti et al. (1963).

The present investigation is concerned with some of the biochemical and histochemical properties of an ATPase which may be involved in potassium transport by the rectal papillae of Calliphora.

**MATERIAL AND METHODS**

Female Calliphora erythrocephala Meig. (3-day-old adults) were used throughout this study and were cultured as described elsewhere (Berridge, 1966).
ATPase localization in blowfly rectum

Biochemical methods

The rectum was dissected out of the abdominal cavity and individual papillae were cut out from the rectal pouch with a sharp razor. This procedure reduced contamination from the surrounding muscle and flattened epithelium.

Papillae were homogenized in 0.35 M sucrose solution buffered at pH 7.4 with 30 mM tris-HCl. The homogenate was centrifuged at 2000g for 10 min to remove the larger particles; the supernatant was further centrifuged at 20000g for 30 min. The precipitate was washed twice with a tris/sucrose solution. The composition of such pellets was examined by electron microscopy, after routine fixation, embedding and sectioning, and resembled a microsomal fraction. For the biochemical analysis of ATPase, the pellet was resuspended in 30 mM tris (pH 7.4). The incubation medium contained Mg2+ (2 mM), Na+ and K+ (20 mM each), tris buffer (30 mM) and tris-ATP (2 mM), made up to a final volume of 0.1 ml. Incubations were carried out at 37 °C and the phosphate released after 30 min was measured by the method of Fiske & Subbarow (1925). Since the amount of enzyme present is low, activity has been expressed simply as Pi released/number of papillae. The effect of pH on enzyme activity was tested using 30 mM tris-HCl buffer in the alkaline range and 30 mM imidazole-HCl buffer in the acid range.

Histochemical methods

The rectum was immersed for 30 min in 2.5% glutaraldehyde buffered at pH 7.2 with 0.05 M sodium cacodylate and containing 0.15 M sucrose. The tissue was washed at 4 °C in several changes of 0.05 M sodium cacodylate (pH 7.2) containing 0.3 M sucrose. Before incubation in a substrate medium, the material was transferred either to a solution containing 0.08 M tris-maleate and 10% sucrose (pH 7.2) or to one containing 0.08 M imidazole-maleate and 10% sucrose (pH 6.6) and kept in that solution for 1 h, in order to equilibrate the tissue to the pH conditions of the respective incubation media. Each conical papilla was then cut deeply but incompletely with a sharp razor blade. Since the cells of the cortical epithelium are very tall and arranged in a single layer, such a cut, made obliquely to the altitude of the cone, passed through a large number of them, thus permitting intracellular access to the incubation medium without altering significantly the spatial arrangement of cell structures within the tissue. A few cells in the medulla also generally lay in the plane of the cut. In some instances, papillae with such single cuts were incubated directly before fixation in glutaraldehyde. In other controls a brief fixation in 6% hydroxyadipaldehyde in place of 2.5% glutaraldehyde was used (Tice & Smith, 1965).

Papillae were incubated for 30 min at room temperature (< 20 °C) in various media described below (Table 1), washed for 15 min in either 0.08 M tris-maleate/sucrose (pH 7.2) or 0.08 M imidazole-maleate/sucrose (pH 6.6) solutions (depending on the pH of the incubation medium), post-fixed in osmium tetroxide solution with sucrose, and embedded in Araldite as described previously (Gupta & Berridge, 1966). The incubation media were modified slightly from the one given by Wachstein & Meisel (1957). The magnesium concentration was lowered from 10 mM/l. to 5 mM/l.;
Table 1. Composition of the incubation media used during the histochemical localization of nucleoside phosphatase in the rectal papillae of Calliphora (based on the procedure described by Wachstein & Meisel, 1957)

<table>
<thead>
<tr>
<th>Medium</th>
<th>Tris-ATP (mm)</th>
<th>Tris-maleate, pH 7.2 (mm)</th>
<th>Imidazole-maleate, pH 6.6 (mm)</th>
<th>MgSO₄ (mm)</th>
<th>CaSO₄ (mm)</th>
<th>Na₂SO₄ (mm)</th>
<th>K₂SO₄ (mm)</th>
<th>Pb(NO₃)₂ (mm)</th>
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<tbody>
<tr>
<td>(a)</td>
<td></td>
<td>80</td>
<td>5</td>
<td></td>
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<td>10</td>
<td>3.6</td>
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<tr>
<td>(b)</td>
<td>Control, pH 6.6</td>
<td>--</td>
<td>80</td>
<td>5</td>
<td></td>
<td>10</td>
<td>3.6</td>
<td></td>
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<tr>
<td>(c)</td>
<td>Mg²⁺-ATP, pH 7.2</td>
<td>80</td>
<td>80</td>
<td></td>
<td>10</td>
<td>3.6</td>
<td></td>
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</tr>
<tr>
<td>(d)</td>
<td>Mg²⁺-ATP and Na⁺ + K⁺, pH 7.2</td>
<td>80</td>
<td>80</td>
<td>5</td>
<td></td>
<td>10</td>
<td>3.6</td>
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<tr>
<td>(e)</td>
<td>Mg²⁺⁺-ATP, pH 6.6</td>
<td>80</td>
<td>80</td>
<td>5</td>
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<td>10</td>
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<tr>
<td>(f)</td>
<td>Ca³⁺-ATP, pH 7.2</td>
<td>80</td>
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<td>5</td>
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<td>3.6</td>
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<tr>
<td>(g)</td>
<td>Ca³⁺-ATP, pH 6.6</td>
<td>80</td>
<td>80</td>
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<td>5</td>
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The other phosphate compounds tested as substrates for the enzyme, adenosine diphosphate (ADP), inosine triphosphate (ITP), guanosine triphosphate (GTP), uridine triphosphate (UTP) and β-glycerophosphate, were substituted for ATP at equimolar concentrations in medium (c).
The effect of calcium on ATPase activity was tested by substituting CaSO₄ for MgSO₄ at both pH 6·6 and pH 7·2. An acid medium was obtained by using an imidazole-maleate buffer (pH 6·6). Since some ATPases are markedly stimulated by Na⁺ plus K⁺ under biochemical conditions, these ions were included in the incubation media. The final composition of the different media used in the present study are given in Table 1.

For thin sectioning, the papillae were oriented so that tissue on both sides of the ‘cut’ was available for examination on the same section. Some of the possible artifacts of enzyme histochemistry, believed to be introduced by cutting thick sections of tissues prior to incubation, have thus been avoided. Thin sections, either without staining or with brief staining in Reynolds’s lead citrate, were studied either in a Phillips EM 200 or an AEI EM 620 electron microscope.

**RESULTS**

**Biochemical observations**

A preliminary study was made to determine the amount of enzyme necessary to obtain a detectable release of phosphate (Fig. 1). Phosphate released was linearly related to enzyme concentration up to 10 papillae/tube; a concentration of 5 papillae/tube was used in subsequent experiments.

The effect of pH on ATPase activity is shown in Fig. 2. As with some other transport ATPases (Kinsolving et al. 1963), maximum enzyme activity occurred at pH 8·0.

![Fig. 1. Relation between phosphate released and the concentration of adenosine triphosphatase. The reaction mixture contained Mg²⁺ (2 mM), Na⁺ plus K⁺ (20 mM each), tris-HCl buffer pH 7·4 (30 mM) and tris-ATP (2 mM).](image-url)
Fig. 2. Relation between ATPase activity and pH.

Fig. 3. ATPase activity in relation to the concentration of Mg$^{2+}$ (●) and Ca$^{2+}$ (○).
Figure 3 illustrates the effect of divalent cations on the ATPase of rectal papillae. Addition of only 2 mM Mg\(^{2+}\) caused an almost 10-fold increase in enzyme activity, but further addition of Mg\(^{2+}\) inhibited the enzyme. Calcium had a similar but much smaller effect (Fig. 3); it is conceivable that the preparation was not entirely free from contamination by a Ca\(^{2+}\)-activated myosin ATPase from the rectal muscles. The concentration of Mg\(^{2+}\) (2 mM) required for a maximum activation of the enzyme is the same as that of the substrate ATP; this stoichiometric relationship has previously led to the suggestion that a Mg\(^{2+}\)-ATP complex participates in the enzyme reaction (Skou, 1957; Post & Sen, 1965).

An attempt was also made to determine the effect of those monovalent cations (Na\(^{+}\) and K\(^{+}\)) which are known to be essential for maximum activity of 'transport ATPases' (Kinsolving et al. 1963). At least under the conditions of the present experiments, addition of Na\(^{+}\) plus K\(^{+}\) did not cause any appreciable increase in enzyme activity. Furthermore, enzyme activity was not inhibited by ouabain (10\(^{-9}\) or 10\(^{-4}\) M).

Further biochemical analysis of the enzyme was not attempted, partly because of the difficulty of obtaining substantial quantities of suitable subcellular fractions from small tissues such as rectal papillae, and partly because it has not been possible to eliminate completely the contamination from surrounding muscle, medullary tissue and ordinary rectal epithelium, all of which may have ATPases of different properties. However, this preliminary biochemical study does establish some properties of the predominant enzyme; namely its pH and Mg\(^{2+}\) requirements, its insensitivity to ouabain and the absence of a marked stimulatory effect of Na\(^{+}\) plus K\(^{+}\). It has already been noted that transport ATPases must necessarily be associated with the plasma membrane. The following histochemical study supports this assumption and provides clues as to the major sites of ATPase activity in rectal papillae.

**Histochemical observations**

The distribution of reaction product was identical in fresh tissues and after brief fixation in glutaraldehyde, but structural preservation was better in the latter and resembled the preservation obtained in earlier morphological studies (Gupta & Berridge, 1966a, b; Berridge & Gupta, 1967). Therefore only the results from pre-fixed tissue will be described here.

The histochemical reaction has been considered specific only when the electron-opaque deposits of the reaction product were completely absent from corresponding sites in the tissue incubated under identical conditions in media without the substrate. Mg\(^{2+}\)-ATP. Small (50-500 Å) electron-opaque deposits (presumably lead phosphate) were consistently localized on the lateral plasma membranes of the cortical cells of the papillae (Figs. 4, 6, 8). Besides the mitochondria (Fig. 12) and in some cases the nucleus (Fig. 13) there were no deposits on any other structure in the cortical epithelial cells. Maximum reaction with coarse deposits occurred near the cut surface, but here both tissue preservation and specificity of the histochemical reaction were poor. Coarse, non-specific deposits may result from an accumulation of lead phosphate formed by a non-enzymic hydrolysis of ATP (Rosenthal, Moses, Beaver & Schuffman,
Away from the cut surface, however, the deposits were finer (less than 500 Å) and fewer, presumably owing to a retarded infiltration of the incubation medium, but the distribution patterns were highly consistent and reproducible. Very fine deposits (50 Å or less) on membranes lying far away from the cut, were used to determine enzyme localization with precision.

Maximum formation of reaction product was found after incubation in Mg$^{2+}$-ATP as substrate at pH 7·2 for 30 min at room temperature (Fig. 4). Discrete deposits were mostly formed on the infolded stacks of the plasma membrane (Figs. 4, 6), and to a much lesser extent on the rest of the lateral plasma membrane lining the larger intercellular spaces (Fig. 4, arrows). At higher magnifications, all these deposits could be seen to be present on the cytoplasmic surface of the unit membrane (Figs. 6, 8, arrows). Under these conditions of incubation at pH 7·2, no reaction product was found on the mitochondria, nucleus, basal plasma membrane or apical leaflets. When ATP was omitted from the incubation medium or when Mg$^{2+}$ was replaced with Ca$^{2+}$ (Table 1) there was no noticeable reaction product in these cells.

Neither the complete omission nor a variation in the ratio and concentration of Na$^{+}$ and K$^{+}$ ions had any effect on the amount of reaction product on the membrane stacks of the cortical epithelial cells, as reported also in frog skin by Farquhar & Palade (1966). Curiously, however, in the rectal papillae from some flies the apical leaflets reacted positively only when the tissue had been incubated in a Mg$^{2+}$-ATP medium containing Na$^{+}$ and/or K$^{+}$ ions (Fig. 9). Furthermore, in such cases the reaction product was found only on the extracellular surface of the plasma membrane; the cytoplasmic surface covered with the particulate subunits (Gupta & Berridge, 1966a) and other structures in the adjacent cytoplasm were free from such deposits. Irregular non-specific deposits were also present in the subcuticular space and the cuticle.

The only other cells in the rectal papillae which reacted regularly with Mg$^{2+}$-ATP at pH 7·2 were the collagen-secreting cells in the medulla (Gupta & Berridge, 1966b). Reaction product accumulated exclusively on the outer surface of the sinuous plasma membrane of these cells (Figs. 10, 11), even when the individual cells had been cut into halves. In the medulla, however, the picture is somewhat confused by a non-specific binding of lead on the abundant extracellular collagen and mucopolysaccharides (Farquhar & Palade, 1966; Behnke, 1966).

The material incubated in Mg$^{2+}$-ATP at pH 6·6 (Table 1) there was much less reaction product on the membrane stacks than after incubation in Mg$^{2+}$-ATP at pH 7·2; no reaction occurred on the apical leaflets. In several instances even the stacks over large areas of the cells were devoid of reaction product. Coarse deposits (about 100 mμ or larger) were commonly present on the membranes lining the larger intercellular spaces. In contrast to the reaction product on the stacks, however, such coarse deposits were mostly located on the extracellular surface of the lateral membranes.

In addition, very fine particulate deposits were also found in the mitochondria (Fig. 12) and in the nuclei (Fig. 13) of the cortical cells. The deposits in the mito-
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Chondria were formed mainly on the surface of the cristae facing the matrix (Fig. 12). In the nuclei (Fig. 13) the reaction product appeared to be confined to the chromatinic areas and part of the nucleolus. The localization in the mitochondria and the nucleus was not found in tissues incubated in any other medium, including Mg\(^{2+}\)-ATP at pH 7.2.

**ADP, ITP, GTP, UTP and \(\beta\)-glycerophosphate as substrates.** With both ADP and ITP as substrates, deposits of reaction product were found exclusively on the lateral plasma membrane of the cortical epithelial cells (Figs. 5, 7). Under identical conditions of tissue processing and incubation the deposits were smaller and fewer with ADP and ITP as substrates than with ATP (compare Figs. 4, 5). However, the deposits were still formed only on the cytoplasmic leaflet of the lateral plasma membrane (Fig. 7) and not in a reversed position (that is, on the outer surface) as occurs in human prostatic cells when different nucleoside phosphates are employed (Mao & Nakao, 1966). No reaction was found on the apical plasma membranes of cortical cells after ADP and ITP, but these substrates were not tried in media containing high concentrations of Na\(^+\) and K\(^+\).

There was no specific reaction in any of the tissue incubated in media containing GTP, UTP or \(\beta\)-glycerophosphate as substrates.

*Rectal epithelium and rectal muscle.* Very little reaction was observed in the flattened rectal epithelial cells which line the rest of the rectal lumen. It is not certain whether this was due to a relatively low level of ATPase activity or to an inability of the incubation medium to reach the intracellular milieu. The extremely flattened and sinuous nature of this epithelium makes it difficult to cut all the cells; but, as reaction product was no more pronounced in cells lying in the plane of the cut than in intact cells, it seemed that entry of the medium was not the cause. Accordingly, it is probable that there is a low intrinsic activity of the enzyme in the rectal epithelium.

Tubules of the T-system and the sarcoplasmic cisternae of the muscles surrounding the rectum (Gupta & Berridge, 1966b) frequently showed specific but extracellular lead phosphate deposits in Mg\(^{2+}\)-ATP medium at pH 7.2 (compare Tice & Engel, 1966; Rostgaard & Behnke, 1966). Some reaction was also noted on the myofilaments in Ca\(^{2+}\)-ATP medium at pH 6.6, particularly after fixation in hydroxyadipaldehyde (compare Tice & Smith, 1965; but see also Gillis & Page, 1967), but not in any other incubation medium tried. Muscle ATPases have not been studied in detail, but the results tend to confirm the potential of the histochemical methods to distinguish between different types of ATPases in the same tissue (however, see Discussion).

**DISCUSSION**

The exact mechanism which leads to the formation of deposits in histochemical reactions for phosphatases is not known. It has been assumed that, in the presence of lead, the site of phosphate release during the enzymic hydrolysis of phosphate compounds is marked by the formation of insoluble lead phosphate. Recently, however, the validity of such histochemical reactions employing lead as a trapping agent has been seriously questioned by Rosenthal *et al.* (1966) and Moses *et al.* (1966).
It is important therefore to consider their objections in detail, before attempting to assess the results obtained in the present study.

Rosenthal et al. (1966) have shown that, under the normal conditions of the Wachstein–Meisel method, lead ions can cause a non-enzymic hydrolysis of ATP. Consequently, it has been argued that deposition of lead salts near plasma membranes may be fortuitous and have no bearing on the localization of membrane phosphatases (Moses et al. 1966). During the course of incubating tissues in the Wachstein–Meisel medium, we consistently observed that a flocculent white precipitate gradually develops in the incubation medium and, according to the observations of Rosenthal et al. (1966), this precipitate is probably lead phosphate. It is curious that neither Rosenthal et al. (1966) nor Moses et al. (1966) mention this formation of a precipitate in their incubation media, even though phosphate is being released in the presence of lead. Conceivably these deposits of lead phosphate could penetrate fixed tissues via the intercellular channels and give the impression of a positive reaction at the electron-microscopical level. In this respect, the particles of lead phosphate could act as a tracer; and indeed, the appearance of ‘lead gum’ deposits (used by Yodaiken (1966) as a tracer) in the intercellular spaces between liver cells is similar in appearance to the reaction product (lead phosphate) in certain tissues incubated in the Wachstein–Meisel medium (Wachstein & Besen, 1964; Kaye & Pappas, 1965; Kaye & Tice, 1966; Kaye et al. 1966; Shiose & Sears, 1966). Any lead phosphate which penetrates into the intercellular spaces may even be bound to specific sites on the extracellular surface of the lateral plasma membrane. Such a process may have resulted in the large accumulation of phosphate found in tissue sections by Moses et al. (1966).

In the present study, electron-opaque deposits measuring 100 mμ or more have been observed covering the damaged membranous structures at the cut surface and sometimes within the larger intercellular spaces. The amount of such deposits is largely controlled by the degree of washing following the incubations. In their size and appearance, such deposits closely resemble those illustrated in low-magnification electron micrographs produced by Moses et al. (1966) as evidence of a non-enzymic reaction. Similarly, such a process may account for the reaction product visualized on the outer surface of the apical leaflets; the observation that opaque deposits accumulate within the cuticle and in the subcuticular spaces strengthens this possibility (Fig. 9). It remains to be shown, however, that the fine reaction product (deposits of less than 500 Å) formed highly selectively in intimate association with one or the other unit membrane (Tice & Engel, 1966; Farquhar & Palade, 1966; the present study) could arise without any participation of cell structures. Moreover, it is difficult to envisage how the precise localization of reaction product on the stacks of lateral plasma membrane in the present study could have arisen by a non-enzymic process. In Figs. 4–8 the deposits of lead phosphate are located exclusively on the intracellular surface of the membrane; deposits are never found on the extracellular surface, as might be expected if they were randomly formed by a lead-catalysed hydrolysis of ATP. It is conceivable, therefore, that deposits of lead phosphate in incubated tissues can originate from two independent processes. First, the coarse deposits may arise by a non-enzymic hydrolysis of ATP, and may accumulate in
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interstitial spaces and give the impression of a positive reaction on the cell surface, especially as seen in the light microscope. Secondly, enzymic activity may account for the finer intracellular deposits of lead phosphate which are intimately associated with the unit membrane and other sites. If high-resolution electron microscopy is employed, it should be possible to distinguish between these two processes. Therefore it is concluded that the Wachstein-Meisel technique for demonstrating ATPases can still provide useful information not obtainable by any other available method.

The precise intracellular localization of reaction product on the lateral plasma membrane of the cortical epithelial cells of rectal papillae is in accord with the theoretical predictions concerning the sites of phosphate release in vivo. While most models and physiological observations on membrane transport indicate that phosphate is released intracellularly (Hokin & Hokin, 1960; Judah & Ahmed, 1964; Whittam, Wheeler & Blake, 1964; Post & Sen, 1965; Opit & Charnock, 1965), nearly all the histochemical studies published so far show that lead phosphate deposits on the outer surface (Kaye & Pappas, 1965; Farquhar & Palade, 1966; Kaye & Tice, 1966; Kaye et al. 1966; Shiose & Sears, 1966). Most histochemists have ignored this inconsistency. Farquhar & Palade (1966) suggest that in amphibian skin the phosphate which is released to the outside of the cells may be replaced by the phosphate which is stored as calcium phosphate deposits in the dermis. However, such an explanation may not be applicable to other tissues, such as kidney tubules (Wachstein & Besen, 1964), ciliary epithelium (Kaye & Pappas, 1965; Shiose & Sears, 1966), cornea (Kaye & Tice, 1966) or gall bladder (Kaye et al. 1966). If we are to assume that the accumulation of lead phosphate marks the site of enzymes hydrolysing ATP, the intracellular localization of reaction product in rectal papillae agrees with theory and lends further validity to the histochemical procedures employed in this study.

Histochemical methods for localizing phosphatases may also give erroneous results if enzyme activity is altered or destroyed during fixation. In an assessment of the total ATPase activity lost in tissues fixed in aldehydes and incubated in Wachstein-Meisel media, Moses et al. (1966) found that 94% of the total Mg-activated ATPase was destroyed in the processed material as compared to the activity in fresh tissue homogenates. However, in similar experiments on sarcoplasmic reticulum, if the tissue was fixed for brief periods (less than 2 h) and the incubations were carried out at 17 °C, Tice & Engel (1966) found only 55% inhibition of the enzyme activity. The experimental conditions followed in the present study closely approach those used by Tice & Engel. It is emphasized that Moses et al. fixed their tissues in stronger aldehyde solutions for longer periods and carried out the incubations at a higher temperature (37 °C) than in the present study. The preservation of some other enzyme systems in glutaraldehyde has been discussed by Quiocho & Richards (1964).

With the hazards of an erroneous interpretation in mind, the present results may be discussed in terms of an earlier hypothesis on fluid reabsorption by the rectal papillae of Calliphora (Berridge & Gupta, 1967). The basal plasma membrane of the cortical epithelial cells, facing either the haemolymph or the infundibular space (Gupta & Berridge, 1966b), is thought to have a minor role in the net transport of ions, and in this respect resembles the inward facing, ATPase-free membrane in
amphibian skin (Farquhar & Palade, 1966) and rabbit gall bladder (Kaye et al. 1966). This basal plasma membrane may be freely permeable to ions, but on account of its relatively small surface area (about 2% of the total surface area of the cell) it may be considered largely insignificant for the major transport processes.

The main sites of active ion transport in rectal papillae are believed to lie on the stacks of infolded membranes which open into the intercellular spaces (Berridge & Gupta, 1967). Since the lateral plasma membrane makes up about 80–90% of the total cell surface in the cortical epithelium of rectal papillae (Berridge & Gupta, 1967), the Mg\(^{2+}\)-activated ATPase associated with this membrane probably accounts for most of the enzyme found in the membrane fraction prepared from homogenates of this organ. The preliminary biochemical study of this fraction indicated only a slight stimulation of ATPase activity after addition of Na\(^+\) and K\(^+\) and no inhibition with ouabain. Apparently, this ATPase differs markedly from the ATPase stimulated by Mg\(^{2+}\) plus Na\(^+\) plus K\(^+\) found in the sodium-transporting systems of many vertebrate cells (Kinsolving et al. 1963; Glynn, 1964; Skou, 1965). It is to be noted, however, that, even in such sodium-transporting systems, the ratio between the Mg\(^{2+}\)-, Na\(^+\)- and K\(^+\)-activated enzyme and Mg\(^{2+}\)-activated enzyme in homogenates varies according to the experimental conditions employed (Farquhar & Palade, 1966). Furthermore, there is every reason to believe that the potassium-transporting system in various insects (Ramsay, 1953, 1955b; Harvey & Nedergaard, 1964; Haskell, Clemons & Harvey, 1965; Berridge, in preparation) may have properties quite different from those classically associated with sodium–potassium exchange pumps in vertebrate tissue.

In considering the nature of potassium transport in rectal papillae, it is important to remember that the pumps are not directed towards the haemolymph but towards an enclosed space which may have a quite different ionic composition. Furthermore, since the contents of the intercellular spaces continuously flow under hydrostatic pressure into the haemolymph (Berridge & Gupta, 1967), the intercellular spaces could not function as a reservoir of sodium, which would be essential for the operation of a traditional sodium–potassium exchange pump. If the potassium pump is relatively independent of extracellular ions, it may resemble either the linked cation–anion pump found in the gall bladder (Diamond, 1962) or the electrogenic pumps which are thought to occur in frog skin (Bricker, Biber & Ussing, 1963) and toad urinary bladder (Frazier & Leaf, 1963).

The role of the apical plasma membrane in ion transport by rectal papillae is less clear. The possibility that the Na\(^+\)- and K\(^+\)-activated ATPase localized on this membrane is an artifact has already been mentioned. If real, however, the enzyme may participate in supplementary pumps for potassium, especially when the concentration of this ion in the lumen falls far below that in the cells. Potassium uptake across this membrane may occur in exchange for hydrogen and result in acidification of the rectal contents. A coat of particulate subunits found on the cytoplasmic surface of this membrane may be involved with some of these transport processes (Gupta & Berridge, 1966a). Similar subunits have been reported in the rectal glands of other insects (Noirot & Noirot-Timotheé, 1966; Drs J. L. Oschman & B. J. Wall, personal
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It is also to be noted that, in other insects where the concentration gradient between the rectal lumen and haemolymph is probably much greater than in Calliphora, the apical plasma membrane is associated with mitochondria.

In conclusion, the restricted localization of an ATPase on the stacks of lateral plasma membrane further emphasizes the importance of these membranes as possible sites of active ion-transport during fluid reabsorption by rectal papillae. Potassium secretion into the enclosed intercellular sinus is believed to create the osmotic gradient necessary to extract water from the rectal lumen (Berridge & Gupta, 1967).

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REFERENCES


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Figs. 4–13 are electron micrographs of the rectal papillae fixed in glutaraldehyde and incubated in various media containing nucleoside phosphates as substrates for the demonstration of the corresponding enzyme activity.

Fig. 4. With substrate ATP at pH 7.2. Electron-opaque deposits, presumably indicating the sites of ATPase activity, are confined to the lateral plasma membranes of the cortical epithelial cells. The lead phosphate deposits seem to be formed exclusively on the cytoplasmic surface of the membrane-infolds in the stacks (si), as well as on the rest of the lateral plasma membrane (arrows). The rest of the cytoplasm including mitochondria (m) and profiles of tracheoles (tr) do not show any deposits. × 32000.

Fig. 5. The same as Fig. 4 but with the substrate ADP at pH 7.2. The deposits of the reaction product are very small and sparse but are still confined to the cytoplasmic surface of the lateral plasma membrane. Asterisks mark the larger intercellular spaces which are more dilated in this case. × 20000.
Fig. 6. As in Fig. 4, but at higher magnification. The exclusively intracellular nature of the reaction product is particularly clear at the sites marked with arrows. The lumina of the sac-like infolds open into larger intercellular spaces (asterisks). (cyt, cytoplasm; m, mitochondrion.) \( \times 70000 \).

Fig. 7. The same as in Fig. 6, but with ADP as the substrate. \( \times 40000 \).

Fig. 8. A small portion from a field similar to that in Figs. 4 and 6, at a much higher magnification, to show that the deposits of the reaction product seem to develop in association with the cytoplasmic leaflet (arrows) of the unit membrane; asterisks mark the intercellular spaces. \( \times 220000 \).
Fig. 9. With substrate ATP at pH 7.2; the incubation medium in this instance contained high Na⁺ and K⁺ in addition to Mg²⁺. The field shows membrane infolds at the apical surface of the cortical epithelial cells, with a portion of the apical cytoplasm (cyt) and of the cuticular intima (cut). Electron-opaque deposits are present on the infolded plasma membrane but are formed exclusively on the extracellular surface (arrows). Deposits are also found in the subcuticular space (scs) and in the cuticular intima but not in the cytoplasm. ×45,000.

Fig. 10. With ATP as substrate at pH 7.2. A portion of the medulla of the rectal papillae showing the reaction product in the intercellular spaces between the collagen-secreting cells (arrows). The cytoplasmic (cyt) and nuclear (n) structures of the cells do not show any reaction but some deposition has occurred in the extracellular material (ext). (nl, nucleolus.) ×30,000.
Fig. 11. A small portion of the field in Fig. 10, at a higher magnification, to show the extracellular nature of the reaction product. Asterisks mark the extracellular material, while the arrows indicate the intercellular space. (cyt, cytoplasm.) × 50000.

Fig. 12. With substrate ATP at pH 6-8. A small portion of a cortical epithelial cell showing the absence of a reaction product from the stacked infolds (st) at this pH. Fine deposits are now formed in the mitochondria (m), apparently confined to the surface of the cristae facing the matrix. × 65000.

Fig. 13. As in Fig. 12. In addition to the mitochondria, very fine deposits of the reaction product are also formed in the nucleus (n), particularly in the chromatinic masses (ch) and in the nucleolus (nl). No reaction occurs in the nucleoplasm or in the cytoplasmic structures such as ribosomes and endoplasmic reticulum. (ne, nuclear envelope.) × 23000.