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THE IMMUNOLOGICAL DEMONSTRATION OF AN ASSOCIATION BETWEEN MALTASE ACTIVITY AND PROXIMAL TUBULAR CELL BORDERS IN THE RABBIT KIDNEY

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SUMMARY

An immunological investigation has been made of the origin in the renal cortex of a membrane fraction selected for its high maltase acitivity, and also of the effect on the enzymatic activity of antibodies specifically directed against maltase.

1. A particulate membrane fraction, isolated from rabbit renal cortex and carrying high specific activities of the disaccharidase enzymes maltase and trehalase, has been used to raise antibodies in guinea pigs.

2. The antisera produced react with and remove from solution the soluble maltase activity which is released from the membrane fraction by papain.

3. The immune precipitate exhibits unimpaired maltase activity.

4. The antiserum when used in the indirect immunofluorescence technique on fixed sections of rabbit renal cortex reacts only with the apical area of the cells of the proximal tubules. This demonstrates that the membrane immunogens in general and the maltase in particular are confined to this brush border region.

INTRODUCTION

There are considerable difficulties involved in isolation and purification of morphologically recognizable renal proximal tubular brush borders. This is partly due to the ease with which the microvilli form vesicles[1]. Clearly a specific enzyme marker for the brush border would simplify purification procedures so that more detailed studies on the nature of this absorptive cell surface could be carried out. Recently, Berger and Sacktor[2], using rabbit tissue, demonstrated that as morphologically intact brush borders were carefully purified from a homogenate of renal cortex, the specific activities of the disaccharidase enzymes, maltase and trehalase, increased. This suggests that these enzymes are found on the brush border, but does not rule out the possibility that they may be present on membrane fragments from elsewhere in the cell, which are not readily separated from the brush borders.

The investigation described here approaches the problem in a different way. A vesicular membrane fraction carrying high specific activities of maltase and trehalase has been isolated from the rabbit renal cortex by a relatively simple procedure[3].

An antiserum raised against this material and shown to contain antibodies specifically directed against maltase, has been used in the indirect immunofluorescence procedure to localize the exact source of this membrane fraction in the intact renal cortex. It is shown that fluorescence occurs only on the apices of the cells of the proximal tubules and that therefore the membrane fraction, with its high disaccharidase activities, is derived from this brush border-containing region.

MATERIALS AND METHODS

Isolation of the membrane fraction

The details of the isolation of the membrane fraction from the rabbit renal cortex have been described elsewhere[3]. The procedure involves collagenase digestion of fresh chopped renal cortex followed by homogenization, exposure to hypotonic Na_2-EDTA solution and density gradient centrifugation in glycerol. The protein content and enzymic activities of the final fraction are reasonably reproducible. It has a protein content of 46.3 ± 1.5 % (3) and specific activities of maltase, trehalase and alkaline phosphatase of 0.14 ± 0.02 (7), 0.52 ± 0.07 (7) and 0.27 ± 0.02 (4) respectively, where activities are in μmoles substrate hydrolyzed/min per mg protein, ± S.E., with number of samples in parentheses. In the electron microscope it appears as an array of small vesicles, of diameter approx. 350 nm.

Preparation of antisera to the membrane fraction

Four female Hartley albino guinea pigs, of about 800 g, were immunized by subcutaneous injection of a preparation of the above membrane fraction, containing 1.4 mg protein, emulsified in complete Freund's adjuvant. This was distributed in 4 limb sites. The initial injection was followed 4 weeks later by booster injections of further membrane fractions from different rabbits. These were given subcutaneously in aqueous solution and each contained approx. 1.6 mg protein per guinea pig. A total of 4 injections was given at intervals of 19, 26, and 18 days. Blood was collected by heart puncture 5 days after each booster injection and the final bleed out was 14 days after the last injection. The blood was allowed to clot at room temperature for 1 h and then was centrifuged at 4 °C in an MSE Multex centrifuge at 693 × *g*. The supernatant was centrifuged at 4 °C in a Sorvall SS-1 centrifuge at 10 300 × *g* for 20 min. Antisera were stored at —20 °C. Serum from unimmunized guinea pigs was collected and treated in the same way.

Preparation of soluble maltase from the membrane fraction

This was carried out as described previously[3] using a brief incubation with crystalline papain. Papain was removed from the released maltase by passage through Sephadex G-100. The maltase activity was excluded from the gel and was collected and concentrated at 4 °C by means of an Amicon ultrafiltration apparatus, using a PM-10 membrane. Maltase activity was assayed by the method of Dahlqvist[4] and protein by the method of Lowry *et al.*[5] using crystalline bovine serum albumin (British Drug Houses) as standard. The soluble maltase was stored at 4 °C in the Krebs–Ringer bicarbonate–saline buffer (pH 7.4) containing streptomycin and penicillin described by Hillman and Rosenberg[1]. Activity decreased considerably if the solution was frozen and thawed.

This soluble maltase was then used to investigate the anti-maltase activity in the antiserum to the membrane fraction. This was done on 1 % agar plates by immunodiffusion[6] and immunoelectrophoresis. Precipitin reactions were also carried out.

Immunofluorescence

In order to demonstrate membrane fractions in the rabbit renal cortex by the indirect immunofluorescence technique, sections of fixed renal cortex were incubated first with the antiserum raised in guinea pigs against the membrane fraction, and subsequently, after washing, with the fluorescent rabbit antibody directed against the immunoglobulin G (IgG) fraction of the guinea pig antiserum.

Preparation of fluorochrome conjugates

Guinea pig IgG was prepared from normal guinea pig serum by ammonium sulphate precipitation and DEAE-cellulose chromatography[7]. It was characterized as IgG by cellulose acetate electrophoresis using a Beckman Microzone electrophoresis system and by immunoelectrophoresis in 0.1 M barbitone buffer (pH 8.6) using rabbit antiserum to guinea pig serum (Hyland Laboratories).

This guinea pig IgG was used to immunize 2 New Zealand white rabbits, each by subcutaneous injection of 3.2 mg protein emulsified in complete Freund's adjuvant. Injection was in 4 limb sites. The initial injection was followed 5 weeks later by a booster injection of 1 mg alum-precipitated protein into the ear vein. Bleeding from an ear vein was carried out after a further 7 days. Assay with guinea pig IgG (1 mg/ml) by the immunodiffusion technique as described by Holborow and Johnson[8], showed 64 units/ml of precipitating antibody.

The IgG fraction of the antiserum was prepared by the same method as for the guinea pig IgG and its purity confirmed again by immunoelectrophoresis using goat antiserum to rabbit serum (Hyland Laboratories). Conjugation with fluorescein isothiocyanate was carried out as described originally by Nairn[9] with the modifications of Holborow and Johnson[8]. A further modification was to carry out the reaction at room temperature for 2 h and to remove unconjugated fluorescent material on a column of Sephadex G-25 equilibrated with 0.15 M phosphate-buffered saline (pH 7). The molar ratio of fluorochrome to protein in the product was 5.7:1. It was found necessary to centrifuge the preparation before use to remove a small precipitate. A conjugate of normal rabbit IgG prepared in a similar way was found to have fluorochrome: protein ratio of 5.0:1. Immunoelectrophoresis of the conjugates demonstrated that conjugation was complete. Assay of precipitating antibody revealed that activity had fallen to 9 units/per ml.

Preparation of tissue

Kidneys were taken from a New Zealand white rabbit under Nembutal and diethyl ether anaesthesia. The cortices were dissected off and sliced into pieces approx. 5 mm thick. These were placed immediately into 95 % ethanol pre-cooled to 4 °C. Tissue was then processed as decribed by Sainte-Marie[10].

Staining

This was carried out on the fixed sections exactly as described by Holborow and Johnson[8]. The initial incubation was with final bleed antiserum diluted 1 in 4 with

phosphate-buffered saline (pH 7). Control slides were treated with similarly diluted normal guinea pig serum. Other control slides were of antiserum-treated sections subsequently incubated with fluorochrome-conjugated normal rabbit IgG. Mounted sections were viewed through a Wild M-20 microscope using a mercury vapour lamp light source and bright field blue light illumination. Photomicrographs were taken with Ektachrome X film at exposures of 1–2 min. Processing was then at an ASA rating of 250.

RESULTS

Characterization of antiserum

Antibody response to the immunizing membrane preparations was assessed by immunodiffusion of the antisera against papain-solubilized maltase from various membrane preparations. A precipitin line was seen after one booster injection and became better defined after further boosters. Fig. 1 shows the line produced after 4 boosters using an antigen concentration of 0.27 mg/ml. The plate had been allowed to develop at room temperature for 10 days. Clearly there is no reaction between the antiserum and either rabbit serum or rabbit urinary Tamm–Horsfall glycoprotein.

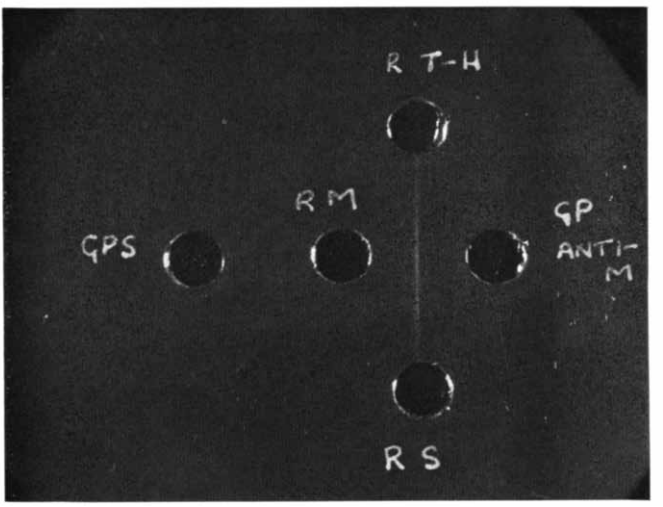

Fig. 1. Immunodiffusion pattern of the papain-solubilized maltase from rabbit membrane vesicles against an antiserum to whole rabbit membrane vesicles raised in guinea pigs. RM, rabbit papain-solubilized maltase (0.27 mg/ml); GP ANTI-M, guinea pig antiserum to rabbit membrane vesicles; GPS, normal guinea pig serum control; R T–H, rabbit Tamm–Horsfall urinary glycoprotein (2 mg/ml); RS, rabbit serum (2 mg protein/ml).

Similarly, the soluble maltase failed to react with guinea pig antiserum to rabbit serum, thereby demonstrating the absence of any serum component. Immunoelectrophoresis of the soluble maltase (0.27 mg/ml) against antiserum to membrane preparation produced a single rather faint anodic arc.

These results demonstrate antibody activity against papain-solubilized material from the membrane preparation. This soluble material also carries high maltase activity (in Fig. 1 the specific activity of the soluble maltase used as antigen was 1.1

μmoles substrate hydrolyzed/min per mg protein). Proof that there are antibodies specifically directed against maltase was obtained by means of a precipitin test.

Precipitation of soluble maltase by the antiserum

The papain-solubilized maltase used had a specific activity of 0.90 μmole maltose hydrolyzed/min per mg protein and had been obtained from a membrane preparation with a maltase specific activity of 0.11. The soluble maltase carried no trehalase activity, in accordance with previous results[3]. The antiserum used was that collected after 2 boosters. It was first thoroughly dialyzed at 4 °C against 0.01 M phosphate buffer (pH 7.0) containing 0.01 % thiomersal (British Drug Houses) in order to remove glucose which would interfere with the maltase assay. Some precipitation of serum proteins occurred during this step; these were removed by centrifugation at 4 °C in the MSE Multex centrifuge for 15 min at 693 × *g*. The supernatant was fully active in immunodiffusion tests against the soluble maltase.

Aliquots of soluble maltase in the Krebs–Ringer buffer, containing 3.25 μg protein, were added to varying amounts of antiserum. Final volumes were adjusted to 0.13 ml with 0.01 M phosphate buffer (pH 7.0) containing 0.01 % thiomersal and the stoppered tubes were incubated at 37 °C for 1 h followed by 4 °C for 48 h. This long period was found necessary for precipitation of the antigen–antibody complex to approach completion. At the end of this period, 0.07 ml of the phosphate buffer was added to each tube and they were centrifuged in the MSE Multex centrifuge at 693 × *g* for 45 min. Aliquots (0.1 ml) of the supernatants were then assayed for maltase activity. The remaining 0.1 ml was agitated gently to resuspend the precipitate and then assayed for maltase activity. The activity in the precipitate was then calculated by subtraction. The results are shown in Fig. 2. It is clear that the antiserum is capable of precipitating the maltase from solution, but that the enzyme retains

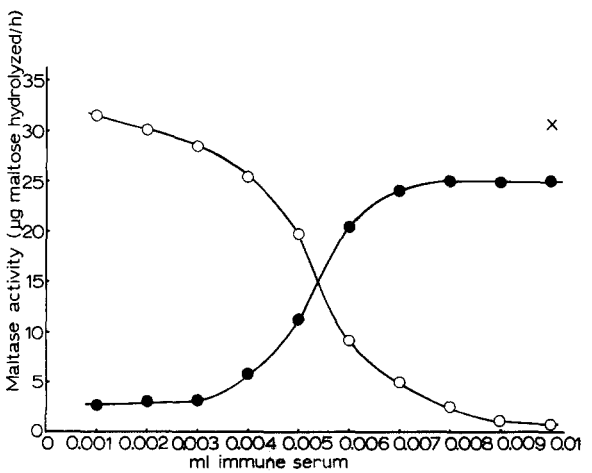

Fig. 2. Precipitation of soluble maltase by increasing amounts of antiserum. Aliquots of papain-solubilized maltase (3.25 μg protein) were treated with antiserum obtained after 2 booster injections. Incubation was for 1 h at 37 °C followed by 48 h at 4 °C. ○—○, maltase activity in the whole supernatant; ●—●, maltase activity in the precipitate; ×, maltase activity after similar treatment with 0.01 ml of normal guinea pig serum. Enzymic activity is expressed as μg maltose hydrolyzed in 1 h.

activity in the precipitated complex. Thus the antibody is directed against the maltase but does not cause appreciable inhibition.

Immunofluorescence

The results of using this antiserum in the indirect immunofluorescence technique on ethanol-fixed sections of rabbit renal cortex are shown in Fig. 3. It can be clearly seen that specific fluorescence is confined to the apical areas of the cells of the proximal tubules. The glomeruli and distal tubules showed a dull autofluorescence which was also seen in controls treated with normal guinea pig serum. Similarly no specific fluorescence could be detected in basement membranes, nor in intracellular areas. Identification of the fluorescent areas with proximal tubular brush borders was confirmed by staining the same section with periodate–Schiff reagent, which reacts with carbohydrate and is known to stain brush borders. Examination of the same area of the slide then demonstrated pinkish brush border areas which were superimposable on the fluorescent areas.

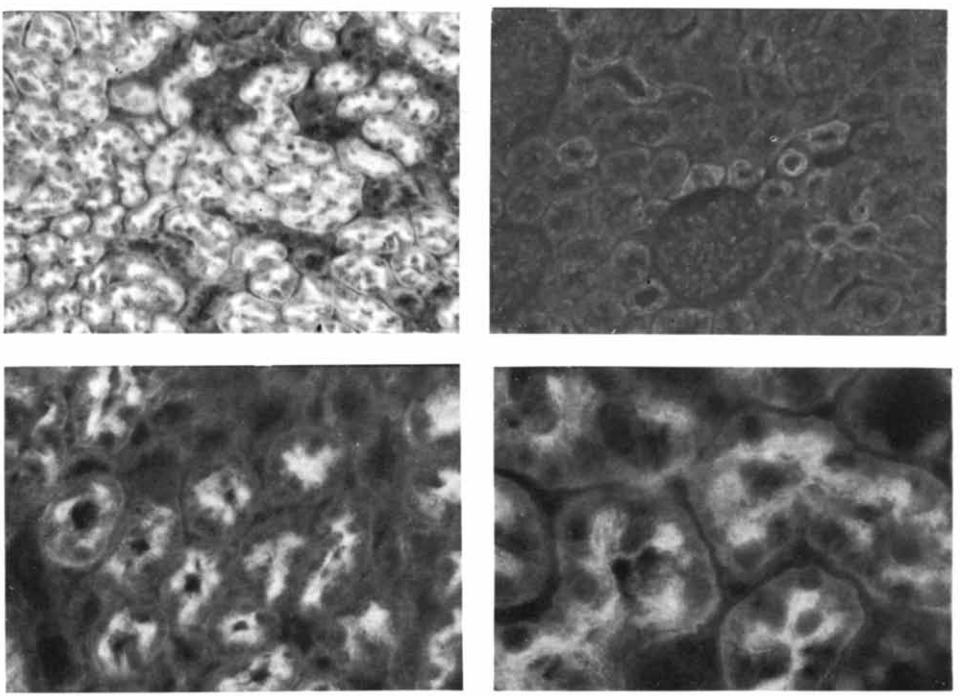

Fig. 3. Sections of rabbit renal cortex treated with antiserum to the membrane vesicles followed by fluorescent antibody to guinea pig IgG. The control was treated with normal guinea pig serum, then with fluorescent anti-IgG. (a) Test × 150; (b) control × 150; (c) test × 300; (d) test × 600.

The whole experiment was repeated using antiserum which had been absorbed with the particulate residue from papain treatment of the membrane preparation. This should render the antiserum specific for substances solubilized by the papain. In fact, 0.5 ml of antiserum was treated with 0.78 mg protein for 1 h at 37 °C, followed by 18 h at 4 °C. The particulate fraction was then removed by centrifugation.

The results of using this antiserum were exactly the same as those using unabsorbed antiserum.

DISCUSSION

The present investigation has shown that a vesicular membrane fraction, isolated from the rabbit renal cortex and identified by its high specific activities of maltase and trehalase, is capable of eliciting the production of antibodies in the guinea pig. At least some of these antibodies are directed specifically against maltase, as seen by the progressive precipitation of soluble maltase by increasing amounts of antiserum. The precipitated maltase–antibody complex is almost fully active enzymically, so that the antigenic sites on the enzyme must be remote from the catalytic site. This is in accordance with results obtained for antibodies to other enzymes[11], where it has been shown that enzymes acting on substrates of low molecular weight are usually not inhibited by their antibodies, whereas the same enzyme acting on a substrate of high molecular weight is inhibited. Presumably antibodies are not formed against the catalytic site due to its universality, and when the substrate is small it becomes less likely that steric inhibition will arise from antibodies covering nearby sites.

It is difficult to assess whether or not maltase is the only immunogenic component of the papain-solubilized material. There is one principal line produce by immunodiffusion and immunoelectrophoresis of the soluble material against the antiserum, although immunodiffusion plates occasionally show two other very faint curved lines (Fig. 1 shows an immunodiffusion plate where the faint lines were most marked), suggesting the presence of more than one soluble reactant.

The same antiserum has been used in the indirect immunofluorescence technique to demonstrate membrane fraction antigens in the renal cortex. Fluorescence was seen only in the apical third of the proximal tubular cells, therefore the membrane fraction must be derived from this region. This apical area includes the brush border, but since fluorescence microscopy has a limited resolution it is not possible to give an absolutely precise localization. However, the membrane fraction is evidently derived from the brush border region. These results also strongly suggest that maltase is only present in the brush border region as there is no reason to suppose that the maltase antigens are not being expressed in the fixed sections. A similar distribution of fluorescence was observed when absorbed serum, rendered specific for the solubilized surface structures, was used.

Such a localization of maltase confirms and complements the results of Berger and Sacktor[2]. Evidently the situation in the kidney is similar to that in the intestine where maltase is also located on the brush border[12]. In the latter tissue, the maltase has a clear role in the terminal digestion of carbohydrate, whereas its function in the kidney is less obvious.

In detailed investigations into some immunochemical properties of the intestinal disaccharidases lactase and invertase, Doell *et al.*[13] were able to localize these enzymes in the brush border region and also to demonstrate that antisera to these enzymes did not cause inhibition of enzymic activity. Similar results have been reported for antisera raised against intestinal sucrase[14] and lactase[15]. A more precise localization of the disaccharidase sucrase in the rabbit small intestine has been carried

out using ferritin labelling of the IgG fraction of precipitating antisera[16]. Ferritin cores were found at a minimal distance of 120 Å from the microvillus membrane luminal surface. It will be of interest to compare the renal and intestinal absorptive surfaces in more detail, particularly as there are obvious differences between them, such as the complete lack of sucrase in the rabbit kidney.

It has been proposed that the urinary glycoprotein first isolated by Tamm and Horsfall[17] and subsequently studied in detail[18,19] may be derived from the renal brush border[20]. The lack of immunological activity between the antiserum to the membrane fraction and rabbit urinary glycoprotein as seen in Fig. 1 of the text does not support this suggestion.

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