Some chemical characteristics of human minor salivary gland secretions

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The minor salivary glands contribute to the composition of whole saliva, but little information has been available about their chemical constituents. Pilocarpine-stimulated labial and palatine secretion from 4 human subjects was investigated by paper and disc electrophoresis, immunochemical analysis, and for content of carbohydrates, amino acids, lipids, hexuronic acids and sulphate. No significant differences were noted between the labial and palatine secretions by any of the methods employed. The minor gland secretions appeared to consist mainly of mucosubstances, possibly with blood group specificity. In addition, three water-soluble components with the characteristics of albumin, alpha-amylase and secretory IgA were seen. The minor gland secretions had an amino acid profile different from those of the major salivary glands and contained higher proportions of carbohydrate. Only one lipid component, with the characteristics of a polar lipid, was seen. Hexuronic acids were not detected in either secretion, whereas both contained sulphate. It would appear that the minor mucous glands contribute to the content of mucosubstances in whole saliva, whereas their content of water-sobuble material is negligible in this respect.

Key-words: Labial and palatine salivary glands; chemical analysis

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The chemical properties of the secretory proteins and glycoproteins of salivary gland secretions may be assessed by chemical or histochemical techniques. A relationship between the results by both techniques has been obtained in the characterization of mucosubstances in the sublingual and submandibular glands (*Eversole*, 1972 a).

Histochemistry allows detailed studies of the types of glandular tissue and secretory material present in the individual salivary glands. It has, however, been difficult to correlate these findings with the composition and origin of the individual macromolecular components of whole saliva. Using chemical techniques alone, the carbohydrate amount of whole saliva was found to be higher than that of the combined individual major salivary gland secretions (*Berggård & Werner*, 1958). A difference in disc electrophoresis patterns of whole saliva and of parotid and submandibular secretions has also been noted (*Hay*, 1969). Irrespective of the technique employed, the main difficulties in correlating data from individual major salivary gland secretions with those

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from whole saliva are probably due to the contribution of the minor salivary gland secretions. In the literature the numerous small glands lining most of the mucosal surfaces of the oral cavity are termed minor, mucosal, intrinsic, accessory or intramural glands. In the present study the term minor salivary glands will be used. *Provenza* (1964) has grouped the minor glands into minor sublingual, lingual, buccal, labial, palatine and glossopalatine. The glands of von Ebner are the lingual glands around the circumvallate papillae and these are stated to be the only minor salivary glands which are serous in nature, the other types being mixed, predominantly mucous or purely mucous glands.

Except for some data on inorganic constituents and total protein concentration (*Dawes & Wood*, 1973) relatively little information about the chemical constituents of the minor salivary gland secretions appears to be available. The present investigation reports some chemical properties of the labial and palatine secretions and correlates the results with the histochemical staining properties of these glands as reported by others.

MATERIALS AND METHODS

Collection of secretion

The secretions were collected from 4 dental students, 20 to 24 years of age, with blood group status A Le (a—b$^+$). Secretory activity was stimulated by oral administration of 10 to 25 mg pilocarpine hydrochloride in 20 ml water. Initially, a dose of 10 mg (per 70 kg bodyweight) was used, which resulted in large individual variations in flow rates. By increasing the dose to 25 mg more material was obtainable from each subject. Collection of secretion commenced 10 min after the subject began to perspire and continued as long as secretion was evident (20 to 90 min).

Palatine secretion was collected from the hard palate and labial secretion from the everted upper and lower lips at the area opposite the lateral and central incisors. Droplets formed during 5 to 10 min were aspirated into 1 ml polypropylene syringes without cannulae (Johnson & Johnson Ltd., UK). Parotid and submandibular secretions were collected from the orifices of their respective ducts into calibrated micropipettes (Carlsberg, Denmark). The secretions were immediately transferred to ice-chilled glass tubes, and stored at —20°C.

Paper electrophoresis

Electrophoresis was performed on Whatman no 1 paper (8 × 20 cm) in a sodium barbitone buffer of pH 8.6 or in a 0.05 M phosphate buffer of pH 6.0. Samples of labial and palatine secretion (200 mg wet weight) were applied on a line and subjected to a constant voltage of 200 V for 5 hrs, the papers were then dried with a hair dryer and the proteins fixed in a forced draft oven at 90°C for 60 min.

The papers were cut to strips of 1 × 20 cm and immersed in staining solutions: 0.2 per cent alcian blue 8GX in 7 per cent acetic acid, 1 per cent toluidine blue 0 in 7 per cent acetic acid, 1 per cent amidoblack 10B or 0.05 per cent Coomassie brilliant blue R-250 in methanol-acetic acid-water (45 : 20 : 9). Strips were also stained with the periodic acid-Schiff method of *Felgenhauer* (1970) and with a spray of 1 per cent ninhydrin in acetone with subsequent development of color at 90°C for 10 min. Alcian blue, toluidine blue and mucicarmine were purchased from Gurr Ltd., UK, amidoblack and ninhydrin from Merck AG, Germany,

and Coomassie brilliant blue from ICI, UK.

Disc electrophoresis

Analyses were carried out in a Buchler apparatus (Buchler Instruments Inc., N.J., USA) with glass columns of 5 × 70 mm. The separating gel was 7.5 per cent acrylamide at pH 9.5, the concentrating gel was 2.5 per cent acrylamide at pH 8.3. The electrophoresis was performed in a TRIS-glycine buffer of pH 8.3, prepared by dissolving 6.0 g TRIS and 28.8 g glycine in distilled water and diluting to 1 liter (*Maurer* 1971).

Aliquots of 100 μl of labial and palatine secretions were added 25 μl glycerol and 1 drop of 0.005 per cent bromophenolblue and applied to the columns. The electrophoresis started at 1.25 mA per gel until the tracking dye reached the separating gel, the current was then adjusted to and maintained at 2.5 mA per gel for the rest of the run.

The gels were stained in 0.05 per cent Coomassie brilliant blue in methanol, acetic acid, water (45 : 20 : 9) for 1 hr and destained in the same solvent overnight. Duplicate gels were stained with 0.2 per cent alcian blue in 7 per cent acetic acid for 1 hr and destained overnight in 7 per cent acetic acid. The gels were stored in 7 per cent acetic acid.

Antisera

Rabbit antisera against human serum, albumin, and colostrum IgA were purchased from Dakopatts, Denmark. Antisera against whole saliva and parotid secretion was raised in rabbits (2—3 kg) receiving 3 weekly injections of 1 ml unconcentrated fluid mixed with the same volume of Freund's complete adjuvant (Difco). The emulsion was deposited subcutaneously and intramuscularly. Additional injections of unconcentrated fluid were given after 1, 2, 7 and 8 months. Blood was drawn by heart puncture 4 days after a final booster dose.

Immunochemical methods

Double diffusion was performed in 1 per cent agarose (Indubiose A 45, L'industrie Biologique Francaise, S.A., Gennevilliers, France) in 0.85 per cent NaCl containing 0.1 per cent NaN_3. Samples of labial and palatine secretions (10 μl) were applied in the central wells and tested against rabbit antisera against human whole saliva, parotid secretion, serum, albumin, and colostrum IgA. The immunodiffusion plates were developed for 48 hrs at room temperature in moist chambers. Unprecipitated proteins were removed by washing in 0.85 per cent NaCl for 3 days, and salts removed by additional washing in distilled water for 1 day. The plates were dried and stained with 0.05 per cent Coomassie brilliant blue R-250 in methanol-acetic acid-water (45 : 20 : 9) for 10 min and destained in several changes of solvent.

Carbohydrates

Lyophilized secretion (50 mg wet weight) was hydrolysed in 2 ml 1.5 N HCl at 105°C for 2 hours in sealed ampoules. The residues were taken to dryness under reduced pressure at 37°C, and extracted with pyridine. Aliquots of the pyridine-extracts, equivalent to 10 μl of the original secretion, and of the reference substances (5 μg each of fucose, mannose, glucose, galactose, N-acetylgalactosamine, N-acetylglucosamine, and N-acetylneuraminic acid) were subjected to thin layer chromatography on 20 × 20 cm cellulose plates of 0.20 mm thickness (MN 300 cellulose, Macherey Nagel & Co., Düren, Germany). The plates were developed

twice by ascending technique with ethyl-acetate, pyridine, acetic acid and water (5 : 5 : 1 : 3) as solvent system. The carbohydrates were detected with a silver nitrate spray reagent (*Trevelyan, Procter & Harrison*, 1950).

Amino acids

Lyophilized secretion was hydrolysed in 2 ml 6 N HCl at 105°C for 24 hours under reduced nitrogen atmosphere, in sealed ampoules. The hydrolysates were taken to dryness under reduced pressure at 37°C. The samples were analysed on a Technicon AutoAnalyzer with Chromobead type B resin, using the 18 hour elution procedure (Technicon AutoAnalyzer manual). Norleucine (0.1 μmol) was added as internal standard. The amino acids were identified by their elution sequence and peak characteristics and were quantitated on a Technicon Integrator/Calculator model AAG.

Lipids

Pilot experiments showed that the mucous minor gland secretions were rendered water-soluble by adding 10 volumes of 0.1 per cent sodium dodecyl sulphate with 0.005 M mercaptoethanol, or by incubation with 0.1 per cent (w/w) trypsin-TPCK (Worthington, US) in a NH_4HCO_3 buffer pH 8.5 for 2 hours at 37°C. In these instances small lipid droplets appeared in the solution, sedimented slowly and coalesced.

To investigate this aspect better, lipids were extracted from lyophilised labial and palatine samples with chloroform-methanol (2 : 1, v/v). Plates (20 × 20 cm) of 0.2 mm thickness were prepared by mixing 40 g silica gel (Sil H nach Stahl, Merck AG) with 90 ml 1 mM Na_2CO_3. Before chromatography the plates were activated at 110°C for 1 hour.

The lipids were analysed in two solvent systems. In petrolether - diethylether - acetic acid (90 : 10 : 1), the lipids extracted from the secretions were cochromatographed with phosphatidylcholine, cholesterol and tripalmitate as references. In chloroform-methanol-water (65 : 25 : 4), the lipids were tested against phosphatidylcholine, phosphatidylinositol, and phosphatidylserine. The reference substances were of reagent grade (Koch-Light Lab. UK).

The developed chromatograms were subjected to iodine vapour, to a spray of 0.2 per cent dichlorofluorescein in 96 per cent ethanol or to a orcinol-sulphuric acid spray (*Krebs, Heusser & Wimmer*, 1969).

Sulphate

Sulphate was determined by the method of *Terho & Hartiala* (1971), using the sodium rhodizonate reagent. The values were calculated relative to the protein concentration, which was estimated after treatment of samples and bovine serum albumin in 1 N NaOH for 1 hour at 100°C (*Lowry et al.*, 1951).

Hexuronic acids

Lyophilized secretion from the labial and palatine glands was hydrolysed in 2 ml 4 N HCl for 4 hours at 100°C in sealed freeze drying ampoules. The samples were taken to dryness in a Rotavapor at 37°C and the residues dissolved in distilled water. Glucuronic acid (reagent grade, Sigma Co.) was used as standard. The method of *Nir* (1964) employing a naphthoresorcinol reagent was used.

RESULTS

Collection of secretion

The present technique allowed the collection of 50 to 300 mg labial and 50 to

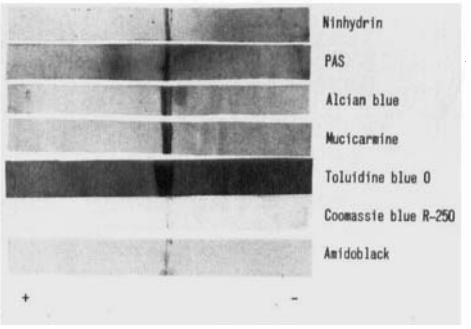

Fig. 1. Paper electrophoresis of palatine secretion. Electrophoresis was performed on Whatman no. 1 paper at 200 V for 5 hrs in a sodium barbitone buffer of pH 8.6. Note diffuse PAS-positive bland with anodic mobility.

1100 mg palatine secretion (wet weight) from each subject. When pilocarpine was administered two or more days in succession, its effect decreased noticeably.

Paper electrophoresis

The mucosubstances of the labial and palatine secretions showed a single component which adhered to the paper at the origin and did not migrate at pH 6.8 or 8.6. The labial and palatine secretions were identical, and stained positively with mucicarmine, alcian blue, Coomassie brilliant blue, toluidine blue, amidoschwarz and ninhydrin. In addition to the PAS-positive material at the origin, a PAS positive component which had anodic mobility at both pH values was observed. None of the other stains showed more components than the material remaining at the origin. No additional components could be observed by altering the electrophoresis period from 2 to 16 hours. A run of palatine secretion is shown in Fig. 1.

Disc electrophoresis

The labial and palatine secretions showed identical patterns after staining with Coomassie brilliant blue. Faint bands

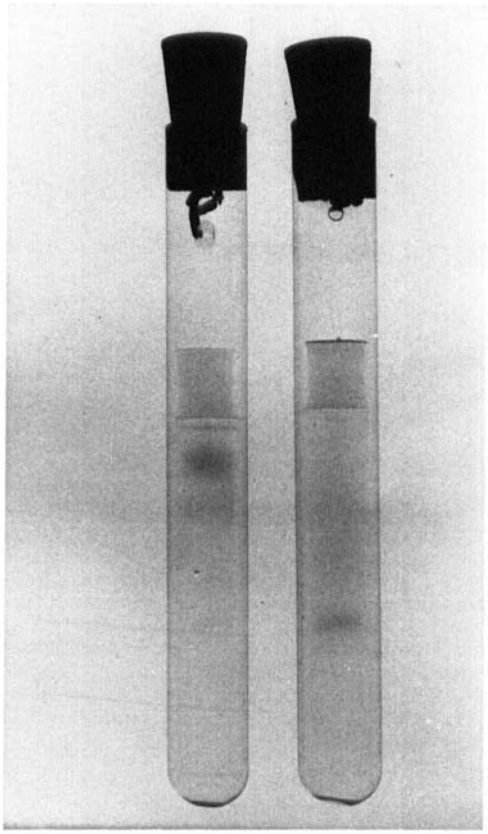

Fig. 2. Anodic disc electrophoresis of labial and palatine secretion.
Electrophoresis was performed in a 2.5 per cent polyacrylamide stacking gel with a 7.5 per cent separating gel in a tris-glycine buffer of pH 8.3. 100 μl palatine (left) and 100 μl labial (right) secretion was applied. The gels were stained with Coomassie brilliant blue. The mucosubstances were originally retained on the surface of the stacking gels, but fell off during the destaining procedure.

corresponding in electrophoretic mobility to IgA, alpha-amylase and albumin were the only components in the gels (Fig. 2) The mucosubstances were retained on the surface of the upper 2.5 per cent stacking gel, and were also stained, but were lost during the handling of the gels in destaining procedures. Alcian blue in duplicate gels only stained the mucosubstances on top of the gels, in these gels no other components were seen.

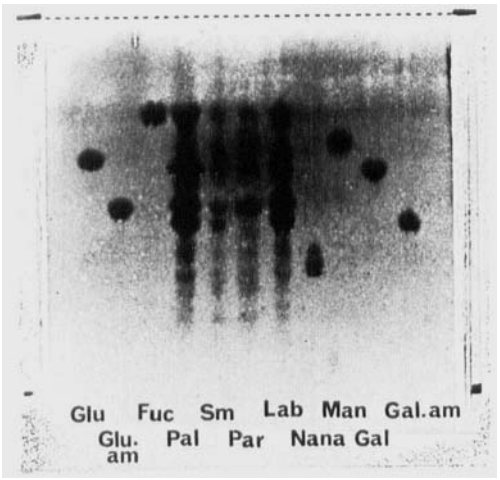

Fig. 3. Thin layer chromatography of carbohydrates in hydrolysed salivary secretions. Amounts corresponding to 10 μl of the original secretions and 5 μg of reference substances were applied. Glu=glucose, Glu.am=N-acetyl glucosamine, Fuc=fucose, Pal=palatine secretion, Sm=submandibular secretion, Par=parotid secretion, Lab=labial secretion, Nana=N-acetyl neuraminic acid, Man=mannose, Gal=galactose, Gal.am=N-acetyl galactosamine.

Immunochemical analysis

The labial and palatine secretions exhibited the same immunochemical pattern, except that the precipitation lines of the labial secretion were somewhat fainter than those of the palatine secretion.

Both secretions showed precipitation arcs against anti-colostrum IgA and anti-albumin, two arcs against anti-serum and three arcs against anti-parotid secretion and anti-whole saliva.

The secretions thus appeared to contain two serum components, IgA and albumin, and an additional component of salivary origin, probably alpha-amylase.

Carbohydrates

Thin layer chromatography of the hydrolysed secretions (10 μl of each) indicated a higher content of carbohydrates per ml secretion in the labial and palatine glands than in the parotid and submandibular glands. Fucose, galactose, N-acetylglucosamine and N-acetylgalactosamine dominated quantitatively in the two minor gland secretions. In addition N-acetyl neuraminic acid and mannose were seen in small amounts (Fig. 3).

Amino acid analysis

The amino acid profiles were similar for the labial and palatine secretions, with treonine, serine, glutamic acid and proline as the major constituents (a total of 42—44 residues per 100). Both secretions contained more of the acidic than of the basic amino acids, and the sulphur-containing amino acids represented a total of 5 to 7 residues per 100 (Fig. 4). The values for N-acetylglucosamine and N-acetylgalactosamine relative to 100 amino acid residues were 15.0 and 11.4 in the labial secretion, and 15.1 and 11.9 in the palatine secretion, respectively.

Lipids

Thin layer chromatography of the extracted materials gave a single spot with dichlorofluorescein in the first system, (petrolether-diethylether-acetic acid) corresponding to a polar lipid — it did not migrate.

The second system (chloroform-methanol-water) also showed only one spot which had an R_f value corresponding to phosphatidylinositol. It gave a negative reaction to sulphuric acid-orcinol and stained positively with dichlorofluorescein and iodine vapour.

Sulphate

Sulphate amounts varying from 8 to 30 μg sulphate per mg protein were found in both labial and palatine secretions.

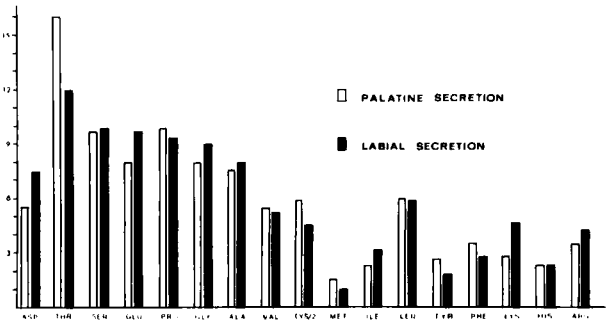

Fig. 4. Amino acid profiles of labial and palatine secretions. The values represent the amounts recovered after hydrolysis in 6N HCl at 105°C for 24 hrs.

Hexuronic acids

Hexuronic acids were not detected in either secretion in the amounts tested (100 to 250 mg secretion, wet weight).

DISCUSSION

The present observations were based on secretions induced by the administration of pilocarpine. *Dawes* (1966, 1967) compared the composition of human saliva secreted in response to pilocarpine and to a gustatory stimulus, and although the concentration of ions and protein varies, electropherograms indicate that the proportion of the individual proteins is unaltered. In dog fundic mucosa *Ley et al.* (1969) found pilocarpine stimulation to alter the proportion of some proteins, but not their composition. *Brandtzaeg* (1971) found that the relative proportions of parotid proteins was changed during stimulation. The concentration of glandular specific proteins such as alpha-amylase increases many more times than that of proteins primarily derived from extra-acinar sources, such as albumin and immunoglobulins. The present results may therefore be influenced by variations in the protein amounts owing to pilocarpine stimulation.

The labial and palatine glands were chosen as representatives of the minor glands for two reasons — they represent the mixed, predominantly mucous (labial glands) and the pure mucous (palatine glands) variety of minor glands. Secondly, the collection of secretion from these glands, was considered to be possible without risking undue mixing with secretions from the major salivary glands, when care was taken during the collection period.

In general, the present results indicated a high degree of similarity between the human labial and palatine secretions. No significant differences in the composition of the secretions with regard to electrophoretic patterns, staining characteristics, immunochemistry or in analysis of amino acids, carbohydrate, lipids, sulphate and hexuronic acids were seen. The labial and palatine secretions do, however, differ in that lysozyme activity is demonstrable only in the labial secretion (*Hensten-Pettersen*, 1975). The amounts of this enzyme are so small (<4 μg/ml) that it would not noticeably affect any of the analyses performed in the present experiments. The results will therefore be discussed together for both labial and palatine secretions, in the following denoted as minor gland secretions.

Zone electrophoresis on paper or in polyacrylamide gels are obviously not the methods of choice in studies of mucous

secretions, but did indicate that the mucosubstances were the major components of the minor gland secretions. Anionic disc electrophoresis revealed only three water-soluble components, which were present in small amounts. Similar experiments with parotid and submandibular saliva have shown 18 to 21 components (*Caldwell & Pigman*, 1965). Since the total protein concentration of the minor and major salivary gland secretions are of the same order of magnitude (*Dawes & Wood*, 1973), the main proportion of protein in the minor gland secretions may be accounted for as water-insoluble mucosubstances, responsible for the high viscosity of these secretions. The small amounts of water-soluble proteins in the minor gland secretions can hardly explain the differences in electrophoretic patterns found between the major gland secretions and whole saliva (*Hay*, 1969). The relatively high concentration of carbohydrates, may, however, be of importance for the values of total carbohydrate in whole saliva, as suggested by *Berggård & Werner* (1958).

The single lipid present in the minor gland secretions was a polar lipid substance. In contrast, phospholipids constitute only 4 to 5 per cent of total lipid components in parotid and submandibular secretions (*Mandel & Eisenstein*, 1969). Lipid inclusions have been observed in electronmicroscopic studies on the labial glands (*Tandler et al.*, 1969; *Doggett, Bentinck & Harrison*, 1971), but data about the association of lipids with mucosubstances in saliva appear scarce. A glycolipid with blood group A specificity has, however, been isolated from hog gastric mucosubstances (*Slomiany & Horowitz*, 1973).

The amino acid profiles of the minor gland secretions differed from those of the major glands (*Leach et al.*, 1967). The minor gland secretions contained higher proportions of threonine, serine, cysteine and methionine, and a lower proportion of proline. The parotid basic proline-rich proteins characterized by *Mandel & Ellison* (1963), *Armstrong* (1970) and *Arneberg* (1974) would therefore be present in low concentration or entirely absent in the minor glands.

The minor salivary glands secrete high amounts of blood group substances (*Milne & Dawes*, 1973), and the amino acid profiles of these secretions (obtained from secretors of blood group status A) showed a high degree of similarity to the composition of purified blood group substance A, isolated from pseudomucinous ovarian cyst fluids (*Donald*, 1973). An interesting possibility would be that the main proportion of glycoproteins in the minor glands consisted of mucosubstances with blood group specificity. In studies on the principal glycoprotein of human mixed saliva, *Schrager & Oates* (1971 a, b; 1974) have shown that the carbohydrate sidechains contain galactose, glucosamine, and galactosamine in a ratio of 4 : 3 : 1, and in addition contain carbohydrate residues endowing the glycoprotein with blood group specificity. A low fucose content (galactose/fucose ratio between 4 : 1 and 4 : 2) is associated with Le_a specificity, an additional fucose residue with H activity; galactose with B activity and galactosamine with A activity. Significant amounts of sulphate are present and ion-exhange chromatography further separates the glycoprotein into two fractions, one sulphated and the other nonsulphated. The sulphate group is possibly connected to a galactose residue, a galactose-3-sulphate.

Eversole (1972) found that the mucosubstances present in mixed seromucous/mucous and pure mucous minor salivary

glands have histochemical staining characteristics comparable to those encountered in portions of the major sublingual and submandibular glands. The mucous cells appear to synthesize neutral, carboxylated and sulphated glycans which are present in varying amounts, whereas seromucous demilunes are PAS and alcian blue reactive, and loose alcianophilia after neuraminidase treatment. Variations in mucosubstance staining properties are encountered within glands and in the same type of gland from different subjects.

If the principal glycoprotein of the minor gland secretions exhibits blood group specificity, this would explain some of the variations observed in the histochemistry of the minor salivary glands from different subjects (*Eversole*, 1972 b; *Harrison*, 1974), but presumeably not the intraacinar differences also reported. As suggested by *Tandler et al.*, (1969), the latter differences may be due to a maturation process in the secretory cycle of the minor gland acinar cells.

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