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The Interaction of Concanavalin A with Blood-Group-Substance Glycoproteins from Human Secretions

By A. E. CLARKE and M. A. DENBOROUGH
University of Melbourne Department of Medicine, The Royal Melbourne Hospital, Melbourne, Vic. 3050, Australia

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1. Gastric juice, saliva and ovarian-cyst fluid were fractionated into glycoprotein components by centrifuging to equilibrium in a caesium chloride density gradient. 2. The glycoprotein fractions from the gastric juice of two group O non-secretors, two group O secretors and three group A secretors all formed insoluble complexes with concanavalin A. 3. Fractions showing maximum interaction with concanavalin A had maximum blood-group activity measured by the haemagglutination-inhibition technique. The sulphate content of the gastric glycoproteins was unrelated to the capacity to interact with concanavalin A. 4. No interaction was found between concanavalin A and the glycoprotein fractions from any of the saliva or ovarian-cyst-fluid samples tested, implying that there is a structural difference in blood-group-substance glycoproteins in gastric juice when compared with those in saliva and ovarian-cyst fluid. 5. The protein components of each of the secretions tested, gastric juice, saliva and ovarian-cyst fluid, interacted with concanavalin A.

The secretions of about 80% of individuals, known as secretors, contain water-soluble glycoproteins with ABH-blood-group specificity. Most of the remaining 20%, non-secretors, secrete glycoproteins with Lewis (Le^a) activity. These blood-group substances typically contain about 85% carbohydrate and 15% amino acids. The carbohydrate, in the form of short chains, is attached to the protein backbone by *O*-glycosidic linkages involving *N*-acetylgalactosamine and either serine or threonine (Morgan & Watkins, 1969).

The possibility that there are structural differences in blood-group substances from different secretions, unrelated to ABH or Lewis activity, was suggested by Lloyd, Kabat & Beychok (1969) on the basis of differing interactions of the blood-group substances with concanavalin A. This protein, the phytohaemagglutinin from jack bean (*Canavalia ensiformis*), has been shown to precipitate several polysaccharides and the detailed structural requirements necessary for interaction have been at least partly established (So & Goldstein, 1967). Lloyd *et al.* (1969) found that concanavalin A interacted with blood-group substance from the stomach of a group O individual and from hog stomach, but not with blood-group substance from the stomach of a group A individual or from ovarian-cyst fluid.

In the present investigation samples of human gastric juice, saliva and ovarian-cyst fluid were fractionated by density-gradient ultracentrifugation, and the interaction of the glycoprotein and protein components of these secretions with concanavalin A was studied.

MATERIALS AND METHODS

Materials. Pepsin, CsCl and other chemicals (purest grade available) were from British Drug Houses Ltd., Poole, Dorset, U.K.

Determination of protein. Total protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine serum albumin as a standard.

Determination of sugar. Total neutral sugar concentrations were determined by the phenol–H_2SO_4 method (Dubois, Gilles, Hamilton, Rebers & Smith, 1956). In all instances where the protein concentration was higher than 100mg/ml the total neutral sugars and the blood-group activity were measured after precipitation of the protein with 5% (w/v) trichloroacetic acid.

Determination of blood-group substances. The amount of ABH blood-group substances in samples of fractionated secretions was assessed by doubling-dilution agglutination-inhibition tests as described by Denborough, Downing & Doig (1969). Lewis activity was measured by Dr Simmons of The Commonwealth Serum Laboratories, Parkville, Vic., Australia.

Determination of sulphate. Sulphate was determined by a modification of the turbidimetric method of Dodgson & Price (1962). For hydrolysis, 450μl of each fraction was mixed with 50μl of 10M-HCl in a glass ampoule that was

then sealed and heated at 105°C for 5h. The sulphate content of 100μl portions of the hydrolysate was determined by mixing with 300μl of 3.8% (w/v) trichloroacetic acid followed by the addition of 100μl of $BaCl_2$–gelatin reagent (Dodgson, 1961). After 20min at room temperature the extinction at 360nm was measured against a reagent blank in which the sample was replaced by 100μl of 1M-HCl. A control in which 100μl of the hydrolysed sample was mixed with 300μl of 3.8% trichloroacetic acid and 100μl of gelatin solution containing no $BaCl_2$ was prepared for each determination. By this method, amounts of less than 5μg of sulphate could be detected in 100μl samples.

Concanavalin A–glycoprotein interaction. Concanavalin A was prepared from jack-bean meal (defatted; Sigma Chemical Co., St Louis, Mo., U.S.A.) by the method of Agrawal & Goldstein (1967), and stored at 4°C in 1M-NaCl.

The interaction of concanavalin A and glycoprotein was followed by using the turbidimetric assay method of Smith, Smith & Goldstein (1968), modified to give the following reaction mixture: concanavalin A (500μg) in 100μl of 2.8M-NaCl in 24mM-sodium phosphate buffer, pH7.2; glycoprotein in water (200μl) containing 100μg of carbohydrate (as galactose). The reaction mixture was incubated at 25°C for 30min and the extinction at 420nm measured. Blanks in which 100μl of 2.8M-NaCl in 24mM-sodium phosphate buffer, pH7.2, replaced the concanavalin A were run for each glycoprotein sample, in addition to a blank in which water replaced the glycoprotein sample.

Secretions. Gastric juice and saliva were collected from apparently healthy volunteers: two group O non-secretors, a group O secretor and a group A secretor. Gastric juice was collected both in the starved state and for 1h after the injection of 50mg of histalog and neutralized immediately with 1M-NaOH. Histalog, 3-(β-aminoethyl)pyrazole dihydrochloride, an analogue of histamine, was obtained from Eli Lilly and Co., Indianapolis, Ind., U.S.A. Subcutaneous injection of histalog produces a stimulation of gastric secretion similar to that produced by histamine, but has less severe side effects. All saliva was collected at the same time and pooled. Ovarian-cyst fluids, two from group O secretors and one from a group O non-secretor, were collected from cysts removed at operation. The secretions were concentrated by ultrafiltration through 8/32 Visking dialysis tubing, brought to an initial density of 1.46g/ml with CsCl and fractionated by sedimentation in a Beckman–Spinco model L utracentrifuge (rotor 50Ti) at 40000rev./min for 40h at 4°C as described by Creeth & Denborough (1970).

Hydrolysis of gastric-juice fractions. Samples of fractionated gastric glycoprotein (0.5mg/ml) were hydrolysed with 1M-HCl by heating in sealed ampoules in a boiling-water bath for 8h. These conditions were selected to give maximum yields of neutral sugars (Spiro, 1966).

Hydrolysate (4ml) was passed through a column (0.8cm×8cm) of Dowex 1 (X2; formate form; 200–400 mesh) and then through a column (0.8cm×8cm) of Dowex 50 W (X4; 200–400 mesh) and washed through with water. The effluent was collected in a polypropylene beaker and concentrated to about 100μl in a vacuum desiccator over P_2O_5 and NaOH. The concentrated deionized hydrolysate was chromatographed on paper in butan-1-ol–acetic acid–water (10:3:7, by vol.) and ethyl acetate–pyridine–water (2:1:2, by vol., upper phase) (Jermyn & Isherwood, 1949), and stained with alkaline $AgNO_3$ by the method of Trevelyan, Procter & Harrison (1950).

RESULTS

Gastric juice. For each of the eight gastric-juice samples tested the more dense carbohydrate-containing fractions were well separated from the predominantly protein fractions. The patterns of separation of the glycoprotein and protein components of resting and post-histalog gastric juice from a group O non-secretor are shown in Figs. 1(*a*) and 2(*a*) and from a group A secretor in Figs. 4(*a*) and 5(*a*). Similar patterns were obtained from both resting and post-histalog gastric-juice samples from another group O non-secretor and a group O secretor.

Two regions of interaction with concanavalin A, one corresponding to the glycoprotein peak and the other rising to a maximum in the fraction having the highest protein concentration, were found for each of the gastric-juice samples tested. The interaction

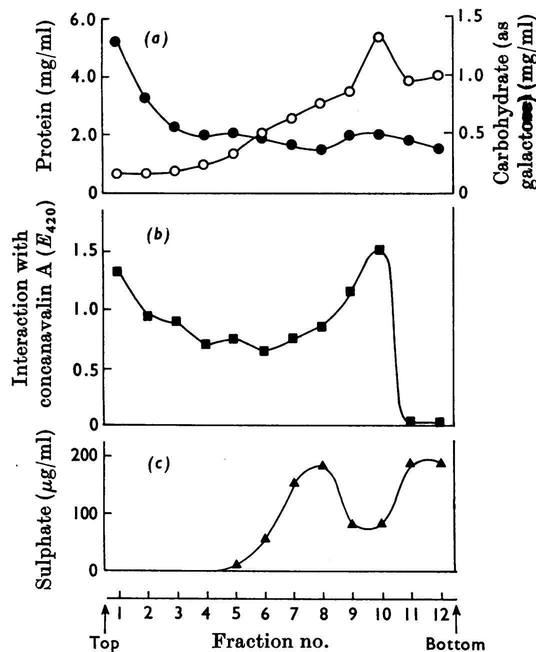

Fig. 1. Fractionation of resting gastric juice (concentrated 4.6-fold) from a group O non-secretor after centrifuging to equilibrium in a CsCl density gradient. The initial density was 1.46g/ml. Sedimentation was for 40h at 40000rev./min in rotor 50Ti of a Beckman–Spinco model L ultracentrifuge. (*a*) Separation of protein (●) and carbohydrate (○); (*b*) interaction with concanavalin A (■); (*c*) distribution of sulphate (▲).

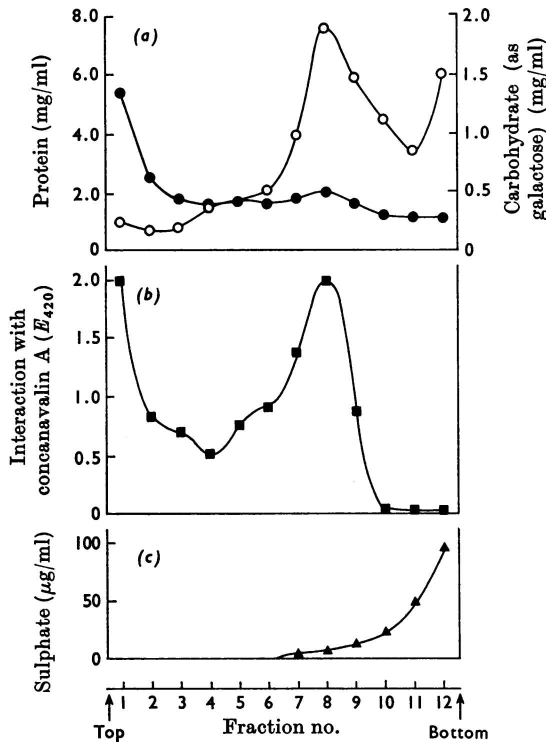

Fig. 2. Fractionation after centrifuging to equilibrium in a CsCl density gradient (details as in Fig. 1) of gastric juice collected for 1h after the injection of 50mg of histalog from the group O non-secretor (as Fig. 1) (concentrated 10.2-fold). (*a*) Separation of protein (●) and carbohydrate (○); (*b*) interaction with concanavalin A (■); (*c*) distribution of sulphate (▲).

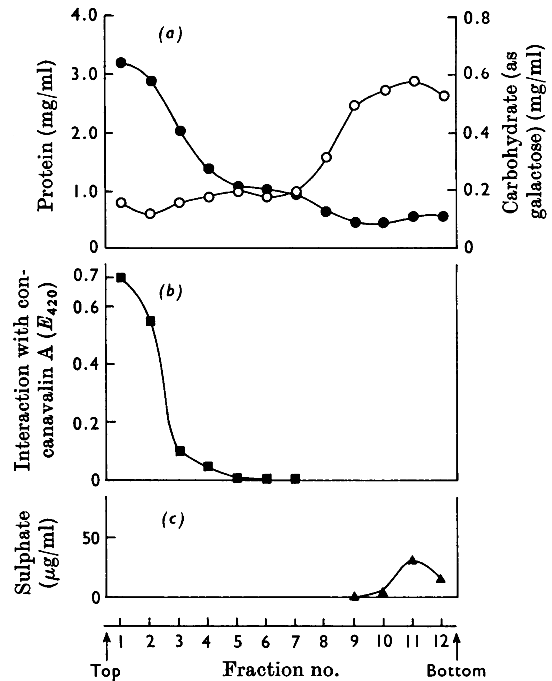

Fig. 3. Fractionation after centrifuging to equilibrium in a CsCl density gradient (details as in Fig. 1) of saliva from the group O non-secretor (as Fig. 1) (concentrated 5.0-fold). (*a*) Separation of protein (●) and carbohydrate (○); (*b*) interaction with concanavalin A (■); (*c*) distribution of sulphate (▲).

of concanavalin A with gastric-juice components from a group O non-secretor is shown in Figs. 1(*b*) and 2(*b*) and from a group A secretor in Figs. 4(*b*) and 5(*b*). The peak of interaction of concanavalin A with glycoprotein corresponds to the peak of blood-group-substance activity of the group A secretor (Figs. 4*b* and 5*b*). Semiquantitative estimations of Lewis activity of the fractions of gastric juice from the non-secretor showed that the glycoprotein fraction having maximum interaction with concanavalin A had 100-fold greater Lewis activity than the protein fraction having maximum interaction with concanavalin A. Similar results were found for gastric juice from the other group O non-secretor and the group O secretor. The extent of reaction with concanavalin A was greater for the gastric glycoprotein fractions from both group O non-secretors than for the group A secretor or the group O secretor.

The sulphate content of the gastric-juice fractions from the group O non-secretor is shown in Figs. 1(*c*) and 2(*c*). There are several groups of sulphate-containing glycoproteins in resting gastric juice, whereas in the post-histalog gastric juice the sulphate content is maximum in the fraction of highest density, indicating that the sulphated glycoproteins of post-histalog gastric juice belong to a more restricted density range. A similar result was found for gastric juice from the other group O non-secretor.

Saliva. The pattern of separation of the glycoprotein and protein components of saliva from the group O non-secretor is shown in Fig. 3(*a*) and from the group A secretor in Fig. 6(*a*). A similar separation was found for salivas of the other subjects tested, another group O non-secretor and a group O secretor. The interaction of concanavalin A with all samples of saliva tested was restricted to the protein fractions. There was no detectable interaction of any of the glycoprotein fractions with concanavalin A. The interaction of concanavalin A with fractionated saliva of a group O non-secretor is shown in Fig. 3(*b*) and with fractionated saliva of a group A secretor in Fig. 6(*b*). The sulphate content of

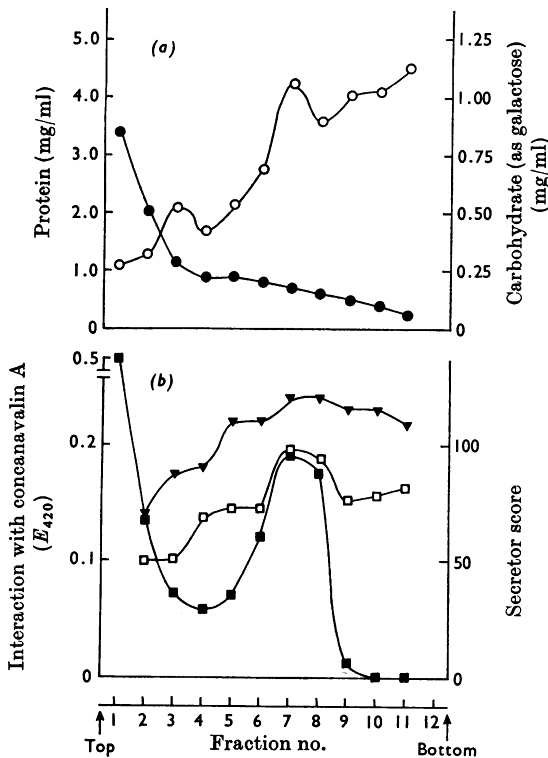

Fig. 4. Fractionation of resting gastric juice (concentrated 4.5-fold) from a group A secretor after centrifuging to equilibrium in a CsCl density gradient (details as in Fig. 1). (*a*) Separation of protein (●) and carbohydrate (○); (*b*) interaction with concanavalin A (■) and blood-group-substance activity: H score (□); A score (▼).

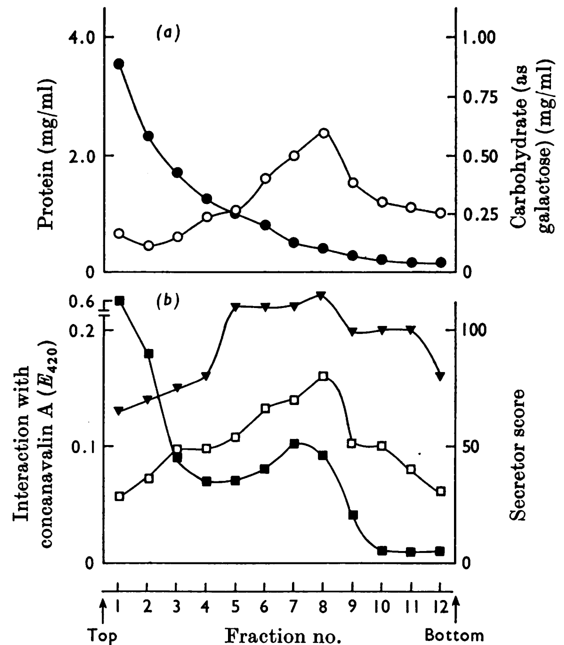

Fig. 5. Fractionation after centrifuging to equilibrium in a CsCl density gradient (details as in Fig. 1) of gastric juice collected for 1h after the injection of 50mg of histalog from the group A secretor (as Fig. 4) (concentrated 7.0-fold). (*a*) Separation of protein (●) and carbohydrate (○); (*b*) interaction with concanavalin A (■) and blood-group-substance activity: H score (□); A score (▼).

saliva is low and is restricted to the glycoprotein fraction, as shown in Fig. 3(*c*).

Ovarian-cyst fluids. In all the ovarian-cyst fluids tested glycoprotein correlating with blood-group activity was well separated from the protein components. A typical pattern of separation of glycoprotein and protein components of one ovarian-cyst fluid, from a group O secretor, is shown in Fig. 7(*a*). As was found for saliva, the reaction with concanavalin A is restricted to the protein fractions (Fig. 7*b*). Similar patterns were obtained with two other samples of ovarian-cyst fluid, another group O secretor and a group O non-secretor; in all three cases the interaction with concanavalin A occurred only in the protein fraction and there was no detectable reaction with the glycoprotein fractions of any of the ovarian-cyst fluids studied. There was no detectable sulphate in any of the ovarian-cyst glycoprotein fractions.

Hydrolysis of fractionated secretions. From the fractionated gastric juice of a group O secretor the two fractions corresponding to the peak of reactivity with concanavalin A and coincident with the secretor-score peak were selected for hydrolysis. Two neutral reducing sugars, which ran as galactose and fucose in two solvent systems, were detected in the hydrolysate. Hydrolysis of whole gastric juice showed four neutral reducing sugars corresponding to galactose, fucose, glucose and mannose in the solvent systems used.

DISCUSSION

In both gastric juice and saliva, blood-group substances form a major part of the total glycoproteins secreted (Denborough, Clarke & Presser, 1970).

The finding that concanavalin A interacts with a glycoprotein fraction from gastric juice but not with any salivary glycoproteins from the four individuals studied, and that the peak of reactivity with concanavalin A coincides with the peak of blood-group activity in the fractionated gastric secretions, suggests a structural difference in the blood-group substance from saliva and gastric juice in the same individual.

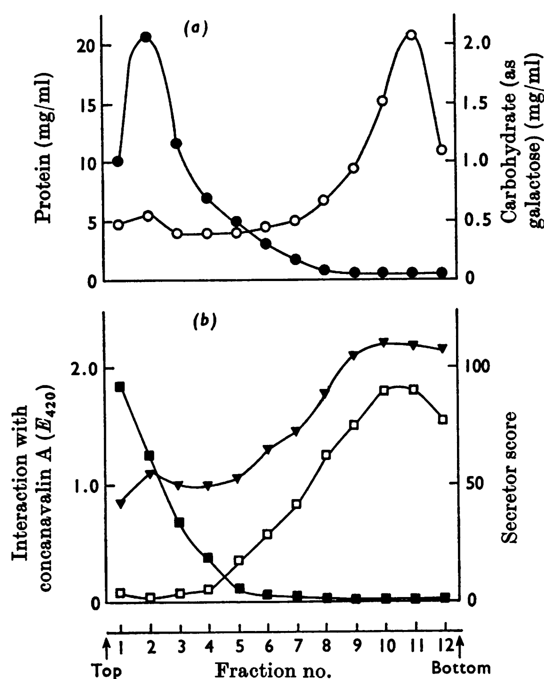

Fig. 6. Fractionation after centrifuging to equilibrium in a CsCl density gradient (details as in Fig. 1) of saliva from the group A secretor (as Fig. 4) (concentrated 8.7-fold). (*a*) Separation of protein (●) and carbohydrate (○); (*b*) interaction with concanavalin A (■) and blood-group-substance activity: H score (□); A score (▼).

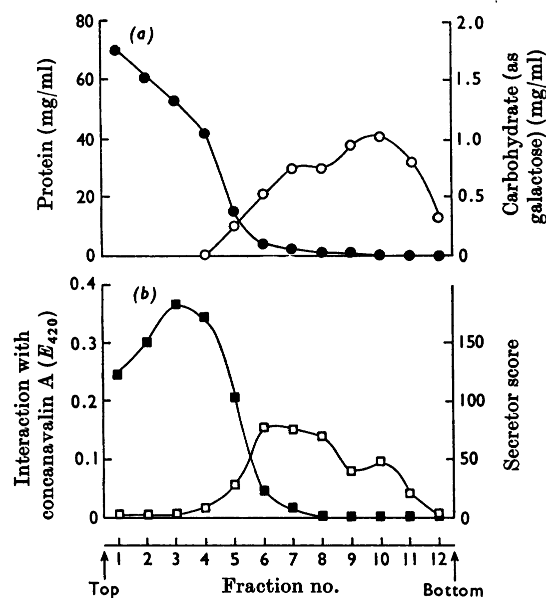

Fig. 7. Fractionation of ovarian-cyst fluid from a group O secretor after centrifuging to equilibrium in a CsCl density gradient (details as in Fig. 1). (*a*) Separation of protein (●) and carbohydrate (○); (*b*) interaction with concanavalin A (■) and blood-group-substance activity: H score (□).

The possibility was considered that blood-group substance from gastric juice is, when secreted, chemically identical with the blood-group substance of other secretions from the same subject, but is modified by the action of either acid or pepsin in the stomach, to expose groups in the glycoprotein that would react with concanavalin A. However, neither incubation of a glycoprotein fraction from the ovarian cyst of a group O secretor with 0.2M-hydrochloric acid–potassium chloride at pH 1.5 for 2.5h at 37°C, nor prolonged exposure of the same glycoprotein to the action of pepsin at pH 1.5 at 37°C, produced any change in the reactivity of the glycoprotein with concanavalin A.

Evidence for a structural difference in blood-group substance from different secretions, unrelated to ABH or Lewis activity and based on the presence of a variable number of sugar chains terminating in non-reducing *N*-acetyl-D-glucosamine units, has been presented by Lloyd *et al.* (1969). Of the four sugars present in blood-group substance, namely L-fucose, D-galactose, *N*-acetyl-D-glucosamine and *N*-acetyl-D-galactosamine, only *N*-acetyl-D-glucosamine meets the requirements for interaction with concanavalin A as determined by So & Goldstein (1967), i.e. it has the D-*arabino* configuration at C-3, C-4 and C-6 and an α-configuration at C-1.

Whole gastric juice has been shown to contain the neutral sugars mannose and glucose as well as fucose and galactose, which are associated with blood-group substance (Schrager & Oates, 1968). Both mannose and glucose have the required configuration for interaction with concanavalin A, and if they are present in the glycoprotein fraction from gastric juice that interacts with concanavalin A they could contribute to the interaction. However, hydrolysates of the glycoprotein fractions of gastric juice from a group O secretor, which had maximum blood-group-substance activity and which gave maximum interaction with concanavalin A, showed galactose and fucose as the only neutral sugars present.

The possibility that the presence of sulphate in some of the fractionated secretions was contributing to the interaction with concanavalin A was also considered. The presence of charged groups such as sulphate and phosphate apparently induces interaction of certain macromolecules with concanavalin A (Doyle, Woodside & Fishel, 1968), and several groups of sulphated glycoproteins are known to occur in gastric juice (De Graef & Glass, 1968). Our results show that neither in resting gastric

juice, where there are several groups of sulphate-containing glycoproteins, nor in the post-histalog gastric juice, where there is a more restricted density range of sulphated glycoproteins, was there any correlation between sulphate content and reactivity with concanavalin A. Sulphate was found to be present in association with the glycoprotein fractions of saliva, but this failed to confer on the glycoproteins the property of reacting with concanavalin A. No sulphate was detected in the ovarian-cyst fluid.

Lloyd *et al.* (1969) reported an interaction of concanavalin A with blood-group substance prepared from the stomach of a group O individual but not with blood-group substance from the stomach of a group A individual. However, in the present study gastric glycoproteins prepared from all the subjects described, as well as another group O secretor and two group A secretors, were found to react with concanavalin A and showed blood-group activity. Glycoproteins prepared from saliva of all the subjects described, as well as another group O secretor and a group A secretor, showed blood-group activity but did not interact with concanavalin A. Similarly glycoproteins with blood-group activity prepared from ovarian-cyst fluids of two group O secretors and a group O non-secretor did not react with concanavalin A. Thus the gastric glycoproteins contain a determinant, apparently unrelated to ABH or Lewis activity and unrelated to sulphate content, which is not present in glycoproteins having blood-group activity from saliva or ovarian-cyst fluid.

An interaction of concanavalin A with the protein components of all the secretions studied, namely gastric juice, saliva and ovarian-cyst fluid, has been shown (Figs. 1*b*–7*b*). This interaction is likely to be due, at least in part, to the plasma proteins that are present in these secretions (Schultze & Heremans, 1966). Nakama & Suzono (1965) have demonstrated, by gel diffusion, several bands of interaction of concanavalin A and whole serum, and there have been several reports of interaction of particular serum proteins with concanavalin A (Nakamura, Tanaka, & Murakawa 1960; Goldstein, So, Yang & Callies, 1969; Morse, 1968).

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