Blood Group Substances in Canine Gastric Mucus and Acid Secretion.*† (26424)

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Gastric secretion is considered a major source of blood group substances(1); secretions from cow, horse, pig and man have yielded considerable information in this connection. Investigations into the chemistry of the macromolecular constituents of gastric secretions from dogs(2,3) and of dogs' erythrocyte-isoantibody reactions(4) have not yielded information about canine blood group substances. Because of the availability of methods for collecting and fractionating a variety of uncontaminated canine gastric secretions(5), and for studying such materials in canine as well as in human erythrocyte-isoantibody systems, the present investigation of reactions of canine gastric secretions in both these immunological systems was undertaken.

Methods. Canine gastric secretions. An acid gastric mucus was obtained from mongrel Heidenhain stomach pouch dogs, following topical application of acetylcholine. Acid gastric juice following stimulation by histamine and mecholyl together was obtained by drainage from pouches, and by oro-gastric intubation from dogs with unoperated stomachs.

Enrichment for mucopolysaccharides. The acetylcholine mucus was processed by peptic digestion, followed by phenol extraction of the dialyzed digest. The criterion for mucopolysaccharide enrichment was a lowering of the N:hexosamine molar ratio. Endogenous pepsinogen contained in the mucus was activated by adjustment to pH 2 with dilute HCl, and the mixture was incubated under toluene at 37°C for one week; following this, it was dialyzed, reacidified and incubated for an additional week. This treatment solubilized almost all of the insoluble gel, and after dialysis and alcohol precipitation afforded a moderate degree of mucopolysaccharide enrichment (about 3-fold). From 3-10% of the organic substances initially present in the mucus was insoluble in water and in sodium acetate at pH 8 after peptic digestion; the insoluble debris exhibited molar ratios of 6.8 for N:hexosamine and 4.2 for N:reducing power, but it was set aside and not worked up further because its insolubility rendered it unsuitable for hemagglutination-inhibition studies. The soluble fraction was lyophilized, then subjected to phenol extraction(6). The phenol-soluble (PS)‡ fraction was subjected to ethanol precipitation, using concentrations up to 25% alcohol in presence of sodium acetate. The phenol-insoluble (PI) residue and the PS alcohol precipitates, were washed with alcohol and ether prior to assay.

Chemical assays. Nitrogen was determined by a micro-Kjeldahl procedure(7). Hexosamine was determined by the method of Blix(8) after hydrolysis in 2N H_2SO_4 for 4 hours in a boiling water bath. Reducing power(9) also was determined on the hydrolysate. Hexuronic acid and fucose determinations were made on the unhydrolyzed enriched mucopolysaccharide according to the methods of Dische(10,11). Paper electrophoresis of the enriched products was performed with the Spinco vertical electrophoresis apparatus in borate buffer $\Gamma/2 = 0.1$, pH 9.0, 200 volts, 6½ hours. Strips were stained by PAS(12) and by toluidine blue(13).

Canine immunological systems. Antisera:

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‡ Abbreviations: N= nitrogen; PI = phenol-insoluble; PS = phenol-soluble.

Isoantibodies for the dog blood group antigens A, C, and D were prepared and employed as described by Young *et al.*(4,14). Antisera for hemagglutination inhibition studies were employed both undiluted and at appropriate dilutions with isotonic saline. *Test red cells*: Three per cent suspensions of red cells in isotonic saline were used for study of the canine C and D systems; red cells suspended in saline or in fresh autogenous serum were used for studies of the canine A system. *Test substances*: Dialyzed and lyophilized gastric secretions and fractions derived therefrom were hydrated at levels of 2-10 mg/ml in phosphate buffer (pH 7.4) for 2 hours at room temperature prior to testing. *Agglutination inhibition studies*: Agglutination inhibition tests were performed by preparing dilutions of both antibody-containing sera and the hydrated test substances; these were combined in a "block titration pattern" (15). Test samples were assayed at 1:1, 1:10, 1:100 and 1:1000 dilutions. Canine anti-A serum was employed undiluted and at 1:5, 1:15 and 1:45 dilutions. In all systems, tubes containing antiserum and test substance were incubated for 15 minutes at 37°C, 15 minutes at room temperature and 30 minutes at 4°C in succession, and finally returned to room temperature. Then 0.1 ml of the test red cell suspension was added to each tube. The tubes with suspensions in saline were incubated at room temperature, those with suspensions in fresh autogenous serum at 37°C. Indirect antiglobulin tests were performed on all tubes of the A-system tests which did not show agglutination. Canine anti-C and anti-D sera were used undiluted and at 1:4, 1:12 and 1:36 dilutions in saline, and these test systems were incubated at room temperature after addition of red cells suspended in saline. Antiglobulin tests were not employed in studies of the canine C- and D-systems, because these antibodies do not react with the antiglobulin reagents.

Human immunological systems. Antisera: Group O, Rh negative serum diluted 1:4 with isotonic saline was used for tests of the human anti-A system. Serum of a person of "Bombay type" was used undiluted for anti-H studies. Tests for H substance were performed also with Ulex europeus diluted 1:4 with saline (courtesy of New York City Dept. of Health). *Test cells*: Three per cent suspensions of group A_1 red cells in saline were used for studies of the human anti-A system, and 3% suspensions of group O red cells in saline for studies with anti-H. *Test substances*: Gastric secretions and fractions were tested at only one concentration. Indirect Coombs tests were not performed. These substances and saline controls were incubated with human blood group antisera at room temperature for one hour, then 0.1 ml of the appropriate cell suspension was added. Following this, tubes containing complete test systems were incubated at room temperature for one hour and examined.

Results. The data (Table I) show that gastric secretion from 11 mongrel dogs did not exhibit blood group substance activity in any of the canine test systems. Peptic digests of gastric secretion, PI residues, and ethanol-precipitated preparations of the PS fraction were all without inhibitory activity at levels of 2-10 mg/ml. By contrast, all of the above preparations showed activity in human A and H systems. PI residues and PS-25% ethanol precipitates exhibited activity in the human A-test system at levels of less than 4 γ/ml with a 1:4 anti-A serum + A_1 cells, and at the 4 γ/ml level in the human H-test system with undiluted anti-H serum (titer 1:32) + O cells.

Data on the chemical composition of mucopolysaccharide-enriched fractions are presented in Table II. From 27 to 48 mg of PI residue, and 11 to 17 mg of PS-25% ethanol precipitate, were obtained from 50 ml of acetylcholine mucus. These products were essentially of the neutral mucoid variety; the molar ratios hexosamine : hexuronic acid, determined on pooled specimens because of an insufficient supply of individual specimens, were 8.4 for the PI residue, and 14.2 for the PS-25% ethanol precipitate. The increase in mucopolysaccharide content as a result of fractionation was accompanied by

TABLE I. Examination of Canine Gastric Specimens for Hemagglutination Inhibition.

Dog	Canine blood type	Gastric specimens		Human inhibition systems†		
		Mu*	Acid	Anti-A	Anti-B	Anti-H
326—pouch	A_1	+	+	Ae	N	Ae
381—unop'd	A_1		+	Ae	N	C
390— "	C		+	C	N	C
392— "	C		+	C	N	C
334—pouch	A_1C	+	+	P	N	C
251— "	A_1C	+	+	C	N	Ae
340— "	A_1C	+	+	C	N	C
388—unop'd	A_1C		+	C	N	Ae
363—pouch	A_1CD	+	+	Ae	N	C
379— "	A_2C	+	+	C	N	C
394—unop'd	A_2C		+	C	N	Ae

Dialyzed and lyophilized gastric specimens were used at levels of 6-10 mg/ml.

* Mu = acetylcholine-stimulated anacid mucus.

† Degree of agglutination inhibition: C = complete; Ae = almost complete; P = partial; N = none. All gastric specimens were tested for hemagglutination inhibition with the canine inhibition systems, Anti-A, Anti-C and Anti-D; no agglutination inhibition was found in any of the specimens.

an increase in blood group substance activity; this was demonstrated by a reduction in the minimum quantity of substance necessary to inhibit hemagglutination in the Ulex system from 100 γ for the untreated mucus to 1 γ for the 2 enriched fractions.

Paper electrophoresis in borate buffer of the PI and PS-25% ethanol precipitate revealed one PAS positive zone which migrated a short distance on the anode side. The PI fraction exhibited 2 anodic metachromatic zones when stained with toluidine blue O, the lighter-staining of which coincided with the PAS positive zone; the PS-25% ethanol precipitate exhibited no metachromatic zones with 1 mg of material on the strip. The presence of metachromatic zones in the PI-residue suggests contamination of the blood group substances by small amounts of acid mucopolysaccharide.

Discussion. These results show that canine gastric secretions do not exhibit blood group substance activity when assayed in the available canine test systems. Employment of the block-titration technic favors establishment of mutually optimal concentrations of antibody and soluble blood group substances. Although a relatively small population of dogs was employed in our investigation, the animals studied reflect all of the common blood group combinations of this species.

The purified fractions as well as the untreated secretions from which they were derived exhibited blood group substance activity in test systems for human A and H substance. Lack of reaction with the canine systems notwithstanding, a biological test based on hemagglutination inhibition in human A and H systems may aid in differentiating and characterizing various preparations derived from canine gastric secretion.

Several possibilities other than absence of canine blood group factors may be advanced to explain the lack of activity in the canine test systems. It is possible that the

TABLE II. Chemical Characteristics of Mucopolysaccharide-Enriched Fractions of Acetylcholine Mucus, Following Peptic Digestion.

	Mean molar ratios (stand. dev.)		
	N:Hexosamine	N:Reducing power	Hexosamine:Fucose
Phenol-insoluble fraction (5 specimens)	3.3 (1.2)	2.1 (.4)	4.1 (.6)
Phenol-soluble fraction precipitated with 25% ethanol (5 specimens)	4.8 (1.7)	3.9 (1.3)	3.9 (.7)

small amount of water-insoluble debris excluded in the present work may have possessed this activity. Mild means for solubilizing this fraction would be required to investigate this aspect further. Also, it is conceivable that agglutination of canine erythrocytes by appropriate antisera depends on the presence of a complex antigen composed of protein components, either alone or in combination with mucopolysaccharide(s). If protein components as well as mucopolysaccharide were necessary to complete the antigen, then gastric mucopolysaccharide alone would not be expected to inhibit the hemagglutination reaction. Further investigation will require liberation, fractionation and concentration of canine erythrocyte surface proteins and protein-mucopolysaccharide complexes.

Summary. Canine gastric secretions—both fluid acid and viscous non-acid—and hexosamine-rich fractions derived therefrom, were assayed for blood group substance activity in canine and in human test systems. None of the specimens tested was active in the canine test systems, but all exhibited A and H activity in the human test systems; no human B activity was detected. Hexosamine-rich fractions obtained by peptic digestion and phenol extraction of canine gastric secretion were largely of the neutral mucoid type, and were active in human A and H systems at the microgram level.

1. Kabat, E. A., Blood Group Substances, 1956, Chap 3, Academic Press, Inc., New York.

2. Horowitz, M. I., Hollander, F., *Fed. Proc.*, 1959, v18, 71.

3. ———, Abst. Am. Chem Soc., Divn. Biol. Chem., Boston, Mass., April 7, 1959, 34C.

4. Young, L. E., O'Brien, W. A., Miller, G., Swisher, S. N., Ervin, D. M., Christian, R. M., Yuile, C. L., *Trans. N. Y. Acad. Sci.*, 1951, v13, 209.

5. Janowitz, H. D., Hollander, F., Jackson, C., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 578.

6. Morgan, W. T. J., King, H. K., *Biochem. J.*, 1943, v37, 640.

7. Jenden, D. J., Taylor, D. B., *Analyt. Chem.*, 1953, v25, 685.

8. Blix, G., *Acta Chem. Scand.*, 1948, v2, 467.

9. Nelson, N., *J. Biol. Chem.*, 1944, v153, 375.

10. Dische, Z., *ibid.*, 1947, v167, 189.

11. Dische, Z., Shettles, L. B., *ibid.*, 1948, v175, 595.

12. Köiw, E., Grönwall, A., *Scand. J. Clin. Lab. Invest.*, 1952, v4, 244.

13. DiFerrante, N., *J. Clin. Invest.*, 1957, v36, 1516.

14. Christian, R. M., Ervin, D. M., Young, L. E., *J. Immunol.*, 1951, v66, 37.

15. Kabat, E. A., Mayer, M. M., *Experimental Immunochemistry*, 1948, 90, Charles C. Thomas, Springfield, Ill.

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Anticonvulsant Properties of 1-(1-phenylcyclohexyl) piperidine·HCl and Certain Other Drugs. (26425)

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1-(1-phenylcyclohexyl) piperidine · HCl (PCP) was found to be an extremely active agent in prevention of electrically induced tonic extensor seizures in mice(1). In man PCP has been shown to induce interceptive sensory deprivation at low doses and general anesthesia at high dose levels(2,3). This investigation dealt with a comparison of the anticonvulsant properties of PCP, Dilantin, phenobarbital and barbital in convulsions induced by sound, by pentylenetetrazol and by electroshock.

Materials and methods. The experiments on audiogenic seizures were conducted on DBA/2 male mice, 28 to 35 days old, obtained from the Roscoe B. Jackson Memorial Laboratory. The age range between 4 to 5 weeks was found to be the critical period for audiogenic seizure susceptibility in this strain of animals.

The apparatus consisted of a small insulated plywood chamber (40″ x 32″ x 30″) with a hinged door at the front. In it were wired a 4″ electric fan and 2 electric door