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Dog Immunoglobulins

I. IMMUNOCHEMICAL CHARACTERIZATION OF DOG SERUM, PAROTID SALIVA, COLOSTRUM, MILK AND SMALL BOWEL FLUID

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Summary. The levels of immunoglobulins A, M and G were measured in dog serum, colostrum, milk, parotid saliva and small bowel fluid using the single radial immunodiffusion method. All the external secretions except early colostrum, by contrast with serum, were found to be rich in IgA with small quantities of IgM and IgG. Exocrine immunoglobulins were partially characterized by gel filtration.

INTRODUCTION

In the last 10 years it has become apparent that IgA is quantitatively the predominant immunoglobulin in the external secretions of many species (Heremans, 1968). Whilst there is evidence that the virus neutralizing activities of IgA antibodies play a role in viral infections (Ogra, Karzon, Righthand and MacGillivray, 1968), the situation is quite unclear with regard to bacterial infections at secretory surfaces (Eddie, Schulkind and Robbins, 1971).

It has been shown that dogs have immunoglobulins analogous to human IgA (Vaerman and Heremans, 1968). During the course of a continuing study of intestinal immune processes in the dog, we have had occasion to measure the immunoglobulin levels in the various classes found in serum or external secretions from dogs. Although similar measurements on some secretions have been published before (Vaerman and Heremans, 1969b; Reynolds and Johnson, 1970d; Ricks, Roberts and Patterson, 1970), the present data add to these and, in particular, provide new information concerning the *in vivo* concentrations and rates of appearance of immunoglobulins in the small bowel lumen. We present the first quantitative values for dog parotid saliva.

MATERIALS AND METHODS

Animals

Dogs. These were adult mongrels of either sex, weighing 5–35 kg. Saliva was collected from dogs weighing 15 kg or more.

Goats. Anti-globulin antisera were raised in goats caught on Manunda Station in the semi-arid zone of South Australia.

Sample collection

Blood serum. This was obtained from the antecubital vein, allowed to clot at 37° for

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1 hour and then stored at 4° overnight to facilitate clot retraction. Following this, the serum was drawn off, centrifuged at 2000 rev/minute for 10 minutes, and stored.

Parotid saliva. This was collected through cannulae from anaesthetized dogs stimulated with prostaglandin. Specimens were collected on ice, centrifuged at 15,000 ***g*** for 20 minutes and stored. 'Immune' saliva came from parotid ducts into which, 2–4 weeks before, $2–5 \times 10^9$ live washed *Vibrio cholerae* 569B had been deposited.

Whole saliva. During the collection of parotid saliva a greater volume of other oral secretions was collected.

Colostrum and milk. These were obtained by gentle hand milking and were defatted and decaseinated as described by Knop, Breu, Wernet and Rowley (1971). Three of the four dogs had received injections of *Vibrio cholerae*, without adjuvant, into the perimammary areas. All assay values were corrected to the original volume.

Small bowel fluids. Two techniques were employed for collection of this material. In the first, samples were obtained from dogs which had not eaten for 20 hours but had had free access to water, by laparotomy under a pentobarbitone anaesthetic and perfusion with saline (25–35°) through Foley's catheters which had been inserted through small orifices cut with cautery 20 cm from each end of the free small bowel and then inflated to give a seal without pallor of the bowel. A single-pass perfusion was performed at 120–200 ml/hour for several hours. The ice-cold samples were centrifuged, dialysed on ice against 0·01–0·02 M phosphate buffer, pH 8·0, then freeze dried and stored at 4°.

To determine the absolute concentrations of immunoglobulins in the small bowel fluid, several fasted dogs were killed by rapid intravenous injection of pentobarbitone. Their abdomens were opened immediately, and the free small bowel ligated and removed. The exterior of the bowel was washed and dried and the contents gently massaged into one end and transferred with a syringe into a beaker on ice. The aspirate was centrifuged immediately at 14,000 ***g*** for 10 minutes at 5° and frozen.

Storage. Samples were stored either at −20° or at 4° with 0·2 per cent sodium azide.

Immunological assays

Single radial immunodiffusion (*SRID*) as described by Mancini, Carbonara and Heremans (1965).

Immunoelectrophoresis (*IE*) according to the method of Scheidegger (1955).

Double immunodiffusion analysis according to Ouchterlony (1958).

Antisera

Goat anti-dog IgG. This was raised by immunizing two goats at 2–4 weekly intervals with 1 mg of IgG (Heddle and Rowley, 1975) in Freund's complete adjuvant. All anti-dog globulin sera were raised by multiple intramuscular injections. After 8 and 11 weeks the goats were bled out and the sera absorbed as described below.

Goat anti-dog IgM. IgM (Heddle and Rowley, 1975) contained, on IE against anti-whole dog serum (Miles Laboratories), traces of non-immunoglobulin protein of α mobility. However, when run in IE against the reference anti-IgM serum and our un-absorbed goat anti-dog IgG, single lines of identical mobility were obtained. The precipitin lines formed with the latter antiserum were used to immunize a goat. The principles of the above technique have been described (Goudie, Horne and Wilkinson, 1966). The antiserum was absorbed as follows.

Goat anti-dog IgA. This was prepared in the same manner as goat anti-dog IgM and absorbed as follows.

Absorption of antisera. All three antisera showed strong cross-reactions (presumably due to light chain reactivities) with other dog immunoglobulin classes when tested by double immunodiffusion analysis and IE. None of the antisera showed cross-reactions to non-immunoglobulin proteins. Antisera were absorbed in one of two ways. Once rendered monospecific by absorption they are referred to by their heavy chain specificity.

(I) *Using soluble immunoglobulin.* Additions were made of small quantities of heterologous dog immunoglobulins and, following incubation at 37° for 30 minutes and 4° for 24 hours, the antisera were centrifuged at 40,000 ***g*** for 90 minutes at 5°. This process was repeated until no unwanted specificities were detected by double immunodiffusion, IE or SRID. The specificities of the absorbed antisera were further demonstrated by antiglobulin-enhanced haemagglutination in which all antisera enhanced only antibodies of the intended class (Heddle and Rowley, 1975).

(II) *Absorptions with insoluble antigens.* This method has the distinct advantage that no soluble antigen–antibody complexes remain which is of particular importance when antibody preparations are to be used in immunoabsorbent columns (Heddle and Rowley, 1975). Aliquots of each of the unabsorbed antisera were treated with 40 per cent saturated ammonium sulphate and the redissolved precipitate passed through columns of Bio-Gel A-50 to which a heterologous dog immunoglobulin had been covalently linked, using the method of Axén, Porath and Ernback (1967). The dog IgA, IgM and IgG columns had attached to them 3, 11 and 100 mg of the respective immunoglobulin. The absorptive capacities of the columns were regenerated using the thiocyanate ion (Bennich and Johansson, 1971). The goat anti-dog globulin preparations obtained were specific for their respective immunoglobulins.

Reference antisera. Reference antisera to dog $\gamma 1$, $\gamma 2$, μ and α chains were provided by Drs J.-P. Vaerman and J. F. Heremans. Our anti-μ and anti-α sera, in double immunodiffusion against dog serum and dog exocrine secretions (parotid saliva and milk), gave reactions of identity with the respective reference antisera. Serum and exocrine secretions gave reactons of identity with our anti-α serum, even though the latter had been raised to secretory IgA. Our anti-γ serum, in double immunodiffusion against dog serum, gave reactions of partial identity with the reference anti-$\gamma 1$ and anti-$\gamma 2$ sera compatible with the respective class and subclass specificities.

Immunoglobulin standards

These were prepared and assayed as described by Heddle and Rowley (1975). Optical densities were read at 278 nm and an extinction coefficient (E_{278nm}0·1 per cent, 1 cm) of 1·34 was assumed for all immunoglobulins (Vaerman and Heremans, 1970). Standards were diluted by single steps and several aliquots of each dilution were frozen. Aliquots of dilutions of a pool of dog serum were used in SRID in parallel with the purified standards. The IgA standard was secretory IgA (Heddle and Rowley, 1975).

Fractionation of immunoglobulins

Samples of mammary secretion, parotid saliva and small bowel fluid were fractionated by gel filtration and the fractions assayed by SRID.

Fractionation on Sephadex G-200. The sample to be fractionated was equilibrated with the buffer, centrifuged (15,000 ***g*** at 5° for 20 minutes) and 1–2 per cent of the column bed

TABLE 1

SINGLE RADIAL IMMUNODIFFUSION ASSAY FOR DOG SERUM IMMUNOGLOBULINS. RESULTS OF ELEVEN TO FOURTEEN SEPARATE ASSAYS FOR EACH IMMUNOGLOBULIN ON ONE SERUM POOL

	Immunoglobulin (mg/ml)		
	IgA	IgM	IgG
Arithmetic mean	0·44	1·37	8·4
Range	0·38–0·52	1·25–1·48	7·4–10·2
Geometric mean	0·44	1·36	8·3
Standard deviation	0·07	0·09	0·8

volume was loaded. The buffer, 0·2 M NaCl, 0·05 M KH_2PO_4:NaOH, pH 7·6, sodium azide 0·02 per cent, was pumped at such a rate that the column volume passed in 40 hours. Fractions were collected every 20 or 30 minutes.

Fractionation on Sepharose 6-B. This discriminated between the larger immunoglobulins. The buffer and conditions of fractionation were as with the Sephadex columns.

RESULTS

QUANTITATIVE ASSAY FOR IMMUNOGLOBULINS

In Table 1 the results are collected of separate assays (by the SRID method) on one pool of serum from six dogs.

SERUM VALUES

The results of SRID assays on the sera of fourteen mongrel dogs are assembled in Table 2 with data of Reynolds and Johnson (1970d) for comparison.*

TABLE 2

SERUM CONCENTRATIONS OF DOG IMMUNOGLOBULINS

Authors	Dogs	Immunoglobulin (mg/ml)		
		IgA	IgM	IgG*
Heddle and Rowley (1975)	Mongrels (14)	0·5† (0·2–1·2)	1·7† (0·7–2·7)	9·8† (5·2–17·3)
Reynolds and Johnson (1970d)	Mongrels (20–35)	0·8	1·4	14·4
Reynolds and Johnson (1970d)	Pure-bred (21–37)	0·8	1·6	9·2

* Reynolds and Johnson (1970d) gave IgG values for individual subclasses. For comparison with our own and other data these have been summed.

† Arithmetic mean followed by the range in parentheses.

* Reynolds and Johnson (1970d) gave IgG values for individual subclasses. For comparison with our own and other data these have been summed. The same subclass values have been applied in Tables 6 and 7 to the data of Vaerman and Heremans (1969b) to obtain secretion/serum ratios for IgG rather than IgG subclasses.

COLOSTRAL VALUES

The total immunoglobulin concentration in colostrum fell sharply from 15 mg/ml on the day of parturition to 2–3 mg/ml on the following 2 days. It remained at 1–2 mg/ml for at least 30 days. The change in composition of mammary secretions following parturition was similar in all dogs and is presented in Fig. 1 as the percentage contribution of IgA, IgM and IgG to the total immunoglobulin content.

The distribution of immunoglobulin classes following Sephadex G-200 fractionation of milk (days 5–9 post-partum) from a single dog is shown in Fig. 2, which is quite representative of several such secretions. In the example given the recoveries of the starting material were 90, 60 and 90 per cent for IgA, IgM and IgG respectively.

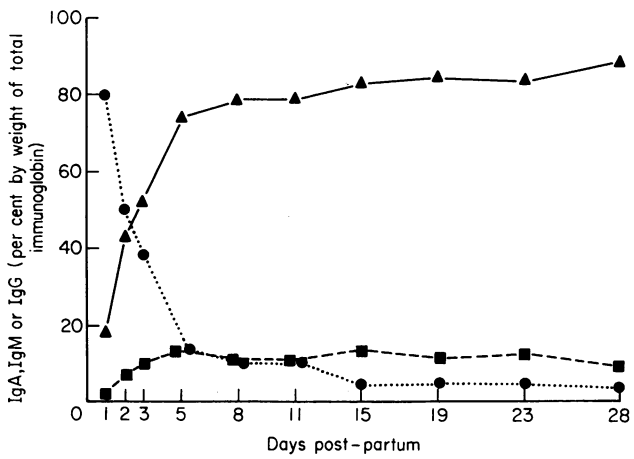

FIG. 1. Immunoglobulins in dog mammary secretions. Each point represents the mean of two to four dogs. IgA (▲——▲). IgM (■ – – ■). IgG (● · · · ●).

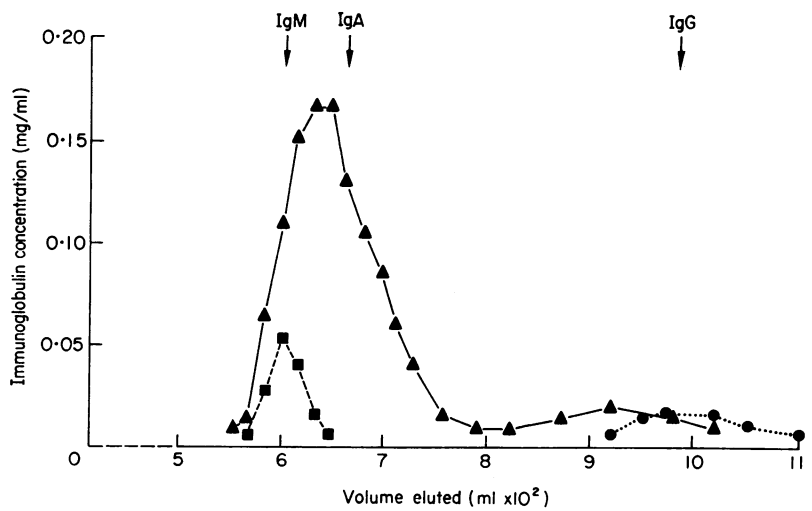

FIG. 2. Gel filtration of dog milk on Sephadex G-200. The elution peaks from fractionation of dog serum on the same column are indicated. IgA (▲——▲). IgM (■ – – ■). IgG (● · · · ●).

SALIVARY VALUES

The results in Table 3 represent four 'normal' samples of parotid saliva and four samples obtained 2 weeks after local immunization. All percentage compositions fell within a remarkably narrow range.

Samples of parotid saliva from these dogs were fractionated on Sephadex G-200 and Sepharose 6-B columns. An example is illustrated in the accompanying paper (Heddle and Rowley, 1975). No immunoglobulin fragments were detected.

TABLE 3

IMMUNOGLOBULIN CONCENTRATIONS IN EIGHT SAMPLES OF DOG PAROTID SALIVA

	Immunoglobulin		
	IgA	IgM	IgG
Arithmetic mean (mg/ml)	0·52	0·033	0·015*
Range (mg/ml)	0·17–1·25	0·005*–0·07	< 0·005*–0·05
Percentage of total immunoglobulin (by weight)	91	6	3
Range (per cent)	89–94	3*–7	< 1*–4

* Approximate value.

SMALL BOWEL FLUIDS

Table 4 gives results on samples obtained by the first technique described in the Materials and Methods section. The initial wash was interrupted when the perfusate became clear; the subsequent perfusion lasted for approximately 1 hour.

In these experiments estimates were made of the total immunoglobulin washed from 1 metre of small bowel in 1 hour (Table 5). The initial wash contained immunoglobulins already present in the lumen at the commencement of the procedure, additional to any entering the loop during that perfusion period. Dog D was perfused for a 3rd hour without fall in immunoglobulins obtained relative to the 2nd hour.

In order to relate the perfusion results on coproantibody composition to normal *in vivo* concentrations, small bowel fluid was collected at autopsy from six dogs and contained (SRID assay) 1·1–8·1 mg/ml total immunoglobulin, with a mean of 3·8 mg/ml. Relative

TABLE 4

COMPOSITION OF SMALL BOWEL PERFUSATES FROM EIGHT DOGS

	Percentage of total immunoglobulin (by weight)*		
	IgA	IgM	IgG
Initial wash	83 (78–90)	11 (4–19)	6 (1*–18)
Subsequent wash	82 (68–91)	8 (3–14)	10 (4–29)

* The results are expressed as the mean followed by the range in parentheses.

† Approximate value.

TABLE 5

IMMUNOGLOBULIN (mg) WASHED FROM SMALL BOWEL PER METRE PER HOUR

Dog	Wash		
	Initial	Second	Third
A	30	5	
B	60	20	
C	60	10	
D	30	10	10

contributions of the immunoglobulin classes were similar to those in Table 4 (means: 80 per cent IgA; 15 per cent IgM; 5 per cent IgG).

Initial small bowel washes from four dogs were fractionated individually on the same Sephadex G-200 column. One of these fractionations is illustrated in Fig. 3. Approximate recoveries of starting materials in the four procedures were, for IgA 70–80 per cent and for IgM and IgG (where estimable) 50–100 per cent. In all fractionations IgA eluted just after IgM but in three cases a second peak, which eluted immediately prior to the first IgG peak and was of similar proportion relative to the major peak as that shown in Fig. 3, was present. When fractions eluting after fraction 40 were pooled and concentrated, small quantities of anti-α reactive material were found in all runs. SRID estimates of this late fractionating IgA-reactive material showed it to comprise <5 per cent of the IgA load. The same concentrated pools of late eluting fractions revealed IgG fragments in all runs. Despite similar sensitivities of the SRID assays, IgM fragments were never detected.

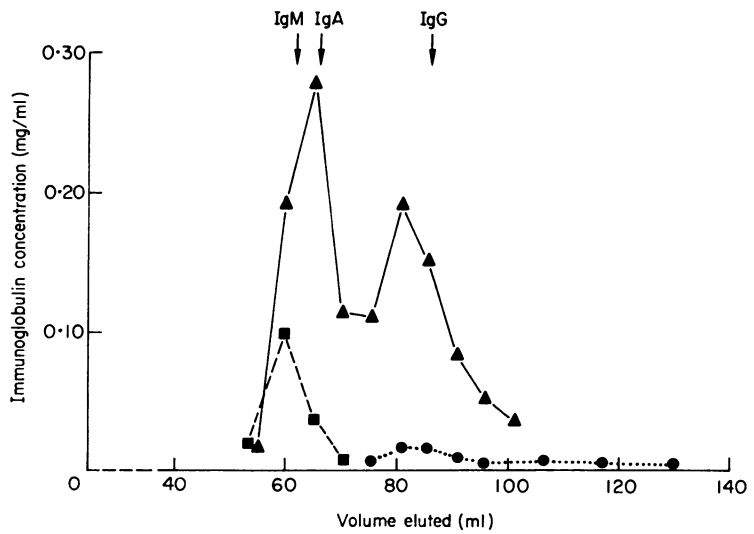

FIG. 3. Gel filtration of dog small bowel washings on Sephadex G-200. The elution peaks from fractionation of dog serum on the same column are indicated. IgA (▲——▲). IgM (■ – – ■). IgG (● · · · ●).

DISCUSSION

The present studies were preliminary to studies on biological efficiencies of different dog anti-*Vibrio cholerae* antibodies (Heddle and Rowley, 1975).

Our anti-α serum, raised against colostral IgA, did not detect secretory piece (SP), as evidenced by failure of the anti-α, secretory IgA precipitin line to spur over the anti-α, serum IgA line. Others working with dog IgA have reported similar results (Vaerman and Heremans, 1969b; Reynolds and Johnson, 1970b). Our antisera appeared comparable to those provided by Vaerman and Heremans.

Table 1 serves as a guide to the accuracy of the SRID technique as used for assessing immunoglobulin concentrations. Values read outside the linear part of the standard curve are indicated as approximate. Non-specific precipitates (Plaut and Keonil, 1969) were observed at high concentrations of some intestinal fluids. They differed qualitatively from precipitin rings, were equally marked on all goat serum SRID plates, including plates made of normal goat sera, and did not cofractionate with the respective immunoglobulins. Sephadex G-200 fractionation of secretions and serum justified the choice of standards, notably the use of a secretory IgA standard. In accord with Johnson and Vaughan (1967) and Vaerman and Heremans (1969b) we found dog serum IgA to be close in size to secretory IgA (Figs 2 and 3).

Our serum IgA concentrations (Table 2) appear to lie between those of Reynolds and Johnson (1970d) and Vaerman and Hermans (1970). A few of our dogs showed quite low serum IgG values, although the means were similar to the literature values.

Vaerman and Heremans (1969b) and Reynolds and Johnson (1970d) studied immunoglobulin concentrations in colostrum over a period of a month from respectively one and four dogs. Ricks, Roberts and Patterson (1970) measured colostral values in six dogs and late milk values in one dog. The results of different authors (Table 6) can be seen to be in agreement. The dramatic changes are the sharp decline in IgG concentrations that occurs within several days of parturition (Fig. 1) and, largely as a consequence, a marked concurrent fall in total immunoglobulin concentrations. Colostrum makes a significant contribution to puppy serum immunoglobulins but absorption of antibodies from the gut appears to decline markedly after the first 12 hours (Gillette and Filkins, 1966).

TABLE 6

IMMUNOGLOBULIN CONCENTRATIONS IN DOG MAMMARY SECRETIONS RELATIVE TO NORMAL SERUM (PER CENT)

Authors	Dogs	Day 1			Days 25–50		
		IgA	IgM	IgG*	IgA	IgM	IgG*
Heddle and Rowley (1975)	2	500	14	160	270	9	1 (approximately)
Vaerman and Heremans (1969b)†	1	1400	46	150	600	54	1
Reynolds and Johnson (1970d)	4	560	50	120	110	10	2
Ricks *et al.* (1970)	6,1	2200	57	300	620	10	3

* Reynolds and Johnson (1970d) gave IgG values for individual subclasses. For comparison with our own and other data these have been summed. The same subclass values have been applied in this table to the data of Vaerman and Heremans (1969b) to obtain secretion/serum ratios for IgG rather than IgG subclasses.

† Given relative to day 6 serum of lactating dog.

Human mammary secretions have been shown to contain antibody activity against a number of enteric bacteria (Shearman, Parkin and McClelland, 1972) which appears to survive passage through the neonate's stomach and intestine (Michael, Ringenback and Hottenstein, 1971). We have observed persistent high titre antibody levels in mammary secretions to *Vibrio cholerae* in a bitch immunized orally. If milk plays a role in the passive immunity of the puppy to local enteric infection, the different compositions of early colostrum and milk may reflect a change in function occurring soon after parturition.

Dog parotid saliva is remarkably rich in IgA (Table 3) whether collected prior to or following immunization. It has proved a fruitful source from which to purify IgA antibodies (Heddle and Rowley, 1975). Whole dog saliva, studied qualitatively by Johnson and Vaughan (1967) and quantitatively by Vaerman and Heremans (1969b) (see Table 7 for comparative values) differs markedly from parotid saliva, having a lower immunoglobulin concentration and, as in man (Heremans, 1968), somewhat higher concentrations of IgG relative to IgA.

TABLE 7

IMMUNOGLOBULIN CONCENTRATIONS IN DOG SALIVA(S) RELATIVE TO SERUM (AS A PERCENTAGE OF SERUM VALUE)

Authors	Saliva	Dogs	IgA ratio	IgM ratio	IgG ratio*
Vaerman and Heremans (1969b)	Whole	6	7·0	<0·04	0·02–0·04
Heddle and Rowley (1975)	Parotid	5	120	2	0·2
Heddle and Rowley (1975)	Whole	3	13	<0·3†	0·05†

* Reynolds and Johnson (1970d) gave IgG values for individual subclasses. For comparison with our own and other data these have been summed. The same subclass values have been applied in this table to the data of Vaerman and Heremans (1969b) to obtain secretion/serum ratios for IgG rather than IgG subclasses.

† Approximate values.

Vaerman and Heremans (1969b) studied small bowel washings from only one adult dog. Reynolds and Johnson (1970d) quantitatively assessed faecal extracts (which they had demonstrated to be qualitatively similar to jejunal washings (Reynolds and Johnson, 1970a)) but it is difficult to relate either sets of data to appearance rates of immunoglobulins or to *in vivo* concentrations in jejunum or ileum. The latter authors reported a mean IgG level exceeding the mean IgA level, yet IgG levels were low in five out of ten dogs (Reynolds and Johnson, 1970d).

The possibility that the results on perfusates might be distorted by contamination with blood was considered. The handling procedures described lysed dog red cells. From the absorption at 577 and 540 nm of a standard lysed red cell suspension, it was evident that the perfusates could not have been contaminated with more than 0·1 per cent of blood, which could have contributed significantly to a minority of IgG values but would have had no effect on IgA or IgM values. The low IgG values observed in all samples (Table 4) argue strongly against either bleeding or inflammatory exudation as significant sources of IgA or IgM.

IgA was the dominant immunoglobulin in dog intestinal perfusates (Table 4), whether 'normal' or 'immune'. Immunofluorescent studies of the dog intestinal mucosa (Vaerman

and Heremans, 1969a) revealed a predominance of IgA-containing cells, particularly adjacent to the lumen.

On fractionation of small intestinal secretions, approximately 60 per cent of the loaded anti-α reactive activity was recovered eluting just behind IgM (Fig. 3). A smaller peak was obtained (also observed by Reynolds and Johnson, 1970a) overlapping the main IgG peak. This (approximately 7S) IgA was probably overestimated by a factor of 3 (Reed and Williams, 1971) and may have contributed to a moderate overestimation of our total IgA.

The significance of the very small quantities of anti-α reactive material eluted after this second IgA peak is not clear (compare the discussion about anti-SP activity), nor is the failure to detect IgM fragments. In agreement with others (Reynolds and Johnson, 1970c; Northrup, Bienenstock and Tomasi, 1970; Reed and Williams, 1971), we have found IgG fragments in intestinal secretions, some of which may have been formed after collection, distorting the quantification of immunoglobulins. IgG is reported to be more susceptible to proteolysis than secretory IgA (Brown, Newcomb and Ishizaka, 1970).

Small bowel fluid from fasted dogs had a total immunoglobulin concentration about a third that of blood serum. If the second perfusion (Table 5) is extrapolated to 24 hours, about 0·25 g of immunoglobulin enters the small bowel per day—a figure compatible with observations on man (Northrup, Bienenstock and Tomasi, 1970). The dog intestine has been reported to be a significant site of IgG degradation (Andersen, Glenert and Wallevick, 1963).

The present studies form the background to further work (Heddle and Rowley, 1975) in which antibodies to *Vibrio cholerae* were raised in serum and secretions, purified and then tested for antibacterial activity in a number of systems. The object of the studies is the elucidation of antibacterial mechanisms at mucous membranes, an interesting aspect of which is the possible antibacterial role of the immunochemically dominant secretory immunoglobulin.

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