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Research Article

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These findings indicate that (a) two groups of IgA proteins can be distinguished on the basis of susceptibility to proteolysis with pepsin, and (b) secretory piece confers, at most, only a minor increase in stability to the IgA molecule against the digestive action of pepsin.

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Two Distinct Groups of Immunoglobulin A(IgA) Revealed by Peptic Digestion

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ABSTRACT Serum IgA M-components, secretory IgA separated from colostrum, and IgA from serum of patients with cirrhosis of the liver were digested with pepsin at pH 4.1. The IgA M-components segregated into two groups on the basis of their relative rates of peptic digestion. Serum and colostrum IgA were digested at a total rate intermediate to that of the two groups of IgA myeloma proteins. It appeared, however, that colostrum IgA may have been initially more resistant to peptic digestion than serum IgA. The variability in the rate of peptic digestion was not related to electrophoretic mobility, light-chain type, or IgA subclass. Experimental conditions related to enzyme to substrate ratio or to the pH of the reaction mixture did not appear to explain the differences found.

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INTRODUCTION

Immunoglobulin A (IgA) found in the secretions bathing the mucous surfaces is distinct in that it contains a protein piece not found in serum IgA (1-4). It has been postulated that this secretory piece may confer some advantage to IgA which makes it more suited for its environment. Suggestions have been made that secretory piece might either facilitate the transport of IgA across the epithelial membrane (1) or stabilize the molecule

against the proteolytic action of enzymes found in these secretions (4).

The purpose of this study was to determine the rate of peptic digestion of colostrum IgA¹ relative to serum IgA. Pepsin was chosen since proteolytic digestion with this enzyme has classically yielded information regarding the structure and function of immunoglobulins (5-8). We found that myeloma proteins have a marked variability in their susceptibility to peptic digestion, that two distinct groups of IgA appear to be delineated by their relative rates of digestion with pepsin, and that colostrum and serum IgA samples were digested at a rate intermediate to these two IgA groups.

METHODS

Materials. Sera containing IgA M-components were preserved with sodium azide and stored at either 4° or -10°C. The IgA M-components were isolated by zone electrophoresis (9) on starch block or Pevikon.² Only those eluates giving a single IgA line in immunoelectrophoresis experiments against antihuman whole serum³ were used. The degree of contamination of IgA proteins Ho and De was elucidated further by measurement of IgG and IgM concentrations using single diffusion in agar gel (10). De and Ho samples from starch block eluates contained 1.0 and 5.6%, respectively, of the total protein as IgG. IgM was not detected in either sample. The maximum possible IgM concentration in the samples was less than 0.8%. IgA protein De from starch block was then put over a Sephadex G200 column to further test purity. About 92% of the protein activity eluted in a major peak just after the void volume. All tubes in the peak gave precipitin reactions in agar gel with specific anti-IgA sera. Another 4% eluted at the point where dialysable polypeptides are found and thus would be removed during preparation for digestion. Thus the total contaminating protein for De was probably in the range

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¹ Since IgA in colostrum may not be representative of secretory IgA from all sites, we prefer colostrum IgA to the term secretory IgA when referring to the 11S IgA separated from colostrum.

² Mercer Chemical Corp., New York.

³ Hyland Laboratories, Los Angeles, Calif. 90039.

of 5% while Ho was somewhat greater. In experiments where the products of peptic digestion were isolated, additional purification of the IgA was obtained by stepwise elution on diethylaminoethyl (DEAE) columns (3). This resulted in reduction of the IgG contamination in sample Ho from 5.6 to 2.5%.

Colostrum was preserved with sodium azide and stored at 4°C until used. One sample was processed immediately after collection to determine the effect of standing. Separation of 11S IgA from colostrum was performed by the method of Tomasi, Tan, Solomon, and Prendergast (3). Colostral IgA prepared in this manner gave a single precipitin line in immunodiffusion experiments against antiserum prepared against whole colostrum. However, antisera produced against these colostral IgA preparations gave two precipitin lines other than IgA after absorption with serum deficient in IgA. These lines had the characteristics described for secretory piece and lactoferrin (11). Thus, a small amount of con-

taminating protein was undoubtedly present in these colostral IgA samples.

Serum IgA was also obtained from patients with far advanced cirrhosis of the liver by the method of Heremans, Vaerman, Carbonara, Rodhain, and Heremans (12). These IgA preparations gave a single IgA line when tested against antihuman whole serum in immunoelectrophoresis experiments.

Antisera were raised in rabbits by giving weekly to bi-weekly 1 ml subcutaneous injections of a 0.2-1.0 mg/ml solution of antigen mixed into an equal volume of complete Freund's adjuvant. Antisera against IgA were rendered specific by absorption with serum deficient in IgA. An antiserum capable of differentiating IgA1- and IgA2-proteins (13-15) was kindly supplied by Dr. Henry G. Kunkel. Antisera against light chains were prepared against Bence Jones proteins or IgG myeloma proteins. These antisera were absorbed with IgG M-components of the opposite light-chain class.

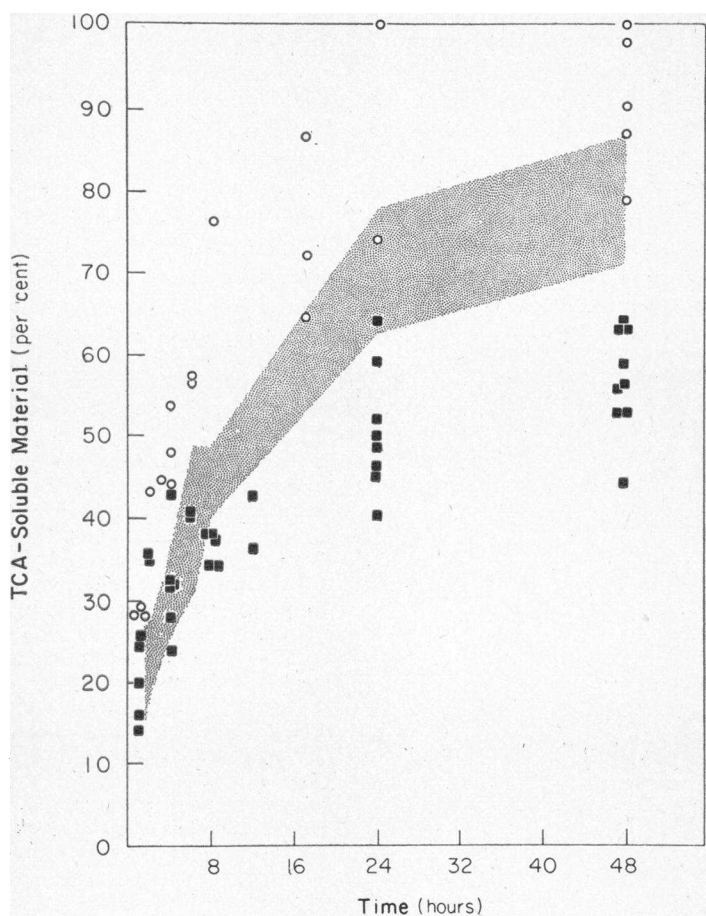


FIGURE 1 Rate of peptic digestion of IgA proteins as judged by the per cent of the original protein activity which became TCA-soluble related to time. O, percentage of TCA-soluble material, individual IgA_(S) M-components; ■, individual IgA_(R) M-components. The cross-hatched area represents the range found for 16 colostral IgA samples from individuals. Values for all 14 IgA M-components are represented at 48 hr. Determinations at earlier times were not performed on all samples but are plotted where done.

TABLE I
Percentage of Digestion of IgA to Small Polypeptides as Judged by Separation by Column Chromatography on Sephadex Gels

IgA Preparation	Digestion time	Percentage of polypeptides
	hr	%
IgA _(R) Ho	8	17
	24	37
Colostrals IgA	8	31
	24	56
IgA _(S) De	3	33
	8	52
	24	60

Abbreviations used are: IgA_(R) Ho, IgA protein Ho which is resistant to peptic digestion, and IgA_(S) De, IgA protein De which is susceptible to digestion.

Methods. Digestion of IgA with pepsin was performed, unless otherwise specified, in 0.01 ionic strength acetate buffer at pH 4.1 and 37°C with an enzyme to substrate protein ratio of 1:100 (16). The digestion reactions were halted at the desired time either by dialysis against phosphate-buffered saline pH 7.4 or by application of the digestion mixture to a Sephadex G200 column equilibrated with phosphate-buffered saline pH 7.4.

Timed digestion curves were performed as follows: first, the IgA protein was adjusted to a concentration of 1 mg/cc. This IgA solution was then dialyzed against 0.01 ionic strength, pH 4.1 acetate buffer until the IgA solution had reached pH 4.1. The final protein concentration was determined and pepsin was added. Serial 0.5-ml aliquots were removed at designated times and 0.1 ml of 25% trichloroacetic acid (TCA) was added to each aliquot. After the TCA-treated samples were allowed to stand at 4°C for 24 hr, the precipitate was removed by centrifugation. Nitrogen concentration of the supernatant was measured and, making a correction for the volume of TCA added, the per cent of nonprotein nitrogen was calculated (17).

The products of peptic digestion of IgA were separated by upward flow in Sephadex G200* (18) on 2.5 × 100 cm columns. In two experiments, Sephadex G100 was used.

Density gradients were prepared using 10 and 40% sucrose (19). Three-tenths ml of sample was layered onto a 4.5 ml gradient and then centrifuged for 16 hr at 35,000 rpm. In some experiments, a mixture of catalase, IgG, and human serum albumin was added to an identical gradient run simultaneously with the sample gradient. These proteins served as 11S, 7, and 4S markers, respectively.

Protein concentration was estimated, unless otherwise specified, by the Folin-Ciocalteu method (20). Analysis of the amino acid content of the IgA proteins was kindly per-

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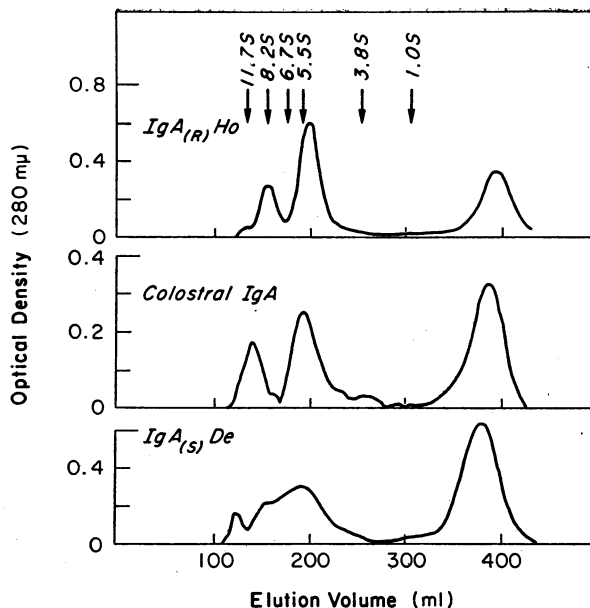


FIGURE 2 Separation of the products of 24-hr peptic digestion of IgA proteins on the same Sephadex G200 column. The elution volumes of substances of known sedimentation coefficients are also given for comparison. The peak eluting between 380 and 400 ml is assumed to be small polypeptide digestion products. The ratio of small polypeptides to protein greater than 1.0S increases from IgA_(R) Ho (top) to IgA_(S) De (bottom) indicating increasing susceptibility to peptic digestion.

formed by Dr. Horace Zinneman. Immunoelectrophoresis, immunodiffusion in agar gel, and electrophoresis on cellulose acetate were performed by standard techniques (21-23).

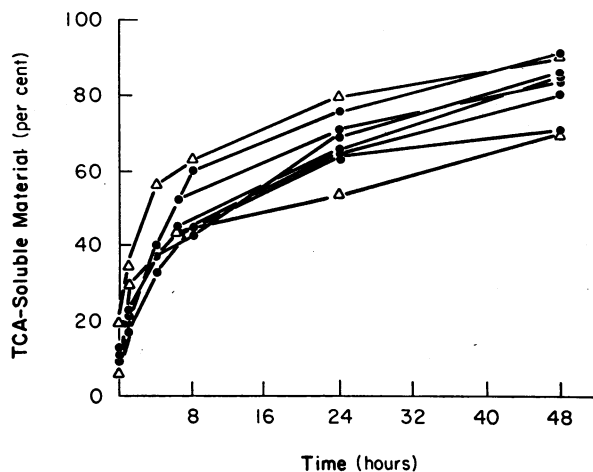


FIGURE 3 Rate of peptic digestion of serum IgA (Δ) and colostrals IgA (●) as judged by the increment in TCA solubility of the original protein. It appears that the serum IgA is more susceptible to peptic digestion during the 1st hr. Thereafter, serum IgA was digested at a rate similar to colostrals IgA.

RESULTS

Timed peptic digestion of IgA proteins. The rate of peptic digestion of 14 isolated IgA myeloma proteins, 5 preparations of colostrum IgA isolated from pooled colostrum, and 16 samples of colostrum IgA from individual mothers was estimated by measuring the disappearance of TCA-precipitable protein from the digestion mixture (Fig. 1). Digestion occurred most rapidly during the first few hours of the reaction. The IgA myeloma proteins segregated into two distinct groups on the basis of the rate of peptic digestion. These groups will be designated IgA_(R) and IgA_(S) for the resistant and susceptible groups respectively. All colostrum IgA preparations were digested initially at a rate that was similar to IgA_(R) proteins. After 6 hr, the digestion rate of colostrum IgA became intermediate to the two groups of IgA myeloma proteins.

The rate of peptic digestion of three of the proteins was studied further by column chromatography on Sephadex G200. Results of these experiments are presented in Table I and Fig. 2. The peaks at the 390 ml elution volume in Fig. 2 represent small polypeptides. As can be seen from the relative areas under the curves and from the data calculated from Folin protein determinations in Table I, IgA_(R) Ho showed the least digestion to small polypeptides while IgA_(S) De was most rapidly degraded. The digestibility of colostrum IgA was intermediate, thus confirming the TCA data.

IgA from the serum of patients with alcoholic cirrhosis was isolated to determine the sensitivity of a spectrum of serum IgA molecules to peptic digestion. Fig. 3 demonstrates two experiments in which the digestibility of six individual samples of colostrum IgA was compared with two serum IgA samples. Colostrum IgA appeared to be slightly more resistant to digestion

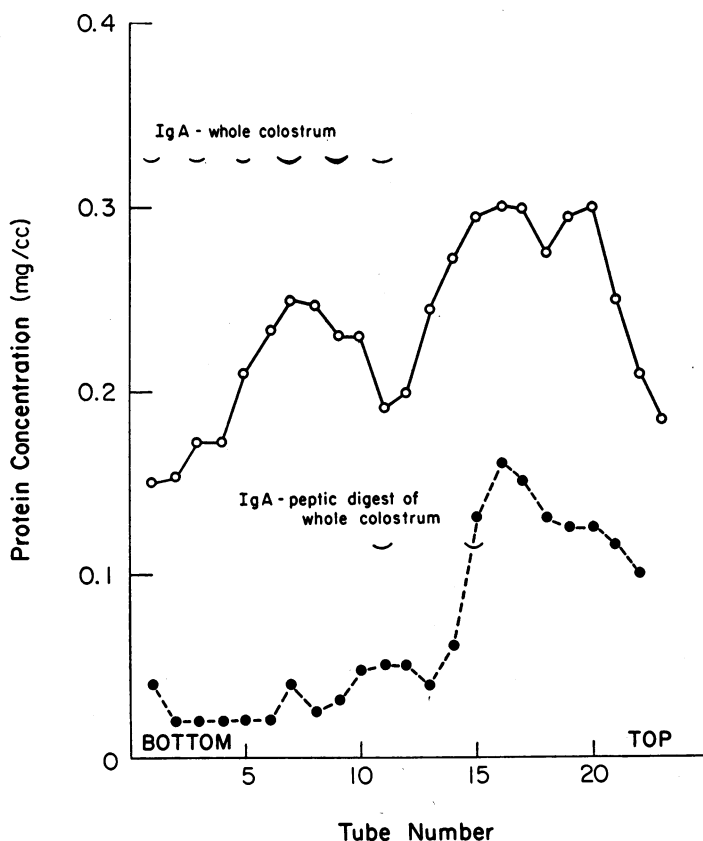


FIGURE 4 Size characteristics of colostrum IgA before and after digestion of whole colostrum with pepsin as judged by separation on a density gradient made of 10 and 40% sucrose. Tube numbers begin consecutively from the bottom of the gradient. O, whole colostrum. ●, peptic digest of whole colostrum. Results of analysis for IgA in Ouchterlony immunodiffusion experiments are shown diagrammatically by precipitin lines.

TABLE II
Light-Chain Type and IgA Subgroup of IgA
Proteins Studied

Group	Protein	L Chain	Subgroup	Mean per cent digestion*
				%
Resistant, IgA _(R)	Ho	K	IgA1	42
	Ru	λ	IgA1	52
	Ke	λ	IgA1	53
	Fr	K	IgA1	53
	Sc	K	IgA2	55
	Iv	K	IgA1	56
	Er	K	IgA1	59
	Ba	K	IgA1	63
	Na	K	IgA1	64
Serum IgA		K, λ	IgA1, IgA2	70, 90
Colostral IgA		K, λ	IgA1, IgA2	71-87
Susceptible, IgA _(S)	Ma	K	IgA2	79
	Ig	K	IgA2	87
	Ra	K	IgA1	95
	Ri	K	IgA1	98
	De	λ	IgA1	100

* TCA-soluble protein after 48 hr of peptic digestion.

than serum IgA during the first hour of digestion. Later values were similar for both serum and colostral IgA and intermediate to that found for the two groups of IgA myeloma proteins.

No apparent correlation was found between known IgA or light-chain subgroups and peptic digestibility. Both IgA_(R) and IgA_(S) groups contained IgA1- and IgA2- proteins as well as IgA proteins of both light-chain types (Table II). No relationship was found between electrophoretic mobility in cellulose acetate and digestibility with pepsin.

Protective effect of colostrum on peptic digestion of colostral IgA. Colostral IgA is fed to infants in whole colostrum, not in the separated state. Thus, although

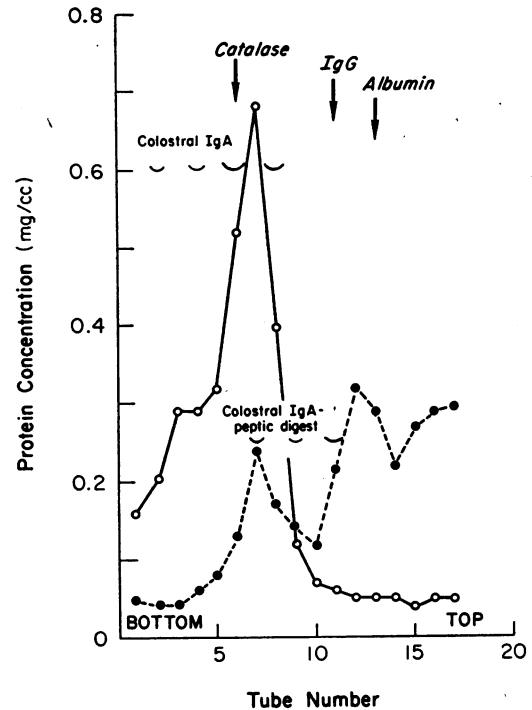


FIGURE 5 Sedimentation of whole- and pepsin-digested colostral IgA in density gradients prepared from 10 and 40% sucrose. O, whole colostral IgA. ●, peptic digest of colostral IgA. Peaks of catalase, human serum IgG and human serum albumin-marker activity are shown by the arrows. Results of analysis for IgA in Ouchterlony immunodiffusion experiments are represented diagrammatically by precipitin lines.

purified colostral IgA is readily digested with pepsin, the possibility remains that other constituents of colostrum exert a protective effect upon colostral IgA. Fig. 4 demonstrates a 10-40% sucrose density-gradient ex-

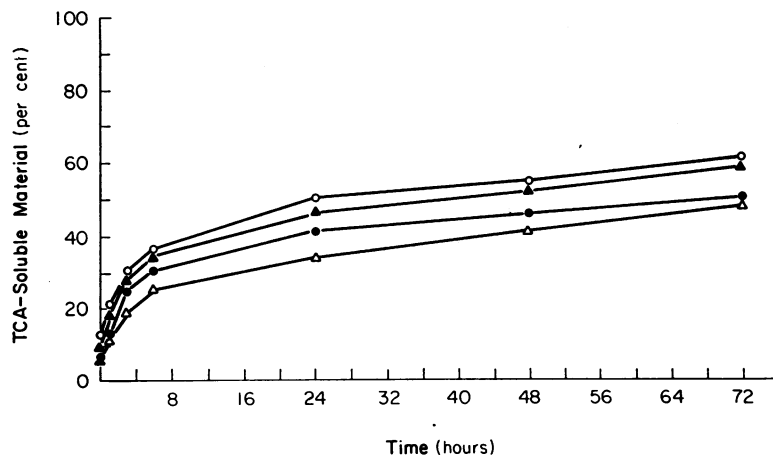


FIGURE 6 Effect of variations in the pepsin to substrate ratio upon peptic digestion of IgA_(R) Ho. Δ, 1:200; ●, 1:100; ▲, 1:50; and ○, 1:33.

periment in which almost all of the 11S IgA had been digested to smaller proteins as judged by immunodiffusion studies in agar gel and by total protein concentration. These findings are comparable with those obtained at the same enzyme to protein ratio for purified colostrum IgA (Fig. 5), suggesting that colostrum IgA is not protected from peptic digestion by other constituents of colostrum.

The effect of treatment of samples and experimental conditions on digestibility of IgA proteins. Neither the treatment of the samples before their use in the study nor the experimental conditions appear to explain these data. The results of digestion experiments were stable both in samples stored for over 6 months at -10°C and in serial samples taken from two patients during a 1 yr period. Repeated freezing and thawing of $\text{IgA}_{(R)}$ IV did not alter susceptibility to peptic digestion. Immediate processing of colostrum yielded IgA that was digested at the same rate as IgA from colostrum stored for up to 1 wk.

If the Folin-Ciocalteu method for the determination of protein concentrations did not measure $\text{IgA}_{(R)}$ and $\text{IgA}_{(S)}$ proteins equally, different enzyme to substrate ratios would have been used for the two groups. Increasing this ratio would have to significantly increase the rate of peptic digestion of $\text{IgA}_{(R)}$ proteins for this proposed variation to explain the differences found. $\text{IgA}_{(R)}$ protein Ho was digested at pepsin to protein ratios which varied from 1:200 to 1:33. Only small differences were found in the rate of digestion (Fig. 6). This appears to exclude the possibility that a suboptimal concentration of pepsin was available to catalyze the proteolytic reaction with $\text{IgA}_{(R)}$ Ho. In contrast, the rate of peptic digestion of colostrum IgA appeared to be significantly influenced by the enzyme to substrate ratio (Fig. 7). However, even when the enzyme to sub-

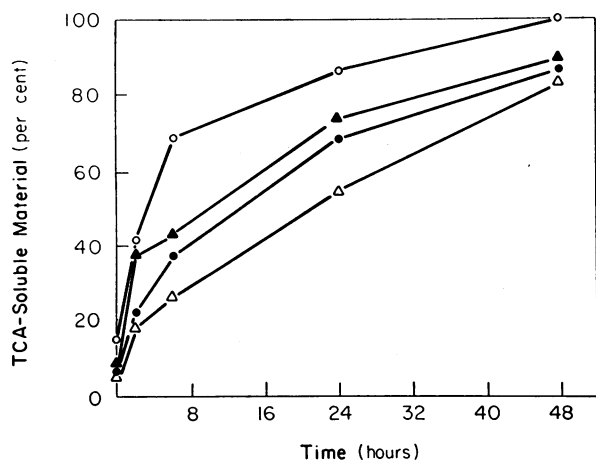


FIGURE 7 Effect of variations in the enzyme to substrate ratio upon peptic digestion of colostrum IgA. Δ , 1:200; \bullet , 1:100; \blacktriangle , 1:50; \circ , 1:33.

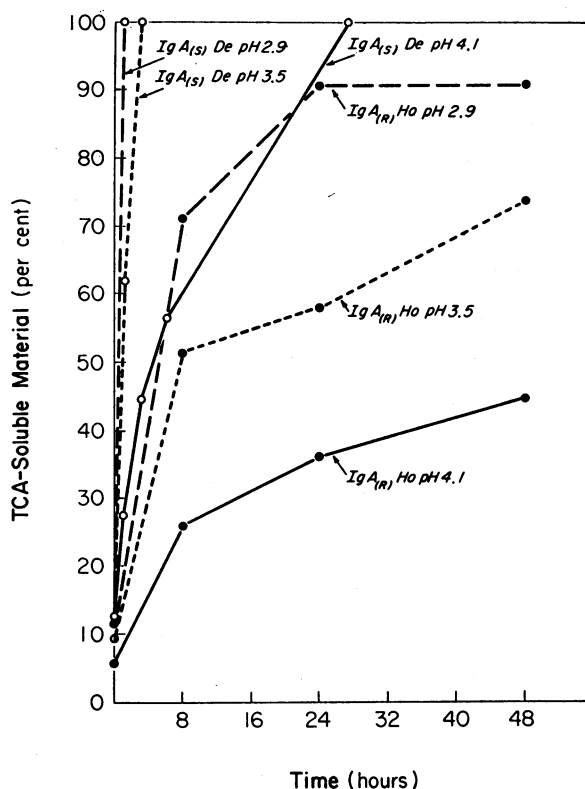


FIGURE 8 Effect of variation in pH on the rate of peptic digestion of IgA proteins as judged by the increment in TCA solubility of the original protein. \bullet , $\text{IgA}_{(R)}$ Ho; \circ , $\text{IgA}_{(S)}$ De; —, pH 4.1; ---, pH 3.5; and —, pH 2.9.

strate ratio was reduced to 1:200, the amount of non-protein nitrogen formed was greater at 48 hr than in any experiments on the $\text{IgA}_{(R)}$ group.

Pepsin has pH optima which vary from 1.5 to 2.5 for different substrates (24). The effect of lowering the pH of the reaction mixture to a more optimal level for peptic digestion was studied (Fig. 8). $\text{IgA}_{(R)}$ Ho, colostrum IgA, and $\text{IgA}_{(S)}$ De all became more easily digested as the pH of the reaction mixture was lowered. $\text{IgA}_{(R)}$ Ho was consistently more resistant to peptic digestion than $\text{IgA}_{(S)}$ De at all pH levels tested. Colostrum IgA was intermediate in susceptibility. $\text{IgA}_{(S)}$ De was completely digested within 1 hr at pH 2.9 so that further comparisons at lower pH levels were not made.

Elaboration of a pepsin inhibitor. A 1:1 mixture of $\text{IgA}_{(R)}$ Ho and $\text{IgA}_{(S)}$ De was digested to study the possibility that a pepsin inhibitor was produced during digestion of $\text{IgA}_{(R)}$ proteins. If an inhibitor was elaborated, the digestion of $\text{IgA}_{(S)}$ proteins should also decrease. The digestion reaction proceeded at a rate intermediate to comparable rates for the $\text{IgA}_{(R)}$ and $\text{IgA}_{(S)}$ proteins tested, thus making the production of significant amounts of a pepsin inhibitor unlikely.

Amino acid content of IgA_(R) Ho and IgA_(S) De. The amino acid composition of IgA_(R) Ho and IgA_(S) De was determined. Since IgA_(S) De has lambda light chains and IgA_(R) Ho is a kappa protein, amino acid differences could be expected on this basis. In spite of this difference, the molar fractions of the individual amino acids were remarkably similar, with differences in only proline and serine being greater than 1% of the total.

Production of antisera against F(ab')_{2a}. Antisera were produced against several separated F(ab')_{2a} IgA proteins in an attempt to raise specific precipitating antisera which could distinguish IgA_(R) from IgA_(S). Antisera against the F(ab')_{2a} from 24-hr digestion experiments had precipitating antibodies directed primarily against light chains and individual antigenic specificities. Antisera against F(ab')_{2a} from secretory IgA digested only 8 hr contained precipitating anti-IgA antibodies. No precipitating antisera against F(ab')_{2a} have been found which distinguish between IgA_(R) and IgA_(S). Attempts to demonstrate antigenic differences between IgA_(R) and IgA_(S) proteins by using antisera produced against the whole IgA molecule were also unsuccessful.

DISCUSSION

These observations demonstrate that IgA M-components vary considerably in their susceptibility to proteolytic digestion with pepsin. It also appears that the 14 IgA M-components studied can be separated into two distinct groups on the basis of their relative rates of digestion. The majority of IgA myeloma proteins are digested at a rate slower than either colostrum or serum IgA. The remainder are, in general, more susceptible to peptic digestion than either colostrum or serum IgA. Precedent for varying susceptibility of immunoglobulins to enzymatic digestion can be found in the relationships between sensitivity to papain and the heavy-chain subclass of IgG (25). It should be noted that relative susceptibility of these proteins to peptic digestion, as measured by both the TCA and the Sephadex G200 methods, may reflect the position rather than the number of peptide bonds hydrolyzed (26). However, the increased total degradation of the IgA_(S) proteins to smaller polypeptides, as shown in Fig. 2, represents the important finding regardless of the mechanisms involved.

Secretory IgA obtained from 21 colostrum samples forms a homogeneous group with regard to peptic digestion. The rate of digestion is similar to that of IgA_(R) proteins for the first 6 hr of digestion. After 6 hr, colostrum IgA is digested at a rate considerably more rapid than IgA_(R) M-components and equal to serum IgA. It appears that colostrum IgA (presumably a heterogeneous mixture of many IgA proteins, most of which

contain secretory piece [27]) may be appreciably more resistant to peptic digestion than serum IgA during the 1st hr of the digestion reaction.

Gamma heavy chains and kappa light chains contain genetically determined differences in amino acid sequences that are inherited in a Mendelian codominant fashion (28-30). These genetic determinants on IgG occur in several regions of the gamma heavy chain (28, 30). If an analogy holds for IgA, genetically determined differences in amino acid sequence at a site of peptic digestion might explain our findings. Secretory IgA was isolated from 16 individual colostrum samples to determine whether differences in peptic digestion of colostrum IgA occurred between individuals. Since all 16 samples were digested at a rate comparable with the secretory IgA obtained from pooled colostrum, it seems unlikely that a simple genetic difference is responsible for our findings. However, in view of the possible complexity of genetic factors that might be involved, our data do not exclude the possibility that genetic differences may be responsible for part or all of the variability found.

Cederblad, Johansson, and Rymo (31) first noted a relative resistance of both serum and secretory IgA, when compared with serum IgG, to proteolytic digestion with papain and trypsin (31). Tomasi and Calvanico have reported that secretory IgA is more resistant than serum IgA to digestion by a variety of enzymes (4). These authors did not study the effect of pepsin. Certainly resistance to proteolytic digestion would confer a biologic advantage to IgA in performing any function that it might have within the gastrointestinal tract. It appears from our data that secretory piece may have a stabilizing effect when peptic digestion of IgA is considered. However, the finding that the majority of serum IgA myeloma proteins are more resistant to pepsin than colostrum IgA suggests that this stabilizing effect is minor. The physiologic importance of this observation, if any, may be confined to the gastric lumen since the pH in the remainder of the intestine is normally above the pH range of pepsin activity.

We also studied the possibility that other constituents of colostrum might inhibit peptic digestion of secretory IgA. Although the results are only semiquantitative, it is apparent that *in vitro* proteolysis of IgA with pepsin proceeds readily in colostrum, as judged both by total protein concentrations and by immunodiffusion experiments. This degree of digestion appears comparable with that of separated colostrum IgA at the same enzyme to substrate ratio.

The variations in susceptibility of IgA proteins to peptic digestion did not appear to be ascribable to the conditions chosen for the experiments. Although both IgA_(R) Ho and IgA_(S) De proteins became increasingly

susceptible to peptic digestion as the pH of the reaction mixture was lowered, IgA_(a) Ho remained more resistant to digestion at all pH levels tested. Peptic digestion of IgA_(a) Ho proceeded at approximately the same rate over a 6-fold concentration of enzyme, thus suggesting that substrate excess does not explain the variance found. Finally, no evidence for the elaboration of a pepsin inhibitor was derived from digestion of a mixture of IgA_(a) Ho and IgA_(s) De.

Classically, subgroups or genetic markers on immunoglobulins have been defined by antibodies having specificity for the site in question. Thus far, we have been unable to demonstrate antibodies directed specifically against either the IgA_(a) or IgA_(s) proteins. Our antisera have been produced largely against F(ab')_{2a} formed during prolonged digestion with pepsin. These F(ab')_{2a} molecules can be shown to be immunologically deficient to F(ab')_{2a} produced during shorter digestion experiments (unpublished data). Therefore, it seems possible that immunization with F(ab')_{2a} obtained earlier during digestion may still yield precipitating antibodies to the sites that determine the ease of proteolysis with pepsin.

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