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Binding of Secretory Piece to Polymeric IgA and IgM Paraproteins *in vitro*

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Summary. By means of immunoelectrophoresis and the immunoselection technique the binding of free secretory piece to serum paraproteins of different classes was studied *in vitro*. Only the polymeric IgA and not the monomeric forms, nor paraproteins of IgG or IgD classes, were found to bind free secretory piece *in vitro*. This property was not dependent on the subclass or the light chain type of the tested paraproteins. In addition higher polymers of IgM with sedimentation coefficients of 25S to 30S also bound the secretory piece, whereas 19S forms did not.

INTRODUCTION

Immunoglobulin A (IgA) is the predominant immunoglobulin in external secretions. It differs from serum IgA by a higher sedimentation coefficient and by an additional polypeptide chain designated as secretory piece, transport piece, secretory component or 'T'-chain. One molecule of secretory piece (SP) is supposed to be bound to two monomeric IgA molecules. In human secretory IgA only a small amount of SP (about 20 per cent) can be released from IgA by dissociation of noncovalent bonds, but the major part of SP seems to be linked by disulphide bridges (Tomasi and Calvanico, 1968; Brandtzaeg, 1970). The site and the binding mechanism of the SP to the IgA is not known. Association with the Fc part of IgA might be expected, because SP appears to have specific affinity for IgA (reviewed by Tomasi and Bienenstock, 1968; Cebra, 1969; Ballieux, Mul, Poen, Stoop, ten Thije and Zegers, 1969) and it was found attached to the α-chains in some external secretions of patients with α-chain disease (Seligmann, Mihaesco, Hurez, Mihaesco, Preud'homme and Rambaud, 1969).

This affinity for IgA is, however, special in some respect. Only IgA in secretions and not serum IgA has been found in association with SP. In addition no myeloma protein in the form of secretory IgA has been found in serum (Ballieux, Stoop and Zegers, 1968; Hobbs, 1968). More recently Thompson, Asquith and Cooke (1969) reported the finding of small amounts of secretory type IgA in the serum of some patients with untreated adult coeliac disease, regional enteritis, ulcerative colitis, and also in nine out of 101 healthy control subjects. Such a finding in patients with intestinal diseases was, however, not confirmed by others (Poen, Ballieux, Mul, Stoop, ten Thije and Zegers, 1969).

The few reported reassociation experiments *in vitro* mention some relatively specific binding of free SP to serum IgA (Tomasi and Bienenstock, 1968; Asofsky and Hylton, 1968; Hanson, Holmgren, Jodal, Johansson and Lönnroth, 1969; Brandtzaeg, 1970) but the results do not seem to be unambiguous. Brandtzaeg (1970) mentions that only a minor fraction of the molecules combined.

Our present study showed that polymeric forms are required for the binding of free SP to serum IgA and IgM paraproteins *in vitro*. Only 10S IgA and the higher polymeric IgM paraproteins were found to bind free SP. This property was not dependent on the subclass or light chain type of these paraproteins.

MATERIAL AND METHODS

Mixed saliva and tears were collected from three patients with selective IgA deficiency. After centrifugation to remove the cells, the material was tested for the presence of free SP and the absence of IgA by immunodiffusion techniques and stored until use at $-20°$.

Sera from thirty-three patients with IgA paraproteinaemia, twelve patients with IgM, twelve with IgG, and four with IgD paraproteinaemia were mainly obtained from the department of Immunohaematology of the University Hospital in Leiden. Most of the sera chosen for this study contained a high level of paraproteins while immunoglobulins of the other classes were decreased or absent.

Immunoelectrophoresis and immunoselection were performed as described previously (Rádl, 1970). For ultracentrifugation in the Spinco E analytical ultracentrifuge sera were diluted six to seven times using phosphate-buffered saline (PBS) of pH 7·4. The sedimentation coefficients were measured at 20° and not extrapolated to zero concentration. Concentrations of components were calculated from the surfaces obtained by planimetry of the schlieren diagrams. Two 10S and one 7S IgA paraproteins were isolated by Sephadex G-200 gel filtration. Reduction and sulphydryl blocking of isolated 10S paraproteins and of all sera which contained small amounts of 10S in addition to a high 7S IgA level were performed according to Ballieux (1963, and personal communication, 1969) using 2-mercapto-ethanol and ethyleneimine (Koch-Light Laboratories Ltd, England). Two IgM paraproteins with major 19S and minor 25S components were isolated from serum by gel filtration through Sephadex G-200. The 19S component in these samples was isolated by repeated zone-centrifugation on sucrose gradients in a Spinco L type preparative ultracentrifuge. Gradients were formed of three equal layers of 40, 25 and 15 per cent (w/v) sucrose in phosphate buffered saline respectively, on which an aliquot of the IgM preparation (< 20 mg/ml) was layered. Centrifugation was performed during 6 hours at 35,000 rev/min at room temperature. Fractions were collected by piercing the tube, scanned spectrophotometrically at 280 nm for protein distribution, then concentrated by ultrafiltration and finally examined by analytical ultracentrifugation.

Specific rabbit antisera against human secretory piece were kindly supplied by Dr R. E. Ballieux and Dr P. J. J. van Munster. Specific antisera against κ and λ light chains, antisera against human serum IgA, and antisera against ovalbumin were prepared in rabbits as described previously (Hijmans, Schuit and Klein, 1969). Specific swine antisera against IgM and IgD and rabbit antisera against human IgG were provided by Sevac Prague. Antitotal horse serum against human serum proteins was obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam and specific rabbit antisera against human albumin, α_2-macroglobulin, transferrin, fibrinogen, haptoglobin and caeruloplasmin were purchased from Nordic Pharmaceuticals and Diagnostics N.V., Tilburg.

Light chains of the paraproteins were typed as described previously (Rádl, 1970). Subclasses of IgA paraproteins were determined by immunodiffusion techniques in the

laboratory of Dr J. F. Heremans and in our laboratory using an antiserum which was kindly supplied by Dr E. C. Franklin.

Incubation of free SP with paraproteins

All sera were diluted with PBS to get comparable concentrations of the paraproteins. Three parts of saliva or tears from patients with selective IgA deficiency were mixed with one part of each paraproteinaemic serum, incubated for 1 hour at 37° and left overnight at 4°.

Testing of SP binding

Two techniques were used to investigate the combination of free SP with a given paraprotein:

(a) *Immunoelectrophoretic examination.* In each plate three samples were tested and compared to the standard solution of free SP, i.e. saliva or tears from patients with selective IgA deficiency. With a specific antiserum against SP the free SP of the standard showed a short symmetric precipitin line with anodic electrophoretic mobility (Fig. 1–1). If the position of the SP precipitin line remained unchanged after immunoelectrophoresis of serum previously incubated with the SP, this serum was considered as not binding (Fig.

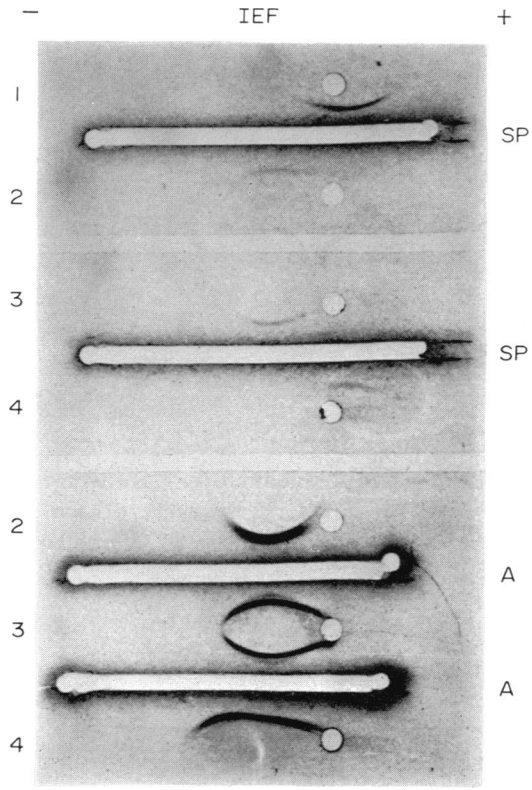

FIG. 1. Immunoelectrophoretic examination of a standard solution containing free secretory piece (1) and three different IgA paraproteinaemic sera after incubation with free SP (2, 3 and 4). The troughs contain specific antisera against secretory piece (SP) and against IgA (A). As can be seen by the change in electrophoretic mobility of the added SP, one IgA paraprotein (3) showed a complete binding, the second one (2) a partial (anodic part just visible) and the third one (4) no binding of the SP.

1–4). In cases where binding took place the precipitin line of the SP was visible in the cathodic part of the plate and its position was the same as that of the abnormal bow of the paraprotein developed with the appropriate class specific antiserum (Fig. 1–3).

(b) *Immunoselection technique.* Double radial diffusion was performed in an agar plate containing an antiserum against determinants specific for the class of paraproteins under investigation. The holes were alternately filled with test samples and specific anti-SP antiserum. A precipitin line between these holes appeared only in cases, where free SP was not bound to a paraprotein and therefore not precipitated by the incorporated antiserum (Fig. 2).

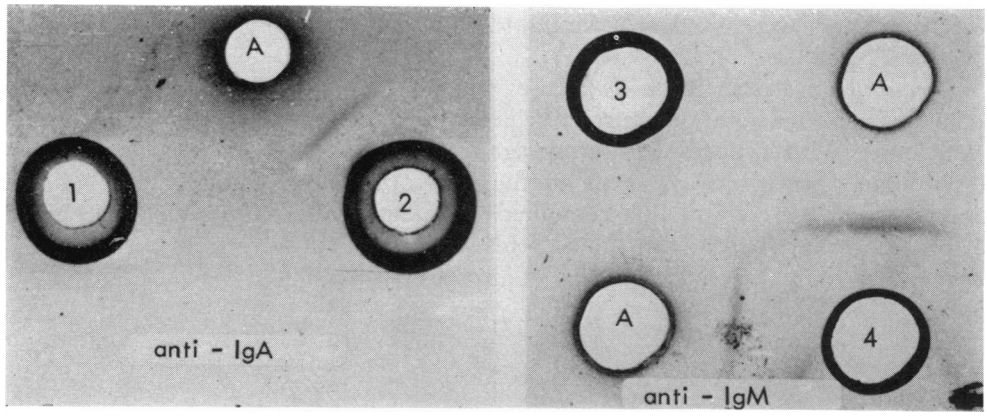

FIG. 2. Immunoselection technique for examination of the binding properties of two different IgA (1 and 2) and two IgM paraproteins (3 and 4) after incubation with the free SP. Antiserum against IgA (the left side) and antiserum against IgM (the right side) was incorporated in the agar. The precipitin line between the holes with antiserum against secretory piece (A) and the holes with test samples appeared only where the SP was not bound to a paraprotein and therefore not coprecipitated by the incorporated antiserum (2 and 4).

In the same way control experiments were performed with all paraproteins and with ovalbumin instead of secretory piece.

RESULTS

All experiments using both immunoelectrophoresis and immunoselection were in agreement with each other and the same results were obtained whether using tears or saliva from any of the three patients with selective IgA deficiency.

PARAPROTEINS OF IgA CLASS

In twenty-seven out of the thirty-four sera the results were unequivocal (Table 1). Eleven IgA paraproteinaemic sera with only a 7S M-component did not bind the free SP whereas fourteen 10S IgA paraproteinaemic sera did.

The third group comprising seven IgA paraproteins with major 7S and minor 10S components gave variable results. When the tests were performed in highly diluted sera, in which the concentration of the 10S component was less than approximately 10 mg/100 ml, no binding of SP was observed, although the abnormal bow of the paraprotein with specific anti IgA antiserum was distinct in immunoelectrophoresis (Fig. 1–/4). In

TABLE 1
BINDING OF THE SECRETORY PIECE TO IgA PARAPROTEINS OF DIFFERENT SUBCLASS, TYPE AND SEDIMENTATION COEFFICIENT

Subclass and type	No. of samples	Sedimentation coefficient				
		7S	10S	Major 7S+small 10S component		
				In high dilution 10S—final concentration below ~ 10 mg/100 ml	In low dilution 10S—final concentration above ~ 10 mg/100 ml	In low dilution after reduction
IgA_1-K	14	6/6−	4/4+	4/4−	4/4+ or ±*	4/4−
IgA_1-L	16	5/5−	8/8†+	2/3†−	3/3†+ or ±*	2/3−
IgA_2-K	3	1/1−	2/2+	—	—	—
IgA_2-L	1	1/1−	—	—	—	—
Total	34	13/13−	14/14+	6/7−	7/7+ or ±*	6/7−

* Partly positive means that after immunoelectrophoresis part of the added SP was found at the position of the IgA paraprotein and part as standard free SP.
† Two different serum samples from one patient—for details see text.
+, Positive, −, negative, ±, partly positive.

low dilution (concentration of the 10S component above 10 mg/100 ml), these sera showed at least a partly positive binding, i.e. part of the SP changed its electrophoretic mobility and part kept its fast anodic electrophoretic mobility (Fig. 1–/2). After reduction and sulphydryl blocking, no 10S components were found in these sera and no binding of the SP to the IgA paraproteins could be observed after subsequent incubation of sera with the free SP. There was only one dubious result. One serum sample of a patient whose disease started with a solitary plasma cell tumour of the jaw showed binding of SP in low and high dilution and even after reduction and sulphydryl blocking of the isolated 7S

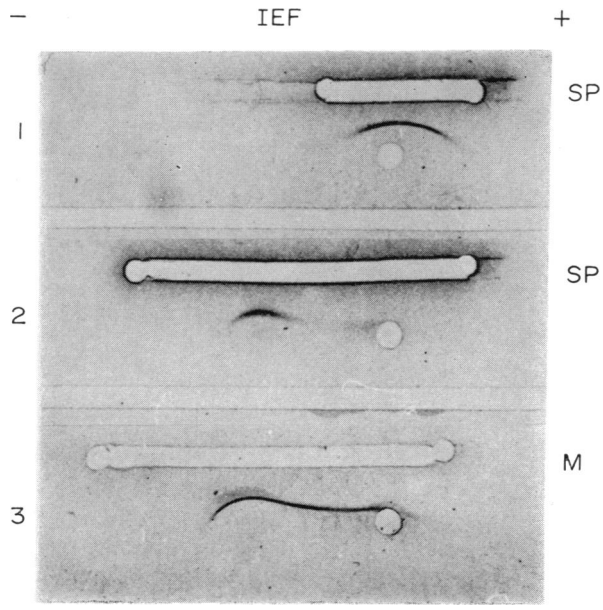

FIG. 3. Immunoelectrophoretic examination of a standard solution of free SP (1) and an isolated IgM paraprotein after incubation with free SP (2). The troughs contain specific antisera against secretory piece (SP) and against IgM (M).

IgA component. There was no more serum for detailed studies. After repeating the examination in a later serum sample, which contained a high amount of 10S paraprotein, the paraprotein, however, behaved in a similar way to the other 10S paraproteins.

The binding of the SP to the IgA paraproteins was not dependent on the subclass or light chain type of the paraproteins, as shown in Table 1.

Three sera were subjected to fractionation. Two isolated 10S IgA paraproteins showed binding of the SP, while the resulting 7S IgA proteins obtained after reduction and sulphydryl blocking (Fig. 4) lacked this property. As can be seen in Fig. 4, the reduced

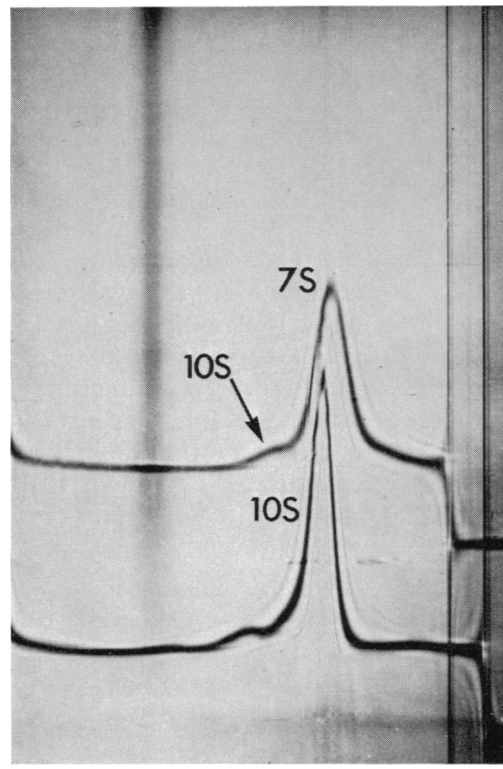

FIG. 4. Ultracentrifugal diagram of an isolated 10S IgA paraprotein (below) and of the same paraprotein after reduction and sulphydryl blocking (above). Some 10S material is still present in the reduced preparation. Sedimentation to the left. (Photographed after 46 minutes, speed 52640 rev/min.)

preparation still contained some 10S material. Consequently in an undiluted sample a partial binding of SP by this preparation could still be observed, while in diluted samples there was no binding. The only isolated 7S IgA paraprotein also failed to bind SP.

PARAPROTEINS OF IgM CLASS

In a group of IgM paraproteinaemic sera, five out of twelve showed binding of free SP, while the remaining seven did not (Table 2). Two IgM paraproteins from sera which bound SF were tested after isolation with the same results (Fig. 3). The attachment of the SP to the paraproteins was not correlated with the light chain type of the paraproteins.

TABLE 2
BINDING OF THE SECRETORY PIECE TO IgM PARAPROTEINS OF DIFFERENT TYPE AND WITH DIFFERENT CONTENT OF HIGHER POLYMERS

Type	No. of cases	High polymers (25S–30S) above ~5 mg/100 ml	High polymers (25S–30S) below ~5 mg/100 ml
IgM–K	10	3/3+	7/7−
IgM–L	2	2/2+	—
Total	12	5/5+	7/7−

+ = positive.
− = negative.

When comparing the sedimentation coefficients, sera containing components with sedimentation coefficients 25S and/or 30S (above 5 mg/100 ml approximately) in addition to a 19S component showed binding of SP, while paraproteins with sedimentation coefficients of 19S and no or only trace amounts of higher polymers (below 5 mg/100 ml) did not. In two isolated IgM paraproteins the 19S IgM fractions were separated from

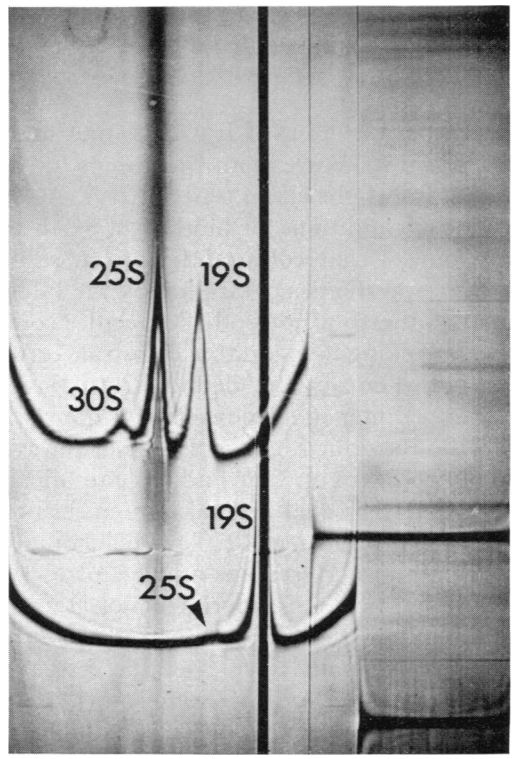

FIG. 5. Ultracentrifugal diagram of a 19S fraction (below) and of a fraction rich in higher polymers (above) prepared from an isolated IgM paraprotein. A trace of the higher polymers is still visible in the 19S fraction. Sedimentation to the left. (Photographed after 18 minutes, speed 52640 rev/min.)

the polymeric forms by sucrose gradient ultracentrifugation (Fig. 5). After incubation with free SP the 19S fractions did not bind SP, while fractions with higher IgM polymers did.

PARAPROTEINS OF IgG AND IgD CLASS

No binding of the SP was observed with the paraproteins of the IgG and IgD classes.

CONTROL TESTS

All control experiments with ovalbumin instead of SP gave negative results with regard to the binding of ovalbumin to any of the tested paraproteins. Neither albumin nor any of the other serum proteins were attached to the tested paraproteins, as shown in immunoelectrophoresis with polyvalent and specific antisera. After incubation of free SP with a pooled normal human serum in a ratio 4 : 1 no binding of free SP could be demonstrated by immunoelectrophoresis or immunoselection. Using this dilution the concentration of 10S serum IgA can be expected to be below 10 mg/100 ml.

DISCUSSION

Our experiments show that only the polymeric IgA and not the monomeric forms, nor paraproteins of IgG or IgD classes, bound free secretory piece *in vitro*. This binding was not dependent on the subclass or the light chain type of the tested paraproteins. In addition we have seen that higher polymers of IgM with sedimentation coefficients of 25 to 30S can also bind SP, while 19S forms cannot.

As regards the results with paraproteins of IgA class one may assume that SP needs for its binding the conformational structure of an IgA dimer (or polymer?), which should be common to both subclasses and light chain types of IgA. A recent study may support the idea that the same structural conditions for binding of SP to IgA are also valid *in vivo*. Lawton and Mage (1969) showed that colostral IgA from rabbits which were heterozygous for light chain allotypic specificities controlled by the b locus consists of molecules bearing one allelic type or the other but not both. Molecules containing both specificities were obtained artificially by mixing b4 and b5 colostral IgA in 5M guanidine and dialysing away the guanidine. The authors deduce from their observations that 11S colostral IgA is synthesized as a dimer in single cells and not derived from 7S IgA.

As already mentioned in the introduction, the results of the reassociation experiments reported in the literature are not clear cut. Our findings can offer a tentative explanation. When using for those experiments a pooled normal human serum or isolated normal IgA (Tomasi and Bienenstock, 1968; Brandtzaeg, 1970), which contains about 10–15 per cent of a 10S IgA component (Heremans, Heremans and Schulze, 1959), only a minor part is therefore available for binding of SP. In experiments of Hanson *et al.* (1969) a pool of six isolated IgA myeloma proteins was used but the sedimentation coefficients of those paraproteins were not taken into account.

In view of our results the demonstration by Thompson *et al.* (1969) of the presence of small amounts of secretory IgA in serum may also be explained as a reabsorption of small molecules of free SP from tissues which produce external secretions followed by bindings to the 10S IgA in the blood.

On the other hand the possibility that some IgA in 7S form also binds SP cannot be excluded. In addition to the first serum sample from our patient with myeloma of the jaw, there is a report from Mestecky, Kraus and Voight (1970) which also points in this direction. These authors found that 43 per cent of colostral IgA molecules in the monomeric fraction, obtained by gel filtration through Sephadex G-200, could be precipitated

by an antiserum against SP. It is difficult to evaluate these data, because no sufficient details are given on the second fraction. The IgA in it was assumed to be monomeric (7S), because it occurred together with IgG and, on double-diffusion, its line of precipitation with anti-α chain serum was straight rather than bent. However, no ultracentrifugation was done to confirm this assumption.

The mode of binding of SP to IgA may be more complex, as might be expected from dissociation experiments of secretory IgA (part bound by noncovalent and part by disulphide bonds) and from a preliminary report of Brandtzaeg (1970) on reassociation experiments, in which two molecular species of 11S IgA were formed, one indistinguishable from native secretory IgA, the second one with a loose structure where all of the known antigenic groups of SP were accessible. More detailed studies of the binding properties are therefore necessary.

Our observation that higher polymers of IgM paraproteins can also combine with SP is somewhat surprising. Up till now, no IgM containing SP was found *in vivo*, although IgM itself can be present in appreciable amounts in external secretions of some patients lacking IgA (Heremans and Crabbé, 1967; Brandtzaeg, Fjellanger and Gjeruldsen, 1968). The latter authors reported that the IgM which was selectively secreted into the parotid fluid of their patients had sedimentation properties similar to serum 19S IgM. Also in the saliva of our patients with IgA deficiency there was a higher concentration of IgM as compared with normal saliva, but SP was not bound to it. In one sample, which was concentrated 20 times, the sedimentation coefficient of IgM was estimated as 19S.

On the other hand higher polymers of IgM were found in appreciable amounts only in sera of patients with macroglobulinaemia of various aetiology (Mattern, Klein, Radema and van Furth, 1967), although they are present in trace amounts in normal human serum as well (see Walton, Rowe, Soothill and Stanworth, 1963). It seems unlikely therefore, that an association of high polymeric IgM with SP also takes place *in vivo*, and if so, that it might play any significant role, for instance for transport of IgM into secretions. In that case one would have to expect only a temporary and loose association, which would dissociate completely after reaching the secretion. The observations reported here, however, are the first instance of any property specifically found in IgM fractions higher than 19S which have hitherto been rather neglected in the literature.

Further studies are necessary to learn more about the nature of the SP binding in our experiments, to see for instance which bonds are formed and whether the same sites of SP and IgA are involved as *in vivo* and whether the same mechanism is present in two proteins as different as IgA and IgM polymers.

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