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Antigenicity of Polypeptides (Poly-α-amino Acids). Physicochemical Studies of a Calcium-Dependent Antigen–Antibody Reaction*

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ABSTRACT: The precipitation of a population of homologous sheep antibodies against α-poly(Glu^{60}Ala^{30}Tyr^{10}) (GAT) has been shown previously to specifically require calcium ions. The effect of Ca^{2+} on the interaction between the "calcium-dependent" antibody and GAT has been studied by ultracentrifugation, the use of [^{14}C]GAT in combination with rabbit anti-sheep γ-globulin, and an immunoabsorbant containing GAT. No interaction occurs unless Ca^{2+} is present. Calcium has no effect on the sedimentation coefficient, hydrogen-exchange kinetics, or fluorescence emission spectra of purified calcium-dependent antibody. In the presence of 0.01 N Ca^{2+}, $s^0_{20,w}$ of GAT increases slightly, [η] decreases by 39%, and optical rotatory dispersion shows small changes. These data all indicate a more compact configuration. A correlation between the ability of Ca^{2+} to induce configurational changes in glutamic acid containing polypeptides and their respective ability to induce calcium-dependent antibody in sheep antisera has been shown. The ability of divalent ions to cause interaction between GAT and calcium-dependent antibody, *i.e.*, ($Ca^{2+} > Sr^{2+} > Mn^{2+} > Ba^{2+} > Mg^{2+}$) corresponds to the order of divalent ion binding to GAT. In addition, when Ca^{2+} is added in increments to calcium-dependent antibody mixed with excess GAT (incomplete antigen), the resultant precipitin curve has the same shape as would be expected were increasing amounts of antigen added to its respective antiserum. These data lead to the conclusion that Ca^{2+} induces unique conformational determinants in glutamyl containing polypeptide antigens. The optical rotatory dispersion data and other considerations preclude the possibility that Ca^{2+} induces helical segments in these antigens.

This, plus the fact that monovalent cations are ineffective, lead to the conclusion that Ca^{2+} acts mainly by bridging two glutamyl carboxyls.

Recently (Maurer *et al.*, 1970) we reported a study of dilution and specific ion effects on the precipitin reaction of several sheep and rabbit antisera directed against synthetic polymers of amino acids. The data presented demonstrated that sheep and rabbit produce two populations of antibody (termed calcium-dependent and calcium-independent antibody) against a random sequence polypeptide,[1] α-(Glu^{60}-Ala^{30}Tyr^{10})$_n$ (GAT), where the precipitin reaction of one of these populations has a specific requirement for calcium ion. Three areas where calcium might have its effect on the precipitin reaction are (1) on a "preexisting" soluble antibody–antigen complex, *i.e.*, the role of calcium as a nonspecific insolubilizing agent; (2) on the antibody; and (3) on the conformation of the antigen, GAT (a highly negatively charged polypeptide). This paper is a report of results of physicochemical studies designed to determine the role of calcium in the precipitin reaction of the calcium-dependent anti-GAT.

In an attempt to further elucidate gross differences in the antigenic determinants of GAT with respect to calcium-dependent and -independent antibodies, we have studied the effects on the precipitin reaction of limited chemical modifications (methylation) of the glutamyl residues of the polypeptide antigen.

Materials and Methods

The preparation, analysis, and handling of antisera, and the purification of calcium-dependent antibody have previously been described (Clark and Maurer, 1969; Maurer *et al.*, 1970). The preparation and properties of the various

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[1] Polypeptide nomenclature used is based upon recommendation of IUPAC-IUB Commission (1968) on Biochemical Nomenclature, Oct 1967. In addition, abbreviations used are: GAT, α-poly(Glu^{60}Ala^{30}Tyr^{10}); GA, α-poly(Glu^{60}Ala^{40}); BBS, sodium borate (0.01 M) buffered saline (pH 7.5); MBSA, methylated bovine serum albumin; G^{60}A^{40}-amide, poly(α-*N*-3-hydroxypropyl-α-glutamide ^{60}Ala^{40}).

polypeptides have been reported (Blout and Idelson, 1956; Katchalski and Sela, 1958). All polypeptides were dialyzed exhaustively in the cold against 0.15 M NaCl or H_2O before use. Radioactive GAT ([^{14}C]glutamic acid) was prepared as was GAT using uniformly labeled γ-benzylglutamic acid *N*-carboxyanhydride. Before use, this preparation was exhaustively dialyzed against distilled water and lyophilized. The specific activity was 44,000 dpm/μg of N. All of the radioactivity in this preparation was precipitable in antibody excess with strong rabbit anti-GAT sera. All chemicals used were of reagent quality.

Ultracentrifugation. Sedimentation velocity experiments were done with a Spinco Model E ultracentrifuge (Beckman Instruments) equipped with scanning absorption optics and multiplexer. Sedimentation coefficients were measured at 20° in 12-mm aluminum centerpiece cells (AN-G rotor). Least-squared values of s were calculated from data obtained from at least six photographs.

Effect of Calcium on Purified Calcium-Dependent Antibody. Sedimentation coefficients were measured in sodium borate (0.01 M) buffered saline (pH 7.5) (BBS) at $CaCl_2$ concentration up to 0.1 M.

Fluorescence emission spectra of purified antibody were measured in an Aminco Bowman spectrofluorometer. Spectra were obtained for excitation at 275 and 290 nm. The concentration of antibody was 0.35 mg/ml. This corresponds to the concentration region where small dilutions of the sample have no effect on the spectra (*i.e.*, just above region where quantum yield is linear with concentration). The effects of calcium were determined by addition of 5 or 10 μl of 1.0 M $CaCl_2$ to 1-ml samples of antibody solution.

Hydrogen-exchange kinetics of purified antibody were measured by the method of rapid dialysis as described by Englander and Crowe (1965). Antibody (8 mg/ml) was tritiated (10–50 mCi/ml) in 0.01 M sodium acetate (pH 6.0) buffered 0.15 M NaCl. "Exchange-out kinetics" were measured in the presence and absence of Ca^{2+} (0.01 M).[2]

Effects of Calcium on Polypeptide Antigens. VISCOSITY. Viscosities of GAT and the other polypeptides studied were measured at 25 ± 0.01° in Cannon-Ubelohde semi-micro suspended level dilution viscometers. Kinetic energy and shear corrections for the viscometers used (zero times 180 to 230 sec) were found to be negligible. Intrinsic viscosities, $[\eta]$, were obtained graphically from intercept values by use of the Huggins equation (1942)

$$\eta_{sp}/c = [\eta] + [\eta]^2 k' c$$

where k' is the Huggins constant and c, concentrations in grams per deciliter.

OPTICAL ROTATION. A Rudolph Model 80 photoelectric spectropolarimeter was used to measure optical rotations. The instrument was modified by the addition of xenon 200-W lamp in conjunction with a Beckman D. U. monochromator. The monochromator was calibrated with the green line of a mercury lamp. Measurements were made at 25° in a 1-dm Heidelberger-type polarimeter tubes fitted with fused silica (quartz) windows. Measurements were made at wavelengths between 260 and 650 nm. Refractive indicies of solvents used were obtained by measurement of refractive index at the sodium D line (Bausch & Lomb refractometer). To obtain the refractive dispersions of solvents, it was assumed their dispersions would follow that of water and, hence, differences observed for the sodium value were added to the dispersion of water.

The effects of calcium on optical rotations of GAT were measured at pH 7.5 in BBS containing either no calcium or 0.05 N $CaCl_2$. Attempts to fit the data obtained to the Moffitt–Yang equation (1956)

$$m' = \frac{a_0\lambda_0^2}{\lambda^2 - \lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2 - \lambda_0^2)^2}$$

by plotting $m'(\lambda^2 - \lambda_0^2)/\lambda_0^2$ *vs.* $\lambda_0^2/(\lambda^2 - \lambda_0^2)$, where m' is the reduced mean residue rotation, did not lead to straight lines regardless of the choice of λ_0.

Because the Moffitt–Yang equation could not be fitted to the GAT data, effects of calcium and pH on the optical rotatory dispersion of a related polypeptide α-$(Glu^{60}Ala^{40})_n$ (GA) were studied. This peptide also produces Ca^{2+}-dependent and -independent antibodies. The solvents for the calcium effects were as above, while pH effects were measured in phosphate- (0.01 M) buffered 0.15 M NaCl.

CHEMICAL DERIVATIVES OF GAT. GAT was extensively methylated by the method of Sri Ram and Maurer (1959) for 24 hr at 5°. The insoluble derivative was tested as an immune absorbant with sheep 2-III (85% calcium independent) and 6-V (90% calcium dependent) antisera.[3] Levels of absorbant used were 5 and 20 times the equivalence concentration of antigen. Absorptions took place for 4 days at 5°. After removal of the absorbant by centrifugation the sera were analyzed for calcium-independent and calcium-dependent antibody from complete precipitin curves by methods described (Maurer *et al.*, 1970).

Cross-reactions of six lightly methylated GAT preparations were studied. The methylated samples were obtained by withdrawing aliquots from the esterification media at 0.5-, 1.0-, 1.5-, 2.0-, 2.5-, 3.0-, and 4.0-hr reaction time. The aliquots were rapidly neutralized at 0° with 0.1 N NaOH to pH 7.0, flash evaporated and exhaustively dialyzed against BBS. Determinations of the degree of methylation by titration could not distinguish the methylated from unmethylated preparations. Further, the hydroxamic acid method (Sri Ram and Maurer, 1958) for determining methylation could not be used due to the insolubility of GAT at acid pH.

GAT–MBSA COMPLEX. An immune absorbant was made by adding dropwise 60 mg of MBSA in 4 ml of H_2O to 4 ml of H_2O containing 190 mg of GAT (Sri Ram and Maurer, 1959). The resulting precipitate was washed repeatedly with cold BBS until the supernatant showed no measurable absorbance at 280 nm. The aggregate was then suspended for 24 hr in normal rabbit sera containing 0.05 M EDTA to allow for any nonspecific absorption of serum proteins. It was washed once with BBS with or without 0.02 M $CaCl_2$ depending on the antibodies to be absorbed.

Results

GAT–Anti-GAT (Calcium-Dependent) Complexes. To determine whether or not antigen–antibody complexes exist in the absence of calcium, three kinds of experiments were performed as follows.

[2] We wish to thank Dr. William Stylos for help with these measurements.

[3] The notation for sheep numbers and bleeding have been defined.

TABLE I: Ability of GAT–MBSA Aggregate to Remove Dependent and Independent Antibodies in the Presence or Absence of Ca^{2+}.

Treatment of Serum	μg of N/ml of Sheep 6-V Serum			μg of N/ml of Sheep 2-III Serum		
	Total	Dependent	Independent	Total	Dependent	Independent
Unabsorbed	285	240	45	111	27	84
GAT–MBSA (5 × equivalence[a]) –0.01 M EDTA[b]	267	245	22	81	30	51
GAT–MBSA (40 × equivalence) –0.01 M EDTA[b]	238	238	0	26	26	0
GAT–MBSA (5 × equivalence)	208	180	28	77	15	62
GAT–MBSA (40 × equivalence)	0	0	0	0	0	0

[a] 5 × equivalence is five times antigen concentration at equivalence. [b] The results obtained for Ca^{2+} removal by extensive dialysis are within experimental error of these experiments where EDTA was used to complex Ca^{2+}.

INTERACTION OF CALCIUM-DEPENDENT ANTIBODY WITH GAT IN ANTIGEN EXCESS. Purified calcium-dependent anti-GAT (50 μg of N/ml) from sheep 6-V in BBS which contained 15 μg of N GAT/ml (10 times the equivalence concentration), was centrifuged at 44,000 rpm in a Spinco Model E. In the same rotor, Spinco AN-D (six place), cells containing the following samples were placed (1) GAT–anti-GAT, as above, containing 0.01 N $CaCl_2$; (2) GAT alone, 15 μg of N/ml; (3) same as 2 containing 0.01 N Ca^{2+}; and (4) anti-GAT, alone, 50 μg of N/ml. Double-sector cells with appropriate buffers as reference were used. After reaching speed each cell was scanned at 280 nm at 16-min intervals.

Figure 1 shows two ultraviolet ultracentrifuge sedimentation patterns obtained for the above. The vertical direction on the patterns depicts absorbance; sedimentation proceeds from left to right. The upper pattern was obtained in the absence of calcium and was taken after a running time of 100 min. The plateaus corresponding to the antibody and the antigen (Figure 1) were identical in shape and in displacement from the meniscus as those for these components run separately (not shown). With the addition of calcium (Figure 1 lower portion), it is seen that the antibody plateau is displaced to the right, the plateau is not well defined and the antigen plateau is lowered. All of these observations indicate the formation of complexes on calcium addition.

INTERACTION OF CALCIUM-DEPENDENT ANTIBODY WITH GAT IN THE PRESENCE OF EDTA AT EQUIVALEVCE. [^{14}C]GAT in the amount required for maximum precipitation (76,500 dpm) was mixed with 1.0 ml of sheep 5-II antiserum to which had been added 200 μl of 0.5 M EDTA. After 72-hr incubation at 5°, with daily mixing, the precipitate of *calcium-independent antibody* was collected on a Millipore filter and washed with phosphate-buffered (pH 7.2) saline (PBS) (Kabat and Mayer, 1961). The filter and precipitate were dissolved in Brays solution employing a solubilizer [NCA (Nuclear-Chicago)], and assayed for ^{14}C. The filtrate of the above was divided into two portions. To one aliquot 100 μl of 1.0 M $CaCl_2$ was added, and to the other rabbit anti-sheep γG was added to precipitate all of the sheep γ-globulin. After incubation, these precipitates were collected, washed, and analyzed as above.

The precipitation distribution of [^{14}C]GAT among independent and dependent antibodies [50:50 by precipitin analysis (Maurer *et al.*, 1970)] and soluble complexes is given in Figure 2. After the removal of precipitating calcium-independent antibody, 6400 dpm/ml of sera ([^{14}C]GAT in the form of soluble complexes) can be precipitated by addition of rabbit anti-sheep γ-globulin to the EDTA containing supernatant. However, addition of calcium to another portion of the supernatant results in the precipitation of 28,600 dpm of [^{14}C]GAT/ml of sera. It should also be noted from the flow diagram that any soluble GAT–anti- (calcium independent) GAT complexes which are known to be present in the first precipitation (Odstrchel and Maurer, 1969) would be carried over in the supernatant used for the second precipitations.

INTERACTION OF CALCIUM-DEPENDENT AND -INDEPENDENT ANTIBODIES WITH GAT–MBSA COMPLEX. The third type of experiment involved an insoluble GAT–MBSA complex (immune absorbant) used as follows. Antisera of sheep 2-III

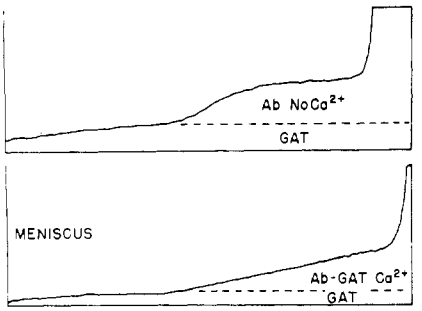

FIGURE 1: Scanner tracings (A_{280nm} *vs.* radial distance) of the sedimentation of calcium dependent antibody with GAT in the presence (lower curve) and absence (upper curve) of Ca^{2+}.

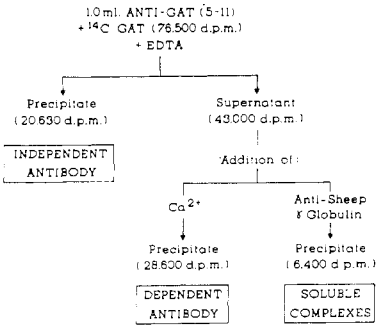

FIGURE 2: Distribution of [^{14}C]GAT among calcium-dependent antibody and independent antibody and soluble complexes.

TABLE II: The Effect of Concentration on the Sedimentation Coefficient of GAT.

Concn (mg/ml)	Sedimentation Coefficient (S)	
	Saline + 0.01 N Ca^{2+}	Saline
0.5	2.7	2.5
1.0	2.75	2.3
2.0	2.8	2.0
s_0	2.7	2.6

and 6-V were absorbed at 5° with the MBSA–GAT aggregate for 4 days, with repeated mixing. Levels of GAT in the absorbing complex were 5 and 40 times the equivalence concentration of GAT. Absorptions were done on normal antisera and on antisera from which calcium had been removed by extensive dialysis or by addition of EDTA (0.01 M). After centrifugation of the MBSA–GAT precipitate, the sera were examined for calcium-dependent and calcium-independent antibody (Maurer *et al.*, 1970).

Table I shows the distribution of calcium-dependent and calcium-independent antibodies after absorption of sheep 6-V and sheep 2-III antisera in the absence of calcium (0.01 M EDTA), with two levels of the GAT–MBSA complex. From the data of sera 6-V, it is seen that none of the *calcium-dependent antibody* was removed even when large amounts of absorbant were used. This very same effect is seen for the *calcium-dependent antibody* of sera 2-III where less of this kind of antibody is present. The absorption data with sera 6-V and 2-III show that absorption with aggregated complex containing five times equivalent antigen removes a slight amount of calcium-independent antibody while nearly all of this kind of antibody is removed with the increased amount of absorbant.

The data of Table I also shows that in the presence of calcium the higher level of absorbant (50 times equivalence antigen concentration) removes completely both kinds of antibody from the sera.

Effect of Calcium on Purified Calcium-Dependent Antibody. Sedimentation coefficients of calcium-dependent antibody and their dependence on concentration were independent of calcium chloride additions (up to 0.1 M) to BBS solutions of the antibody. Single, symmetrical schlieren peaks were obtained in all cases, which yielded least square values of $s_{w,20}$ of 6.5 S.

The fluorescence emission spectra of antibody obtained by excitation at either 275 or 290 nm were unperturbed by calcium (0.005 or 0.01 M). Similarly, the hydrogen-exchange-out kinetics of antibody were unaffected by calcium.

TABLE III: Effect of Added Ca^{2+} on the Intrinsic Viscosity of GAT in Borate-Buffered Saline (pH 7.5).

Ca^{2+} Concn (N × 10^3)	$[\eta]$
0	0.872
2.0	0.808
4.0	0.750
6.0	0.702
8.0	0.657
10.0	0.629

TABLE IV: Effect of Ca^{2+} on the Intrinsic Viscosity of Polypeptide in Borate-Buffered Saline (pH 7.5).

Polymer	Mol Wt	$[\eta]$ (Saline)	$[\eta]$ (Saline + 0.01 N Ca)
α-$(G^{60}A^{30}T^{10})_n$	45,000	0.875	0.629
α-$(G^{60}A^{40})_n$	43,000	1.190	0.922
α-$(G^{54}A^{36}L^{10})_n$	50,000	0.772	0.672
α-$(G^{42}A^{30}L^{28})_n$	50,000	0.515	0.501
α-$(G^{60}A^{40}$-amide$)_n$[a]	50,000	0.451	0.451

[a] α-(γ-*N*-3-Hydroxypropylglutamide^{60}Ala^{40})$_n$.

Effect of Calcium on GAT and Other Antigens. SEDIMENTATION AND VISCOSITY. Tables II and III, respectively, show sedimentation and intrinsic viscosity data for GAT. The sedimentation coefficient of GAT increases from 2.6 to 2.7 S on addition of 0.01 N Ca^{2+}. More striking, however, is the dependence of S on concentration, *i.e.*, ds/dc (not shown graphically). This latter quantity changes on addition of calcium from a slightly positive term in 0.15 M NaCl to a negative one of some magnitude. The data in Table III show a relatively large (39%) decrease in intrinsic viscosity on addition of 0.01 N Ca^{2+}.

The third and fourth columns of Table IV show viscosity data for other polypeptides in the presence and absence of 0.01 N Ca^{2+}. With the exception of the poly(γ-*N*-3-hydroxypropyl-α-glutamide^{60}Ala^{40}) ($G^{60}A^{40}$-amide) (Maurer, 1970), a neutral polymer, calcium causes a decrease in the intrinsic viscosity of all polymers. However, the magnitude of the effect parallels the degree of net nagative charge of the polypeptides (listed in decreasing order of net negative charge).

OPTICAL ROTATION. Figure 3 shows the optical rotatory dispersion curves of GAT, and GA as affected by calcium and

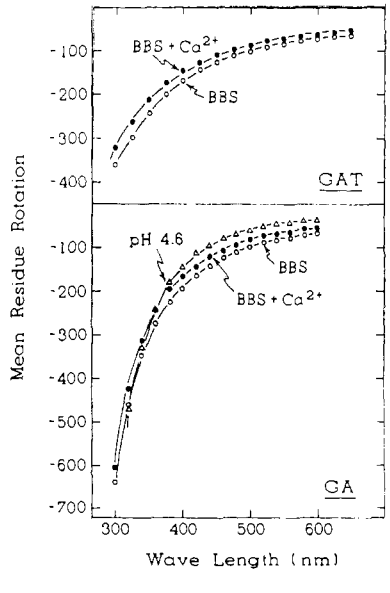

FIGURE 3: The effect of Ca^{2+} on the optical rotatory dispersions of GAT and GA.

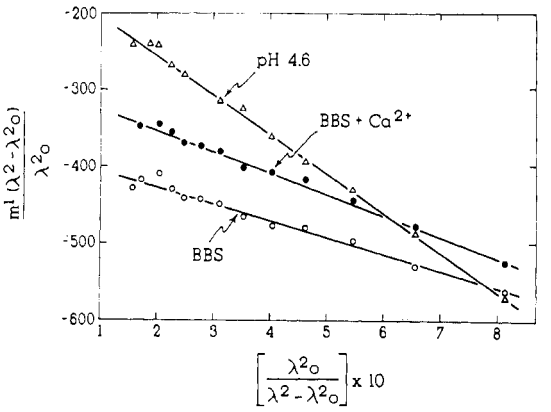

FIGURE 4: Moffitt–Yang plots of GA as affected by Ca^{2+} and pH.

pH. The effect of calcium is to shift all curves uniformly upward, much like a concentration effect. For GA, decrease in pH exhibits a similar effect at wavelengths greater than 370 nm.

Figure 4 shows a Moffitt–Yang plot of the GA dispersion data. It is seen that addition of calcium causes a slight negative increase of the slope relative to the "no calcium" condition. The b_0's for these conditions are -272 and -225, respectively. Assuming a b_0 of -630 corresponding to 100% α helix, then 0.05 N calcium increases the helix content of GA from 36 to 43%. Similarly, decreasing the pH to 4.6 increases the helix content to 84%, as would be expected for this polypeptide.

CROSS-REACTIONS OF CHEMICALLY MODIFIED GAT. Absorption of sheep 6-V (90% calcium-dependent antibody) and sheep 2-III (85% calcium-independent antibody) antisera with the extensively methylated GAT removed no antibody of either kind. This would be consistent with the importance of the free glutamyl group in the antigenic determinants of GAT.

Table V shows the data for per cent cross-reaction (relative to GAT) with the graded methylated GAT's. From the per cent cross-reactions listed in the table, it is seen that limited methylation of the antigen leads to a greater reduction of cross-reaction with calcium-independent antibody than with the calcium-dependent antibody. This effect is shown graphically in Figure 5. In the Figure, the abscissa is scaled in time (*i.e.*, reaction time of methylation for the samples) (see Methods). Based on titration studies, the maximum extent of

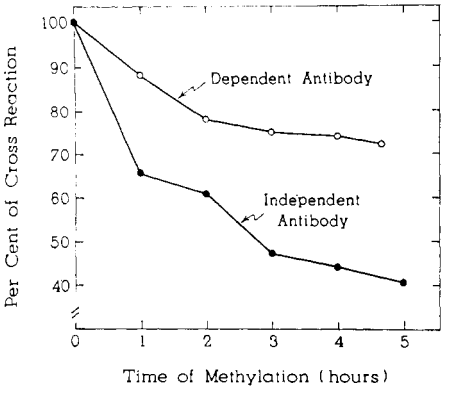

FIGURE 5: Cross reactions of calcium-dependent and -independent antibodies as affected by limited methylation of GAT.

TABLE V: Cross-Reaction of Limited Methylated GAT Preparations with Calcium-Independent and -Dependent Antibodies.

Time of Methylation (hr)[a]	μg of N/ml; 6-V Serum			% Cross-Reaction
	Total	Dependent	Independent	Dependent Antibody[b]
0	282	239	43	100
1	244	211	33	88
2	211	186	25	78
3	204	179	25	75
4	205	178	26	74
5	195	172	23	72
	μg of N/ml; 2-III Serum			Independent Antibody[b]
0	116	26	90	100
1	82	23	59	66
2	71	16	55	61
3	68	16	42	47
4	58	18	40	44
5	50	14	36	40

[a] See Methods and text. [b] Results are calculated for major class of antibody in each serum.

methylation for these samples does not exceed 10% which is equivalent to about 14 residues/molecule.

ABILITY OF SPECIFIC PRECIPITATES (DEPENDENT OR INDEPENDENT) TO ABSORB ANTIBODY OF THE OTHER KIND. To determine if calcium-dependent and -independent antigenic determinants are on the same molecule of GAT or if the GAT preparation contained two antigenic populations, the following experiment was performed. In four precipitin tubes (two control, two experimental) 100 μl of purified sheep 5-II calcium-independent antibody ($A_{280\ nm}$ 0.436) was mixed with an equivalent amount of GAT and incubated for two days; the precipitates were washed once with BBS. To the experimental tubes 100 μl of purified sheep 5-II calcium-dependent antibody ($A_{280\ nm}$ 0.337) was added, plus calcium to give a final concentration of 0.05 N. Similarly a second set of four tubes received 100 μl of the calcium-dependent antibody, plus antigen and calcium. After a 2-day incubation, these precipitates were washed with the borate buffer containing calcium (0.05 N). To the two experimental tubes containing washed precipitates, 50 μl of the calcium-independent antibody solution was added. In both cases the second incubation lasted 2 days, after which the precipitates were given a single wash. Amounts of precipitates were quantitated by dissolving them in 0.1 N NaOH and reading absorbance at 280 nm.

The results of absorption of purified sheep 5-II calcium dependent antibody with the specific precipitate of GAT–calcium-independent antibody and results of the reverse kind of absorption are given in Table VI. Rows one and three give antibody N for the specific precipitates of calcium-dependent (37.7 μg of N) and calcium-independent (44.4 μg of N) antibodies. As seen from rows two and four of the table, absorption of these specific precipitates in purified antibody of the other kind results in significant increase in antibody N. The calcium-dependent antibody specific precipitate increases by

TABLE VI: Ability of Specific Precipitate of Calcium-Dependent or -Independent Antibody to Absorb Antibody of Other Kind.

Specific Precipitate	μg of N	% Increase over Control
GAT, anti-GAT (CD),[a] control	37.7	
GAT, anti-GAT (CD) + Cl	50.2	33
GAT, anti-GAT (Cl),[a] control	44.4	
GAT, anti-GAT (Cl) + CD	62.2	40

[a] CD and Cl designate calcium-dependent and -independent antibody, respectively.

TABLE VII: Relation between Ca^{2+}-Induced Changes in Poly Co- and Terpeptides and Their Ability to Induce Calcium-Dependent Antibody in Sheep.

Polymer	$(\Delta[\eta]/[\eta]) \times 100$ Saline	% Dependent Antibody
α-$(G^{60}A^{30}T^{10})_n$	−39	50.0–90.0
α-$(G^{60}A^{40})_n$	−29	63.5
α-$(G^{54}A^{36}L^{10})_n$	−15	33.0
α-$(G^{42}A^{31}L^{28})_n$[a]	−3	0.0

[a] α-(γ-*N*-3-Hydroxypropylglutamide^{60}Ala^{40})$_n$.

33% after treatment with independent antibody, while the reverse situation gives a 40% increase.

Discussion

Recent studies (Zimmering *et al.*, 1965; Odstrchel and Maurer, 1969; Maurer *et al.*, 1970, unpublished results) have shown that sheep antibodies have certain unique and peculiar properties. Extensive work in our laboratory has demonstrated that sheep antibody can form significant quantities of soluble antigen–antibody complexes even with undiluted sera. Whether this phenomena is peculiar to synthetic random polypeptides as immunogens with sheep antiserum is presently under study. We have studied the possibility of calcium acting on soluble antigen–antibody (calcium dependent) complexes by three independent methods. The results of the ultracentrifuge study and those for the immunoabsorbants are unequivocal. Both demonstrate that no interaction between GAT and its calcium-dependent antibody occurs unless calcium is present. Also, these experiments are completely reversible in that addition of EDTA to the interacting system results in sedimentation patterns identical with the unreactive system (Figure 1, upper). Similarly, EDTA treatment of an immune absorbant which removes both calcium-dependent antibody and -independent antibody results in quantitative release of only the calcium-dependent antibody. The data obtained with the anti-globulin experiment are in essential agreement with the above results. Although 78% of the [^{14}C]-GAT does not interact with calcium-dependent antibody in the absence of calcium at equivalence, it would appear that 22% of the antigen is involved in soluble complexes. One of the most plausible explanations of this discrepancy is that the experiment was initially done such that any soluble complexes formed in the first precipitation (*i.e.*, of calcium-independent antibody) would remain in the supernatant used for the subsequent anti-globulin precipitation and therefore the soluble complexes are most likely of the calcium-independent class. Such a result would agree with other studies on soluble complexes in calcium-independent antibody systems (Odstrchel and Maurer, 1969).

Another important result which would lead to the same conclusion is as follows. In the [^{14}C]GAT antiglobulin experiment that serum used (5-II) has a one to one ratio of independent to dependent antibody. The radioactivity (antigen) determined in the precipitates of independent and dependent antibodies is 20,630 and 28,600 dpm per ml of sera, respectively. This indicates that the combining ratios of both kinds of antibody are most likely the same (about 1 determinant/4500 molecular weight unit of GAT). Based upon the above, if it is now assumed that all or a major portion of the soluble complexes are due to the independent antibody, then the agreement between combining ratios is even better.

Having eliminated the possibility that the action of calcium on the calcium-dependent antibody system involves the precipitation of existing antigen–antibody-soluble complexes, calcium could either affect antibody or GAT and related antigens. Although ionic effects on certain antibodies systems are well documented (Kleczkowski, 1965; Goodman *et al.*, 1951) no specific effects of calcium on isolated antibody have been reported. Yet reports of calcium-dependent antigen–antibody reactions have appeared in the literature (Lachmann and Thompson, 1970; Yokohari and Leon, 1965). Because the antigen combining sites constitute less than 1% of the total amino acid residues of IgG (Karush, 1962), the fact that no changes in sedimentation coefficient of calcium-dependent antibody were observed in the presence of calcium does not necessarily prove that subtle changes are not taking place at the binding site. Our failure to show any effect of calcium on the purified dependent antibody by two techniques, *viz.*, hydrogen exchange and fluorescence emission spectra, which are sensitive to small local changes in conformation (Page *et al.*, 1967; Han and Benson, 1970) is, however, good evidence that calcium does not affect the antibody.

More convincing, in this connection, are the results of effects of calcium on GAT and related antigens. Although the increase in sedimentation coefficient (due to calcium) for GAT is small, nevertheless, it is significant as is the change in its concentration dependence. For extended polyions sedimentation coefficients are not overly sensitive to minor configurational changes. In all cases, where an antigen elicited calcium-dependent antibody, the intrinsic viscosity data show significant decreases as affected by calcium. These data and the change in sedimentation coefficient of GAT are indicative of a more compact structure due to charge neutralization. Table VII shows a correlation between the change in intrinsic viscosity of those polypeptide antigens we have studied and the respective calcium dependency they induce in sheep antisera. Because the molecular weights of these polypeptides are essentially the same, then by virtue of the relation, $h^3 = [\eta]M/2.1 \times 10^{21}$ (Flory and Fox, 1950), where h is the end-to-end distance of the polymer, the changes in intrinsic viscosity listed in the table can be regarded as relative change in configuration of the polypeptides as affected by calcium.

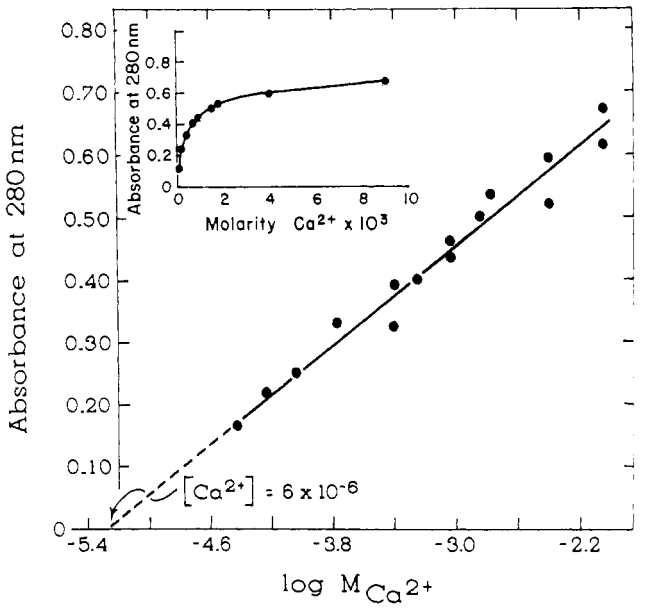

FIGURE 6: The effect of Ca^{2+} concentration on the precipitin reaction of calcium-dependent antibody with GAT at equivalence concentration.

These data strongly suggest that calcium dependency is a function of the ability of calcium to alter the conformation of glutamyl residues in these polypeptides. Thus calcium induces the formation of antigenic determinants which are subsequently recognized by homologous antibody. Additionally, for antigens such as proteins and uncharged or positively charges polypeptides where calcium would have little or no effect on antigen conformation we have never observed calcium dependency in sheep antibodies (Maurer *et al.*, 1970).

The specificity of calcium and other divalent ions for the interaction of calcium-dependent antibody was reported as $Ca^{2+} > Sr^{2+} > Mn^{2+} > Ba^{2+} > Mg^{2+}$ and also it was noted that monovalent cations have no effect (Maurer *et al.*, 1970). Further it was observed that this order correlates with the absolute difference between the anhydrous radius of each cation and that of calcium. As it is well established that ionic site binding to polyions is related to anhydrous cation radii (Strauss, 1962), the above correlation supports our contention that calcium is binding to the glutamyl residues of these antigens. Further, the order of binding for divalent ions to GAT corresponds to the effectiveness of the ions in the GAT–anti-calcium-dependent antibody reaction. (P. A. Liberti, 1970, unpublished observations). A similar order for divalent ion binding to α-polyglutamic acid has been observed by Jacobson (1964). Thus the degree of interaction caused by divalent cations is a function of their binding to antigen which corresponds to the number of determinants formed.

In addition to specific ion effects, we also reported the dependence of antigen–antibody interaction on calcium concentration. These data are shown here in Figure 6, on a semilogarithmic plot (the insert shows the data as originally published). It is noted that the ascending limb of the insert curve has a shape similar to a precipitin curve in the antibody-excess region. Thus the addition of calcium to the system of calcium-dependent antibody and GAT (incomplete antigen) is analogous to adding antigen. Consequently, it follows that if calcium-dependent antibody were mixed with an excess amount of GAT in the absence of calcium, increasing amounts of calcium should result in an increase in precipitate formed to a maximum (as in Figure 6, insert), followed by a decrease, *i.e.*, antigen excess zone. Recent experiments at twice antigen equivalence have substantiated these predictions. Additionally, it should be noted that were calcium exerting its effect by converting antibody into active antibody, under the above conditions, the amount of precipitate formed would increase to a maximum and remain there, since antibody excess could not be achieved.

From the semilogarithmic plot of Figure 6, extrapolation to zero absorbance (precipitin formation) yields a value of 1.2×10^{-5} N Ca^{2+} concentration at the onset of precipitation. For the GAT system this value corresponds to 1 equiv of calcium/80 glutamic acid residues. Since the amount of precipitation plateaus (insert curve) at 0.01 N Ca^{2+}, there are 12 equiv of calcium/glutamic acid residue in the region of maximum interaction. This suggests that on an equivalent basis about 10% of available calcium could be bound to GAT. Thus at the onset of precipitation it would be expected that less than 0.1% of GAT would have a full complement of calcium. Assuming antibody binding to an antigenic determinant involving calcium could stabilize a localized calcium concentration build-up, then less than 0.1% of the system could be interacting. This is consistent with the data and the contention that calcium induces formation of determinants in GAT.

The question of the specific role calcium plays in inducing determinants in GAT and related polypeptides is not easy to answer. From the data on the ability of a specific precipitate of calcium-dependent antibody to bind calcium independent antibody and the reverse situation, it would seem likely that both determinants are on the same antigen molecule (*i.e.*, two populations of GAT do not exist). Whether both determinants are the same (1° amino acid sequence) except that one involves a conformational rearrangement due to calcium is less probable in view of the cross-reaction data with the methylated derivatives. If the determinants were the same except for a calcium rearrangement, it would be reasonable to expect that cross-reactions with both kinds of antibody with the methylated derivatives would decrease in a similar fashion. In fact one might have expected a greater reduction in cross-reaction of the calcium-dependent antibody because methylation of one glutamyl residue may affect *via* Ca^{2+} a second glutamyl residue. Studies by Odstrchel and Maurer (1969) on the tyrosine involvement for the dependent and independent antibodies also suggest the determinants are of different primary amino acid sequence.

The role of Ca^{2+} in inducing changes in secondary structure of GAT and related polypeptides has been studied by physicochemical techniques. Potentiometric titration studies of the effects of divalent ions on polyglutamic acid have led to two opposing conclusions. Jacobson (1964) claims divalent ions *stabilize* helix formation while Kono and Ikegami (1966) contend there are no specific effects. The data we obtain for GA as affected by calcium does show increased helix content and presumably, the effect on GAT would be very similar. Based on one antigenic determinant per 33 residues (4500 molecular weight) for GAT and assuming a determinant of 7 amino acids of most probable composition $\simeq$ 4:2:1 (Glu, Ala, Tyr, respectively), then about 4/20 or 20% of the total glutamic acid could be involved in a determinant. If the calcium-dependency phenomena solely involves a random coil–helix transition of only those glutamyls in the determinant, this would require 20% additional helix content which is not at all consistent with optical rotatory dispersion data where an increase of 6% obtains. Yet the fact that this effect cannot be elicited by monovalent ions indicates that divalent ions

ıteract with at least two glutamyl carboxyls. Thus a likely ɛplanation of this phenomena is that calcium plays its role y bridging two carboxyl groups such that unique local con-ɔrmations are induced but not of a helical nature. Whether ıe uniqueness of this determinant so formed involves one or ıore such calcium interactions is difficult to show.

Despite the fact that the system we have described might be ɛgarded as an immunochemical oddity there are several im-ɔrtant aspects to which attention might be directed. First, ıe system demonstrates that the local environment of the ɔst might alter the structure of the immunogen. Second, and ɛrhaps more importantly, we have described a system which unique in that the antigen–antibody interaction can be ɛgulated simply by the action of small concentrations of cal-um or EDTA. This quality has allowed solution studies of ıe interaction of macromolecular antigen and antibody which ıve heretofore been impractical. Preliminary studies of this ıture have been reported (Liberti *et al.*, 1970).

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