CHARACTERISTICS OF AN IMMUNE SYSTEM COMMON TO CERTAIN EXTERNAL SECRETIONS*

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Plate 2

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It has been demonstrated that γ_1A-globulin is the predominant type of γ-globulin found in human parotid saliva, colostrum, lacrimal secretions (1), and recent evidence suggests nasal and bronchial fluids as well (2–4). The γ_2/γ_1A ratio in this group of fluids is less than 1 whereas in normal human serum this ratio is approximately 6, suggesting that there is preferential secretion of γ_1A-globulin into these body fluids. In an attempt to elucidate the mechanisms involved in the secretion of γ_1A-globulin this protein was isolated from two of these fluids (saliva and colostrum) and some of its properties were compared with those of serum γ_1A. Previous work in this laboratory (5, 6) had suggested that salivary and colostral γ_1A may have a higher sedimentation coefficient than that of the majority of serum γ_1A. The current report contains further chemical and immunological studies on purified preparations which indicate that while salivary and colostral γ_1A are similar to each other, certain of their chemical and immunological properties differ significantly from those of serum γ_1A. Using fluorescent antibody and autoradiographic techniques local synthesis of γ_1A has been shown in the parotid gland. In addition, antibody activity (isohemagglutinins) have been demonstrated in purified preparations of γ_1A derived from these fluids. These studies indicate that the immunoglobulins present in these external secretions differ in chemical and immunological properties from those of serum. The local production of a

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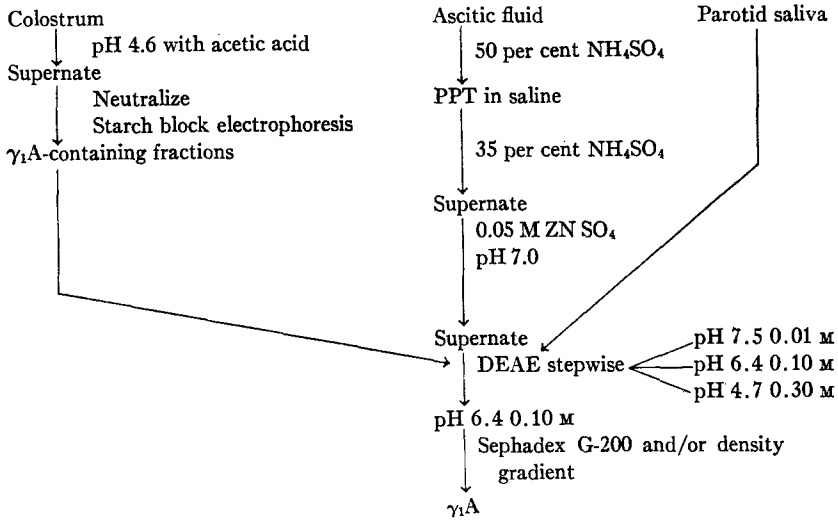
unique type of antibody suggests the existence of an immune system characteristic of certain external secretions whose properties differ, at least in part, from those of the immunological system responsible for the production of classical circulating antibody.

Methods

Collection of Samples.—Parotid fluid was obtained with a Curby parotid cap. Samples of 100 to 200 ml were collected from normal humans and patients with multiple myeloma and concentrated approximately 50-fold by negative pressure dialysis.



TEXT-FIG. 1. Outline of procedure used in the isolation of γ_1A from colostrum, ascitic fluid and parotid saliva.

Parotid fluid samples were obtained from two healthy males whose serum had been found on routine immunoelectrophoresis to lack completely γ_1A. Studies on the sera of these individuals have been recently reported (7). Colostrum was collected with a standard breast pump at the end of the 1st or 2nd postpartum day. The colostral samples were immediately centrifuged in the Spinco model L at 35,000 RPM for 1 hour and the upper fatty layer removed and discarded. Ascitic fluid was collected from three patients with advanced Laennec's cirrhosis by paracentesis. The quantitative determinations for immunoglobulins were performed immediately on the fresh fluids which were then stored at −30°C until used further.

Isolation of γ-Globulin.—The isolation of γ_1A from the various fluids is outlined in schematic form in Text-fig. 1.

Ascitic fluid γ-globulin was precipitated by making the sample 50 per cent saturated with ammonium sulfate. The precipitate was collected, dialyzed against distilled water, and reprecipitated with 35 per cent ammonium sulfate. The supernate of the 35 per cent ammonium sulfate step was dialyzed against saline, made 0.05 M with zinc sulfate, and the pH adjusted to 7 with sodium carbonate as suggested by Heremans (8). After 1 hour at room temperature the solution was filtered through Whatman No. 1 paper and solid ammonium sulfate was added to 50 per cent saturation. The precipitate was removed by centrifugation, dissolved

in distilled water containing 1 per cent EDTA, dialyzed exhaustively against 0.01 M phosphate buffer pH 7.5, and concentrated by negative pressure dialysis. The concentrated sample was then placed on a DEAE cellulose column.

The centrifuged colostrum was adjusted to pH 4.6 with glacial acetic acid and the resulting precipitate (mainly casein) removed by centrifugation. The supernate was immediately adjusted to pH 8.5 with sodium hydroxide, dialyzed against 0.05 M veronal buffer at pH 8.6, and subjected to starch block electrophoresis (9). Following electrophoresis the starch block was cut into ½ inch sections and eluted with phosphate buffer pH 7.4, 0.01 M. The γ_1A-containing fractions of the starch block (tested immunologically with a specific antiserum) were pooled, concentrated by negative pressure dialysis in collodion sacs and placed on a DEAE cellulose column.

DEAE chromatography was performed as previously described (10) using a stepwise gradient of three buffers, (*a*) pH 7.4 0.01 M, (*b*) pH 6.2 0.1 M, (*c*) pH 4.8 0.3 M. In each of the fluids studied the γ_1A was eluted with the pH 6.2 0.1 M buffer, all tubes containing γ_1A were pooled, dialyzed against saline, and concentrated. This material was then placed on a column of sephadex G-200 and eluted with 0.14 M sodium chloride. In some experiments with colostrum the γ_1A-containing tubes of the sephadex gradient were pooled in 3 fractions in order to demonstrate any heterogeneity in the size of the γ_1A. The salivary γ_1A was restricted to a more confined area of the gradient and therefore, only a single pool was made.

Ultracentrifugation.—Analytical ultracentrifugation was carried out in a Spinco model E ultracentrifuge at 52,640 RPM and 20°C. Infinite dilution values were calculated from plots of sedimentation coefficients against concentrations. Protein concentrations were measured by the method of Folin-Ciocalteau or by the Biuret reaction (11). In experiments employing urea as the solvent, the sedimentation constants were corrected to $S^0_{20,w}$ using the equations of Svedberg and Pedersen (12). Solvent viscosities were determined at 20° C in an Oswald capillary viscometer with a water flow time of 110 seconds and densities in a 1 ml A. H. Thomas specific gravity bottle.

Density gradient ultracentrifugation using a 10 to 40 per cent sucrose gradient was performed as previously described (13). In some experiments markers were added of purified preparations of 7S and 19S γ-globulins, the sedimentation characteristics of which had been previously accurately determined in the analytical ultracentrifuge.

Immunology.—Antisera were prepared by immunizing rabbits with subcutaneous injections of antigens in complete Freunds adjuvant at weekly intervals over a period of 2 to 3 months. The antiserum 5L used in many of these studies was prepared by immunization with a purified preparation of 11S colostral γ_1A.

Gel diffusion in agar was performed according to the method described by Ouchterlony (14).

Quantitative determinations of the immunoglobulins were performed on the density gradient and chromatographic fractions according to a method proposed by Heremans which has been previously described (6). In this technique an antiserum specific for the component to be quantitated is mixed with 1 per cent agar and poured into Ouchterlony plates. The test antigen is placed in the well and after incubation for 24 hours at 37°C the diameter of the resulting ring is measured. The concentration of antigen is determined by reference to a standard curve relating ring diameters to concentrations. The preparation of the purified antigens used as standards and of the antisera have been previously described in detail (6).

Dissociation Experiments.—Disulfide reduction was performed as routine using 0.2 M beta mercaptoethanol (βME) in phosphate buffer at pH 7.4. Reduction was allowed to proceed at room temperature for 6 hours. In some experiments incubation at room temperature was carried out for periods of 24 to 48 hours and also following addition of 0.4 M βME in order to determine whether any further reduction occurred. Following reduction the liberated SH

groups were blocked with 0.3 M iodoacetamide. In some experiments the reduced and alkylated proteins were dialyzed against freshly prepared urea (Baker analyzed) for 2 days at 4°C. Enzymatic digestion with papain and pepsin were performed as previously described (15).

Carbohydrate Determinations.—Hexose sugars were determined colorimetrically using the anthrone reaction according to Mokrasch (16). The sialic acid determinations were carried out by means of the thiobarbituric acid method as described by Warren (17) after hydrolysis in $\frac{1}{10}$ normal H_2SO_4 at 80°C. Standard curves were prepared using acetyl neuraminic acid obtained from Worthington Biochemicals Corp., Freehold, New Jersey. Removal of sialic acid from γ_1A was accomplished by using a neuraminidase preparation obtained from the Behringwerke Company (Marburg-Lahn, Germany). Following concentration (50-fold) the enzyme preparations contained approximately 50 units of neuraminidase activity per cc. The activity of the enzyme preparations was tested periodically by determining their effect on the mobility of a purified transferrin preparation.

Electrophoresis.—Immunoelectrophoresis was performed by the micromethod of Scheidegger (18) with 2 per cent agar in 0.05 M barbital buffer at pH 8.2. Starch block electrophoresis was performed in veronal buffer pH 8.6, 0.05 μ as previously described (9).

Fluorescent Antibody Studies.—The fluorescent antibody technique (19) was employed in the study of parotid tissue sections. Antisera to colostral 11S γ_1A (5L) and serum 7S γ_1A were conjugated with fluorescein isothiocyanate (20). The conjugated 5L antiserum was also absorbed with normal serum, thus making it specific for 11S γ_1A. Normal parotid tissue was obtained during surgery and snap frozen in a dry ice alcohol mixture. Sections were cut at 4 to 6 μ and the sections either washed or fixed in acetone before staining with the conjugated antisera. Fluorescein conjugated antisera to 7S γ_2-globulin and other serum proteins served as controls for specificity.

I^{131}-Labeling Studies (21).—γ_1A-Globulin was isolated from the serum of a patient with a discrete (monoclonal) 7S γ_1A-globulin peak by pevikon block electrophoresis (22). The particular protein fraction eluted from the block which was chosen for labeling was shown to contain only γ_1A-globulin by immunoelectrophoresis employing a polyvalent γ-globulin antiserum, a whole human serum antiserum, as well as a specific γ_1A-globulin antiserum. The protein was trace-labeled with I^{131} by the iodine monochloride method of McFarlane (23). After passage through an ion exchange resin and dialysis against saline, the final product contained less than 2 per cent free I^{131}. When the labeled protein was added to the donor's serum, radioimmunoelectrophoresis and starch gel electrophoresis showed the radioactivity to reside exclusively in the γ_1A-globulin band. Density gradient centrifugation of the labeled preparation showed that the distribution of radioactivity coincided almost exactly with the protein concentration in the 7S region of the gradient. The iodinated protein was passed through a 0.22 μ Millipore filter and 35 mg of sterile human albumin was added per ml as carrier protein.

Lugol's solution or tapazole was given to each subject prior to and throughout the course of the study to inhibit the thyroidal uptake of radioiodine. Each subject received 20 μc, 1 mg of γ_1A-globulin–I^{131} as an intravenous injection. Five subjects, one normal and four with various diseases, received the labeled protein. In all cases the preinjection saliva samples were normal in immunoglobulin content. A serum sample was obtained 15 minutes after injection, daily for 4 days, and then every 48 hours for a 2 week period. Complete daily urine collections were also obtained for this period. Three 48 hour stool collections were also obtained from each subject. Parotid secretions and/or whole saliva was obtained prior to injection of the iodinated protein and then daily until no significant radioactivity could be detected. In addition, four of the subjects received normal 7S γ-globulin prepared by DEAE-sephadex chromatography and labeled with I^{125}.

Serum, urine, and saliva samples were placed in glass screw-top tubes and were counted

with an appropriate standard in an automatic well type gamma scintillation counter with a thallium-activated sodium iodide crystal. Stools were collected in paint cans, diluted to a constant volume, homogenized with a paint can shaker, and counted with a standard on a gamma scintillation counter. Less than 3 per cent of the retained I^{131} activity appeared in the stools per day. The protein-bound radioactivity in parotid secretions or whole saliva was determined by addition of 3 ml of 5 per cent albumin to 1 ml of saliva and precipitation with 1 ml of 72 per cent perchloric acid. Aliquots of saliva were dialyzed exhaustively against saline and the residual radioactivity determined. Specific antiserum to γ_1A-globulins was also added to saliva samples in order to precipitate any labeled protein present. In addition density gradient ultracentrifugation was performed on two saliva samples and the distribution of radioactivity in the gradient compared with that of γ_1A (determined immunologically).

Immunoelectrophoresis Autoradiography.—Cultures were made in roller tubes containing tissue fragments (about 50 mg) in 2 ml of a medium to which had been added 2 μc/ml of C^{14}-labeled L-lysine and L-isoleucine. The details of the preparation of the medium are the same as those described by Hochwald *et al.* (24). Following incubation at 37°C for 48 hours the tissues were frozen and thawed 3 times, homogenized in a teflon homogenizer, and the supernatant fluid concentrated 4-fold by negative pressure dialysis. The concentrated culture fluids were subjected to immunoelectrophoresis with normal human serum or an isolated salivary 11S γ_1A-preparation as a carrier. Each slide was run in triplicate and following drying and staining exposed to x-ray film for periods of 2, 4, and 8 weeks. Antisera used included anti-whole serum, specific anti-γ_1A-antiserum, and antiserum against 11S colostral γ_1A (5L), both unabsorbed and absorbed with normal serum.

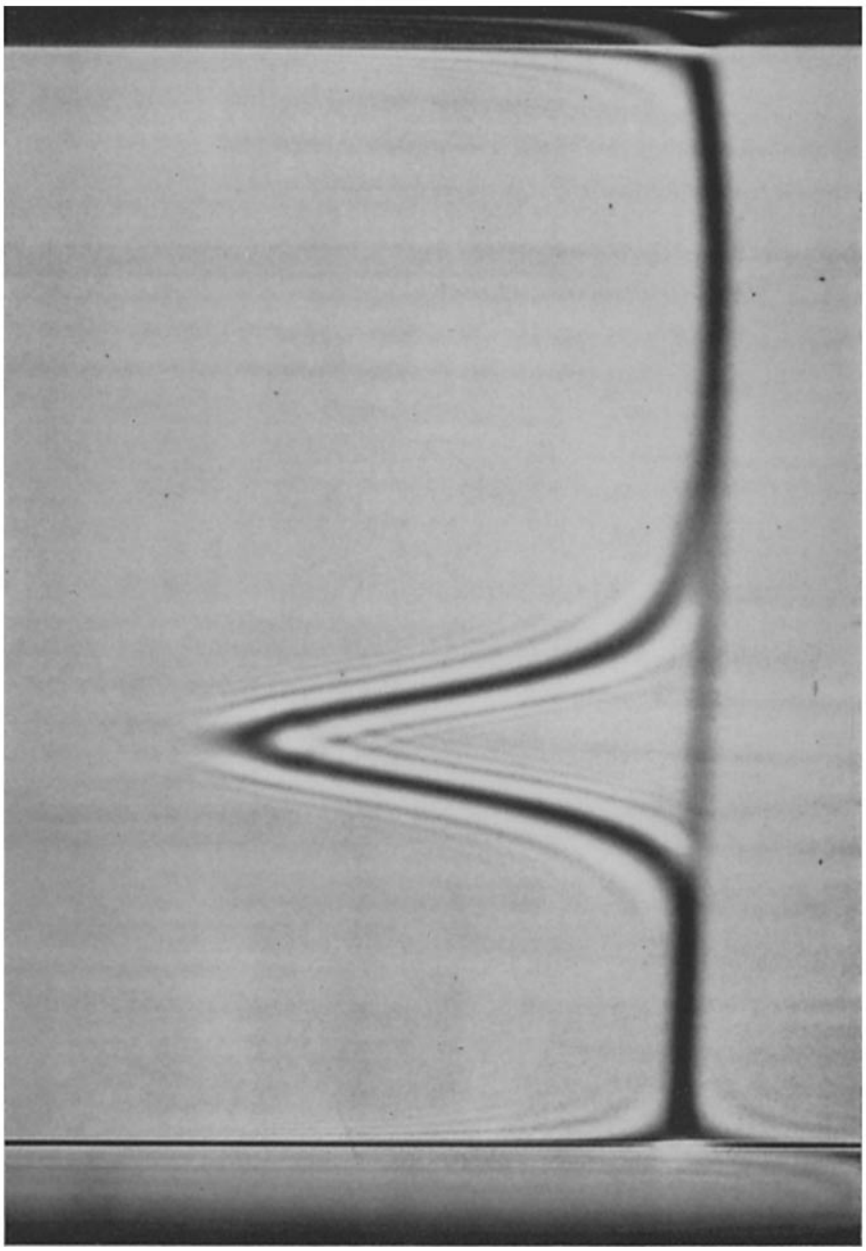
Isohemagglutinin Activity.—Anti-A and anti-B titers were determined on saliva and serum density gradient fractions in the usual manner (11). In some experiments the type(s) of γ-globulin responsible for the hemagglutination were determined by absorption experiments performed in the following manner: Antisera specific for 7S, γ_1A, and γ_1M were fractionated by starch block electrophoresis and the γ-globulin isolated. The antibody containing fractions were concentrated, absorbed with A and B cells, and incubated for 1 hour at room temperature with the serum or salivary density gradient fractions under investigation. The anti-A and anti-B titers were then determined and compared with controls in which saline replaced the antiserum.

Anti-B isohemagglutinins were obtained from a serum having high titers of anti-B antibodies by dissolving the specific precipitate in excess B substance. The antibodies in this serum have been previously shown to be almost entirely of the γ_1A-type and were intermediate (approximately 11S) in sedimentation (25).

RESULTS

Serum and Ascitic Fluid.—There is evidence which suggests that some of the γ_1A-globulin in normal human serum is present in the form of polymers (26). However, density gradient ultracentrifugation followed by quantitative determination of γ_1A on the fractions of three normal sera and the sera from three patients with Laennec's cirrhosis revealed that over 90 per cent of the γ_1A was present in 7S form.

Analysis of ascitic fluid of patients with Laennec's cirrhosis were found to contain γ_2- and γ_1A-globulins in approximately the same ratios as they are present in the patient's serum. Preparation of γ_1A isolated from ascitic fluid were homogeneous both electrophoretically and in the ultracentrifuge. The sedimentation coefficient of the protein shown in Text-fig. 2 was $S^0_{20,w} = 6.9$.



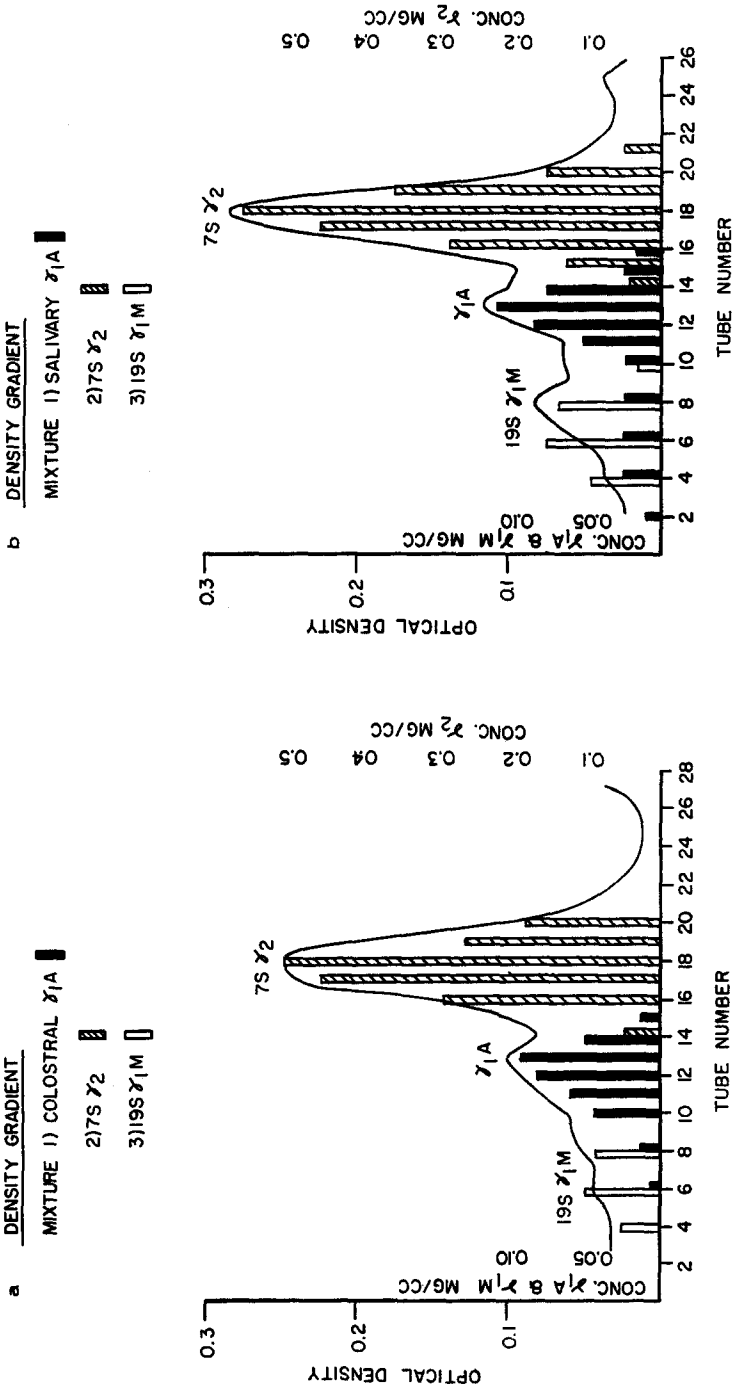
TEXT-FIG. 2. Ultracentrifuge pattern of ascitic fluid γ_1A. Photograph taken 40 minutes after reaching full speed. Sedimentation is from left to right $S^0_{20,w} = 6.9S$

This preparation showed a single band in the γ_1A-area on immunoelectrophoresis although trace amounts of 7S γ_2 were detectable by Ouchterlony analysis. In view of these findings ascitic fluid was used as a ready source of large quantities of γ_1A- and the γ_1A-globulins derived from it were considered representative of the major type (7S) of γ_1A present in serum.

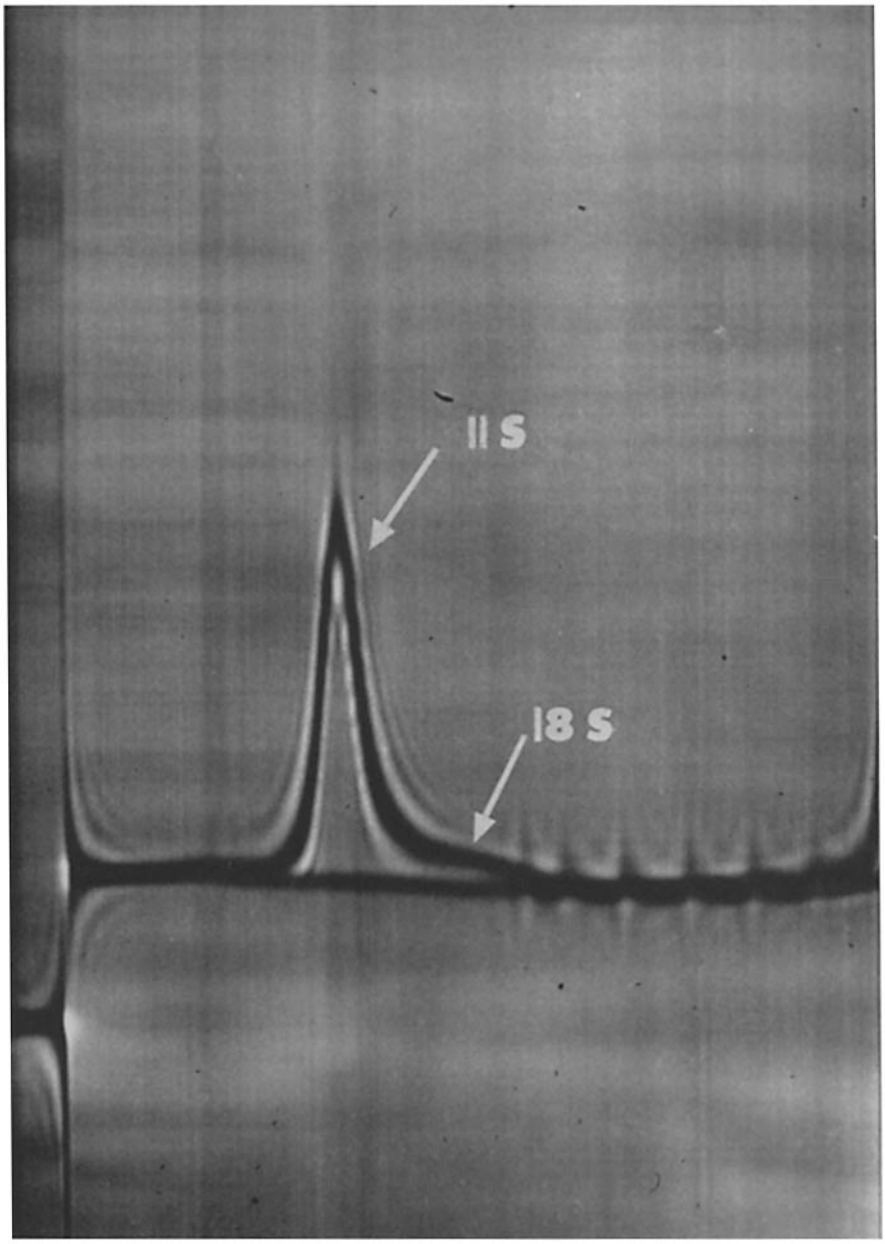
Colostrum.—In the final step in the fractionation of colostrum on sephadex G-200 the γ_1A-containing eluate was divided into three fractions in order to demonstrate any heterogeneity in the size of the colostral γ_1A. Density gradient ultracentrifugation was performed on each of the three fractions and the concentration of γ_1A in the various portions of the gradient determined. The first fraction eluted contains approximately 60 per cent 11S and 40 per cent 18S γ_1A. Fraction II contains about 90 per cent 11S and 10 per cent 18S γ_1A (see Text-fig. 3 *a*). Fraction III contains approximately 75 per cent 7S and 25 per cent 11S γ_1A. From these figures and the protein contents of the fractions it can be estimated that approximately 60 per cent of the γ_1A in the original sample is 11S, 20 per cent 18S, and 20 per cent 7S. Colostral samples from several other individuals showed some variations in the relative amounts of the various polymers but in all cases the 11S was the major component.

Saliva.—The ultracentrifuge pattern obtained on parotid saliva contained two peaks, a major component having an $S^0_{20,w}$ of 11.4S and a minor component usually consisting of from 5 to 10 per cent of the protein with a sedimentation coefficient of approximately 18S (Text-fig. 4). The following evidence was obtained indicating that both the 11S and the 18S components were γ_1A: (*a*) By careful quantitation of the γ_1A it could be demonstrated that the areas under the ultracentrifugal peaks could be explained quantitatively only if both the 11S and 18S components were γ_1A. (*b*) Density gradient ultracentrifugation using markers of known molecular size shows (Text-fig. 3 b) that the major peak of γ_1A is in a position in the gradient consistent with a molecule having a sedimentation coefficient of 11S. Some "tailing" of the γ_1A-concentration towards the bottom of the gradient in Text-fig. 3 *b* probably represents the 18S component. (*c*) Samples of saliva were obtained from two normal individuals whose sera completely lacked γ_1A. The ultracentrifuge patterns of one of these salivas is shown in the lower frame of Text-fig. 5. It can be seen to lack both the 11S and 18S components, which are normally present in concentrated saliva (upper frame, Text-fig. 5).

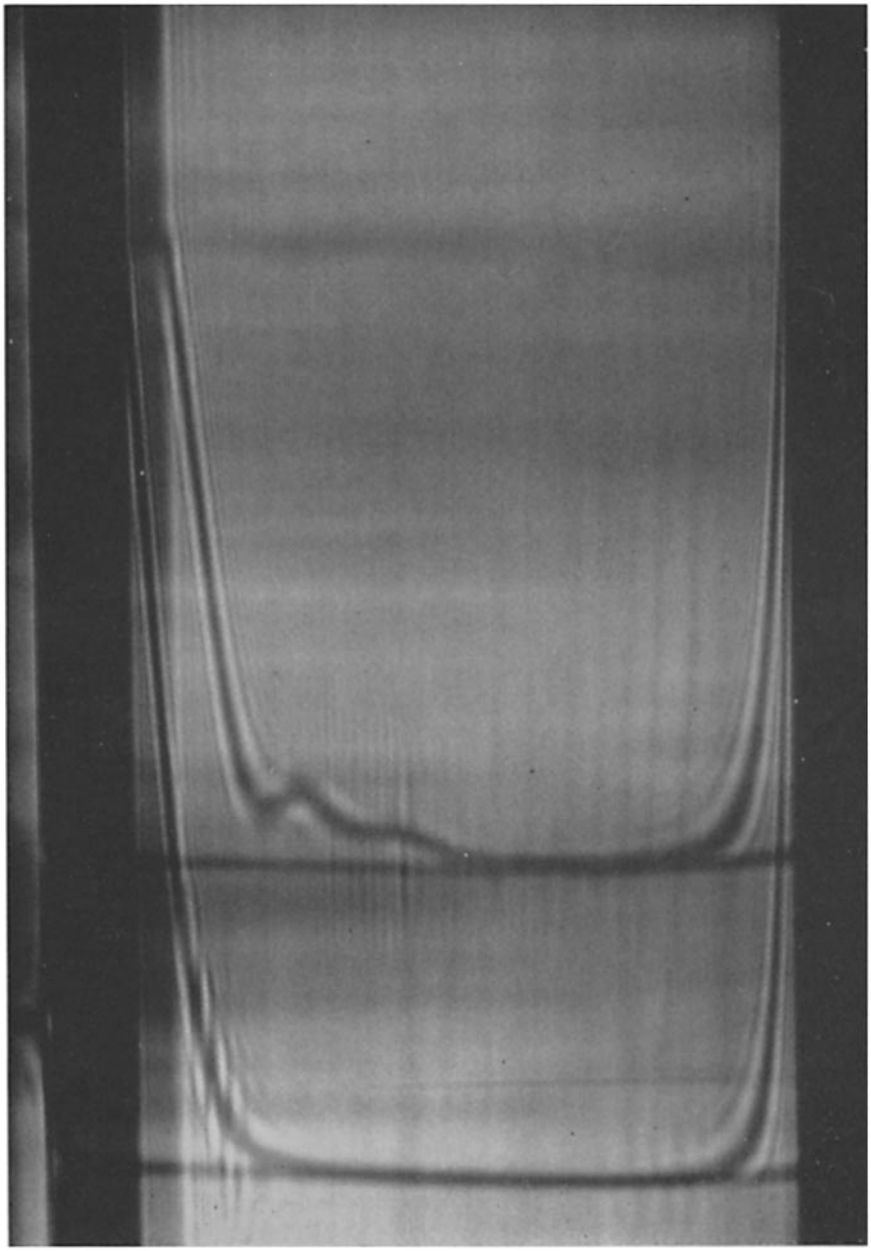
Comparison of Some Chemical Properties of Salivary and Colostral 11S γ_1A with Those of Serum γ_1A.—Some of the properties of salivary and colostral γ_1A compared with those of serum are summarized in Table I. The electrophoretic mobility of salivary and colostral γ_1A is slightly slower than that of serum γ_1A. This finding is consistent with the lower sialic acid content of the 11S γ_1A. Treatment with neuraminidase significantly slowed the mobility of the serum and also the salivary and colostral γ_1A indicating the removal of



TEXT-FIG. 3. Density gradient ultracentrifugation of mixtures of colostral (3 *a*) and salivary (3 *b*) γ_1A with markers of known s rates. On the left ordinate is protein concentration expressed in units of optical density and on the smaller scale the concentration of γ_1A and γ_1M in mg/cc. The colostral sample used was fraction II obtained from the sephadex gradient (see Methods) and the salivary sample used was the preparation shown in Text-fig. 4. The figure illustrates the distribution of the γ_1A primarily in the intermediate regions of the gradients.



TEXT-FIG. 4. Ultracentrifuge pattern of salivary γ_1A. Photograph taken 40 minutes after reaching full speed. Sedimentation from left to right. Major peak has $S^0_{20,w}$ = 11.4S, minor peak is approximately 18S.



TEXT-FIG. 5. The ultracentrifuge pattern of concentrated parotid saliva from normal subject (upper frame) and patient lacking γ_1A (lower frame). Photograph taken 24 minutes after reaching full speed. Sedimentation from left to right. Protein concentration of both samples was 35 mg/cc. Pattern shows the absence of both the 11S and 18S components in the saliva of the individual lacking γ_1A.

at least a part of the sialic acid. Similar results were obtained with three isolated γ_1A-myeloma proteins.

The 11S components in saliva and colostrum behaved identically in all of the studies to be described. The 11S γ_1A, like the 7S γ_1A derived from ascitic fluid, did not dissociate in disulfide-reducing agents, such as beta mercaptoethanol, even following exposure to concentrations as high as 0.4 M for 48 hours. However, following treatment of the reduced protein with 2 M urea partial dissociation and with 4 M urea complete dissociation occurred to 3.5S units. When both the 7S (serum) and 11S (salivary and colostral) γ_1A are reduced and subsequently alkylated with 0.3 M iodoacetamide dissociation occurs. The product, although showing considerable heterogeneity in the ultracentrifuge, has a major component of 3.5S. If the reduced and alkylated preparations are treated with 8 M urea, a single 3.5S peak results. Treatment of 7S and 11S γ_1A with papain in the presence of 0.01 M cysteine resulted in units of 3.7S. Products of similar s rate are obtained with pepsin at pH 4.4 in the presence of 0.02 M cysteine.

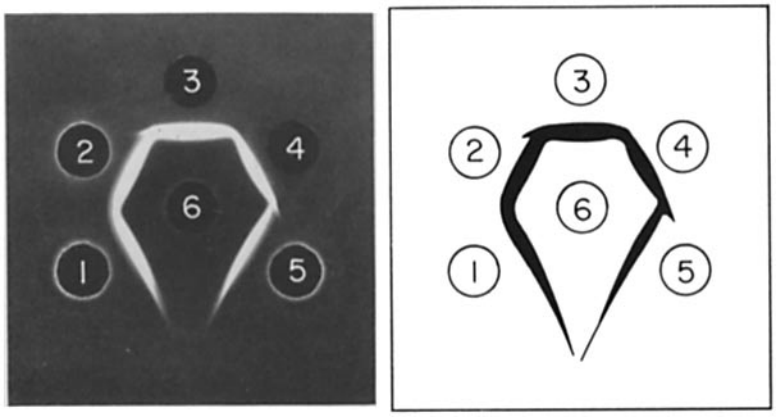
TABLE I

Comparison of Some Properties of γ_1A from Serum and Secretions

	Ascitic fluid (serum) γ_1 A	Salivary colostral γ_1 A
$S^0_{20,w}$	6.9	11.4
Hexose, per cent	5.0	6.2
Sialic, per cent	1.7	1.0
Gm	−	−
Inv	+	+
Groups I and II	+	+
S-S reduction	−	−
Reduction + alk. + urea	3.5S	3.5S

Immunological Properties of Salivary and Colostral 11S γ_1A.—Using an antiserum made against serum, (ascitic fluid) γ_1A, and four different antisera against isolated γ_1A-myeloma proteins, no immunological differences could be detected between serum, saliva, and colostrum. However, using an antiserum made against 11S γ_1A derived from saliva or colostrum (antiserum 5L) immunological differences could be detected. As shown in Text-fig. 6 spurring of the colostral and salivary γ_1A over serum indicates the presence in the 11S γ_1A of antigenic determinants not present in serum. Following absorption of antiserum 5L with normal serum activity remains against saliva and colostrum. The absorbed antiserum failed to react with six normal sera, five γ_1A-myeloma sera and the sera from two patients with Sjögrens syndrome, having large amounts of γ_1A with a broad range of electrophoretic mobilities. Antigenic

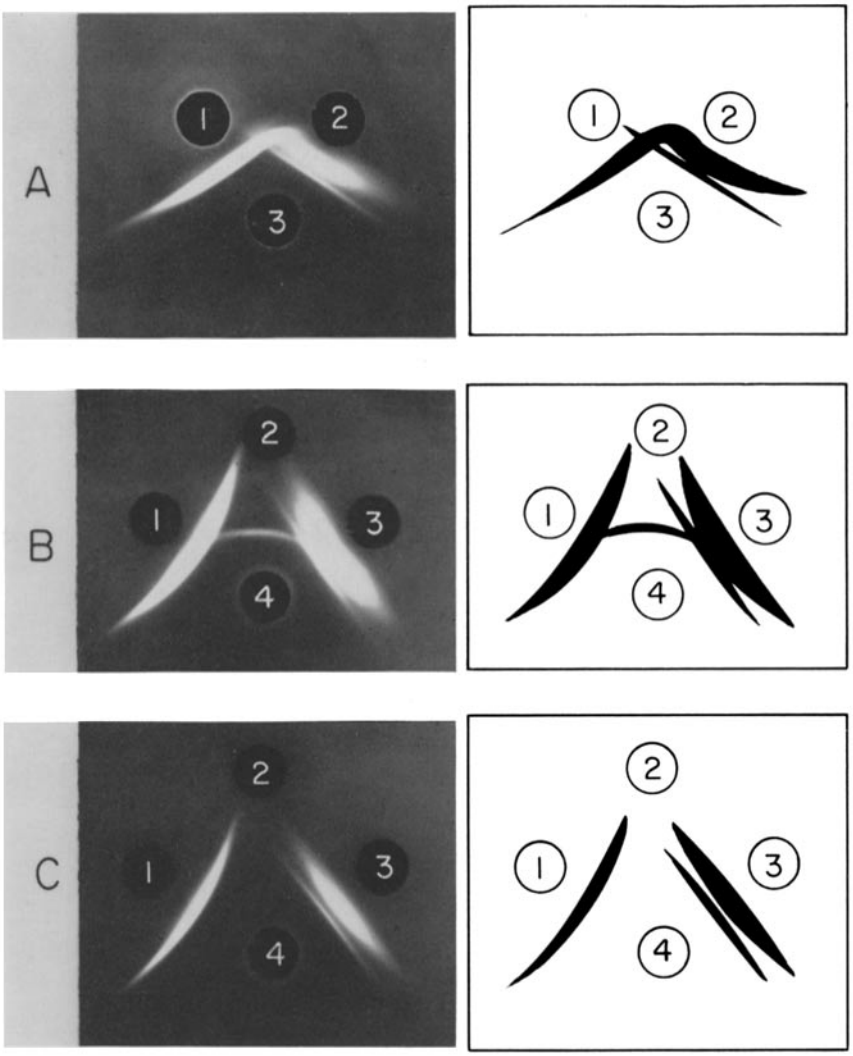
specificity could also be demonstrated in the reduced and alkylated 11S as shown in Text-fig. 7 *A*. The precipitin line nearest well *2* is due to the reaction of antibody with determinants shared by 7S and 11S γ_1A while the inner sharp line is unique to 11S γ_1A as shown by the spurring over the serum. The unabsorbed antiserum 5L gives a single precipitin line with the isolated 11S γ_1A as shown in Text-fig. 7 *B*. Following absorption with fraction II gamma globulin antibodies reacting with L chains are removed, as indicated by the lack of reactivity with the Bence Jones protein, but two precipitin bands still remain with the reduced and alkylated 11S preparation (Text-fig. 7 *C*).



TEXT-FIG. 6. Ouchterlony plate illustrating the immunological specificity of salivary and colostral 11S γ_1A. Well *1*, normal human serum; *2*, ascitic fluid 7S γ_1A; *3*, salivary 11S γ_1A; *4*, colostral 11S γ_1A; *5*, normal human serum; *6*, anticolostral 11S γ_1A.

Evidence that the specificity of the salivary and colostral γ_1A is associated only with the higher polymers present in saliva and colostrum is suggested by the immunological studies on the γ_1A fractions derived from colostrum. Text-fig. 8 shows that while the 11S and 18S γ_1A-polymers are immunologically identical both have been shown to spur over the 7S colostral γ_1A. The inner faint band near well *1* is due to slight contamination of the 7S preparation with 11S γ_1A. No immunological differences have as yet been detected between 7S colostral and 7S serum γ_1A. The γ_1A-polymers present in serum lack the specificity of the salivary and colostral polymers since the 11S γ_1A from colostrum spurred over 4 γ_1A myeloma polymers (s rates 9S to 12S) and a preparation containing an 11S γ_1A anti-B antibody isolated from serum by specific precipitation with B substance (see Methods).

Fluorescent Antibody Localization of Salivary γ_1*A.*—When fluorescein-conjugated anti-11S γ_1A (antiserum 5L) was applied to parotid tissue sections, two types of staining were seen. Cells showing striking cytoplasmic fluorescence



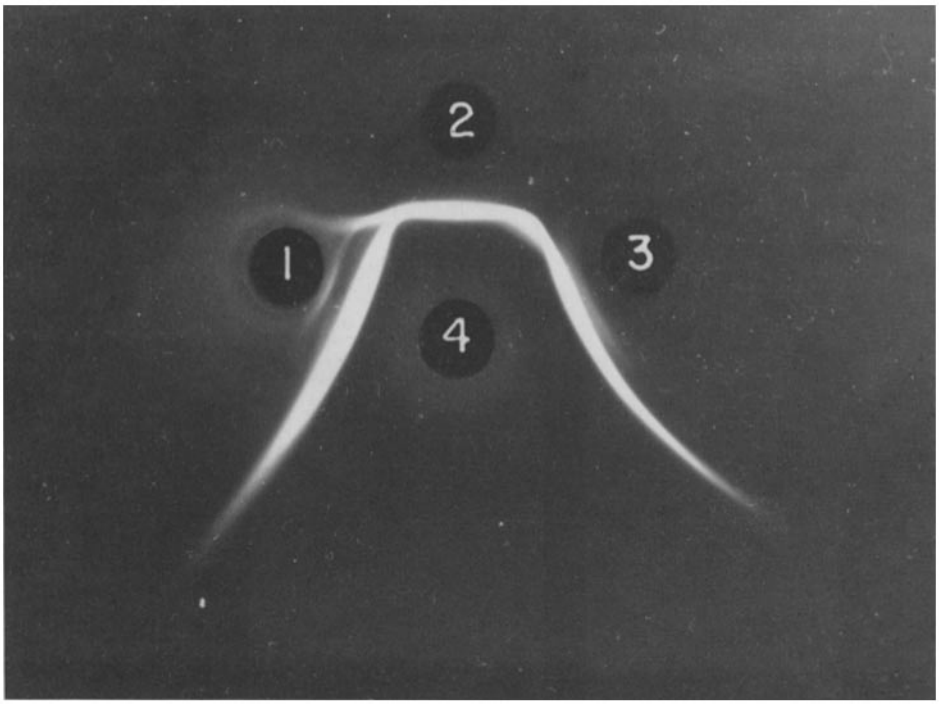
TEXT-FIG. 7. Ouchterlony plate showing the specific component of the 11S colostral γ_1A in the reduced and alkylated protein.

A. Well *1*, normal human serum; *2*, reduced and alkylated colostral 11S γ_1A; *3*, anticolostral γ_1A absorbed with fraction II γ-globulin.

B. Well *1*, colostral 11S γ_1A; *2*, group II Bence Jones protein; *3*, reduced and alkylated colostral 11S γ_1A; *4*, anticolostral γ_1A unabsorbed.

C. Well *1*, colostral 11S γ_1A; *2*, group II Bence Jones protein; *3*, reduced and alkylated 11S γ_1A; *4*, anticolostral γ_1A absorbed with fraction II γ-globulin.

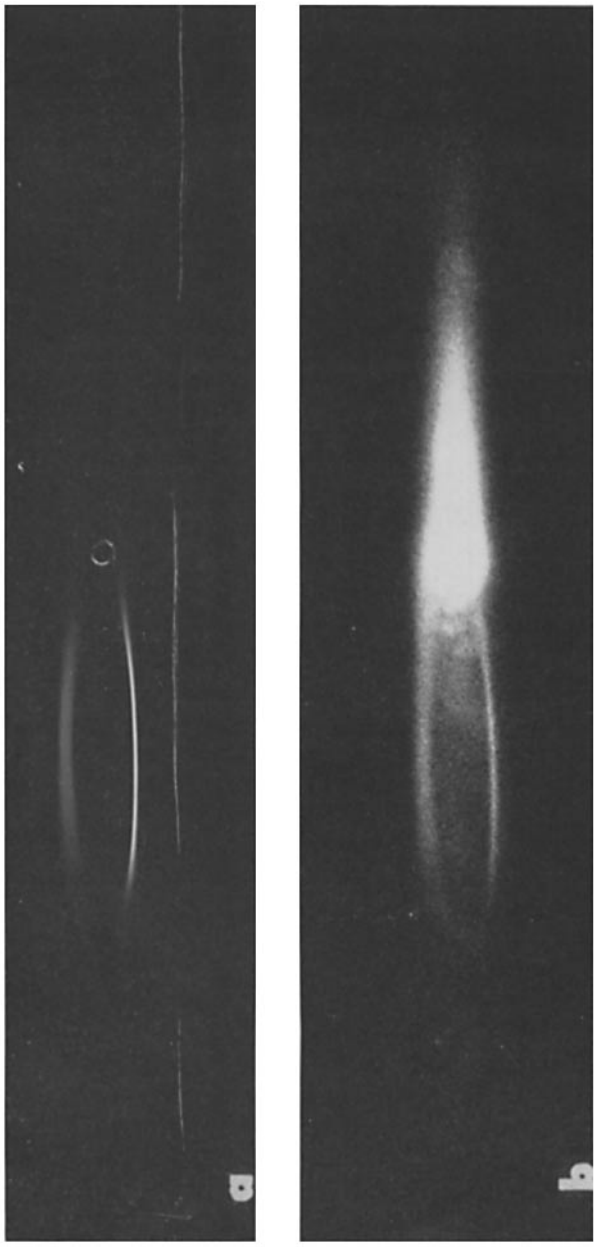
were seen in the interstitial tissue between the acini. Some of these cells contained large amounts of cytoplasm and had eccentric nuclei (Figs. 1 *a* and 1 *c*) and were probably plasma cells. Other interstitial cells also showing cytoplasmic fluorescence could not be definitely characterized. The second type of tissue staining present in the cytoplasm of the epithelial cells was scattered randomly



TEXT-FIG. 8. Immunological relationships of the γ_1A polymers isolated from colostrum by the procedures outlined in Text-fig. 1. Well *1*, 7S colostral γ_1A. This preparation contains small amounts of 11S γ_1A as evidenced by the faint inner line nearest well *1*. *2*, 11S colostral γ_1A; *3*, 18S colostral γ_1A; *4*, anticolostral γ_1A unabsorbed. The 11S and 18S γ_1A-fractions show immunological identity and the 11S spurs over the 7S γ_1A.

and was not uniform except in the acini adjacent to the ductules (Figs. 1 *c* and 1 *f*). In this region, there was uniform fluorescence of almost all glandular cytoplasm.

When antiserum 5L was absorbed with normal human serum staining of the interstitial cells was entirely removed, leaving only the glandular cytoplasmic staining. Further evidence that the glandular cytoplasmic stain was specific for salivary 11S γ_1A was provided by the conjugate of antiserum against serum 7S γ_1A. With the latter conjugate, there was staining of the interstitial



TEXT-FIG. 9. Immunoelectrophoresis radioautography of parotid tissue culture fluid.

A. Immunoelectrophoresis of parotid tissue culture fluid. Center well contains culture fluid to which isolated salivary 11S γ_1A has been added as a carrier. Upper trough contains antisera against 11S γ_1A, lower trough contains antisera against serum γ_1A.

B. Radioautograph of *A*.

cell cytoplasm but no staining of glandular acinar cells. The fluorescein-conjugated 7S γ_2-antiserum showed no staining of parotid tissue.

I^{131}-Labeled γ_1A studies.—The metabolic behavior of the I^{131}-γ_1A-preparation will be discussed elsewhere (21). Briefly, the mean biological survival (T ½) was 6 days and 42 per cent of the total body γ_1A-globulin was calculated to be in the intravascular compartment. The plasma radioactivity was almost completely protein-precipitable, whereas the urinary radioactivity was all free I^{131}.

Radioactivity appeared in the saliva in significant amounts beginning about 15 minutes following the injection of the I^{131} γ_1A. The counts per milliliter of

TABLE II

Effect on Anti-B Agglutinins after Absorption with Specific Antisera

Sample	Saline control	Prior absorption with		
		Anti-γ_1 A	Anti-7S	Anti-γ_1 M
L. T. saliva	3+	0	2+	3+
J. C. saliva	3	Tr.	3	3
D. D. saliva	4	0	1+	4
L. C. saliva	4	0	1+	4
S. Z. colostrum	3+	0	3+	3+
L. D. colostrum	4	0	3	4
L. H. serum*	3	2	2+	0

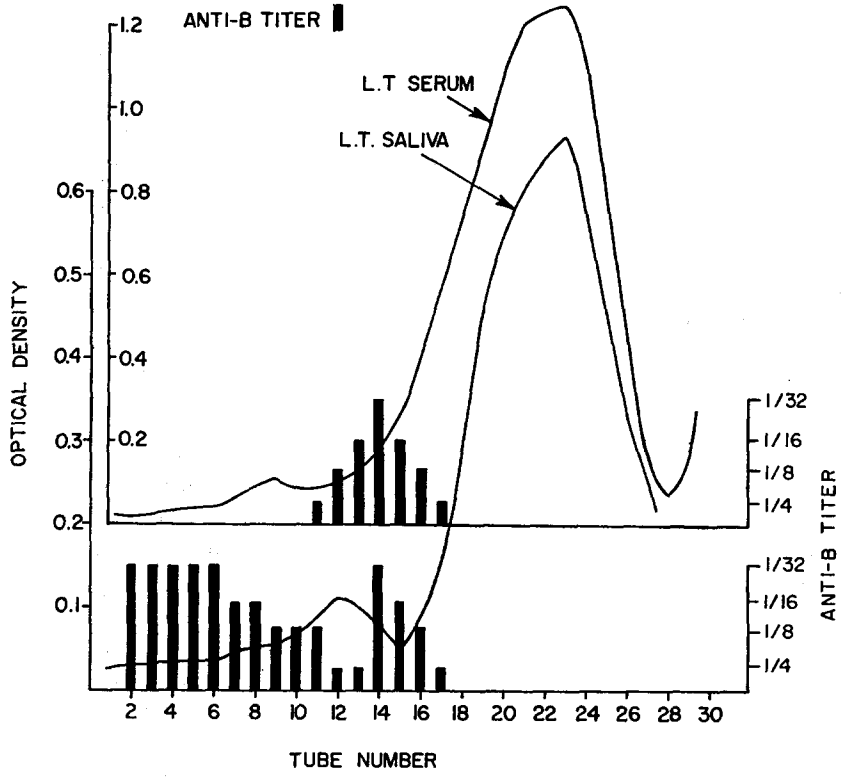
* Serum completely lacked γ_1A; agglutinins found only in 19S region on density gradient ultracentrifugation.

saliva increased rapidly to a maximum at 3 days and then slowly decreased, becoming negligible after 2 weeks. The following evidence was obtained indicating that the radioactivity in saliva was not bound to protein: (*a*) In all samples obtained during the study no significant radioactivity was found in the protein fraction precipitated by perchloric acid. (*b*) Using a specific anti-γ_1A-antisera no radioactivity was detected in the washed precipitate of four saliva samples. (*c*) Radioactivity was entirely dialyzable in six samples obtained at various time intervals after injections. (*d*) Density gradient ultracentrifugation showed that the I^{131} was primarily restricted to the top tubes of the gradient. The small number of counts found in the γ_1A-containing area of the gradient (less than 5 per cent of the total) were completely dialyzable.

These studies suggest that the radioactivity present in the saliva was associated with free iodine or with small dialyzable molecules or fragments of molecules. Thus no evidence of transport of intact γ_1A from serum to saliva was obtained. Similar studies performed in four subjects using I^{125}-labeled 7S γ_2 likewise showed no evidence of transport of the γ_2-molecule.

Immunoelectrophoresis Autoradiography.—Text-fig. 9 shows the *in vitro* incorporation of labeled amino acids into the γ_1A by normal parotid tissue. In the slides incubated for 2 weeks a prominent γ_1A-band and a single α-globulin band were visible on the radioautographs. In slides incubated for longer periods there was additional labeling of several α- and β-globulin bands. In all slides the γ_1A was most prominent and evidence of trace amounts of 7S γ-formation was found only after 8 weeks exposure to x-ray film. Whether the several bands in addition to the γ_1A which were labeled represent the synthesis of

ANTI-B TITERS ON DENSITY GRADIENT FRACTIONS OF NORMAL SERUM AND SALIVA FROM SAME INDIVIDUAL (L.T.)



TEXT-FIG. 10. Density gradient ultracentrifugation of serum and saliva from normal subject L.T. Anti-B hemagglutination titers (black bars) on right ordinate and protein concentration expressed as OD on the left ordinate. The anti-B agglutinins are distributed in the intermediate portions of the gradient in the serum and in both the intermediate and "19S" regions in the saliva.

other serum proteins or the binding by the serum proteins added as carriers of labeled material synthesized by parotid tissue has not been elucidated.

Antibody Activity in Saliva and Colostrum.—Anti-A and Anti-B antibodies were demonstrable in the saliva of individuals of appropriate blood groups. Colostrum also contained isohemagglutinins sometimes in very high titers (*e.g.* 1:8000), often exceeding markedly the serum titers.

Table II illustrates the effects of prior absorption with an antiserum specific for each of the types of immunoglobulins. The anti-B antibodies in whole

TABLE III

Parotid Saliva from γ_1A-Myelomas

Patient	S Coefficient of myeloma protein	γ_1 A per cent of total protein
N. C.	7	3.5
S. S.	7	2.8
S. W.	7	3.4
M. C.	7	1.1
L. S.	7	1.7
J. M.	7	1.4
M. S.	11	1.2
A. V.	11	1.3
Average		2.0
Average, normal (18 cases)		1.9
Range, normal (18 cases)		0.8–3.0

saliva and in isolated γ_1A preparations from colostrum were inactivated by prior absorption with an anti-γ_1A-antiserum but not by an anti-7S or anti-γ_1M. Density gradient ultracentrifugation of several salivas showing high titers of anti-B showed that activity was present only in the intermediate portions of the gradient following closely the distribution of the γ_1A. However, as shown in Text-fig. 10, in a few salivas anti-B activity was found in both the 19S and intermediate portions of a density gradient whereas the serum contained only intermediate sedimenting antibody. In this experiment the whole concentrated saliva contained no demonstrable γ_1M and the bottom fractions of the gradient were inhibited by prior incubation only with an anti-γ_1A-antiserum and not by incubation with an anti-7S or anti-γ_1M-antisera. In this case therefore the antibody activity in the bottom fractions of the gradient was associated with the higher γ_1A-polymers. Similar results have been obtained with several other salivas.

Myeloma Proteins in Saliva.—The occurrence of myeloma proteins in the

salivas of eight patients with γ_1A-myeloma were studied (Table III). In two patients the predominate serum protein was 11S and in six patients it was 7S, although polymers of other sizes were often present in smaller amounts. In none of the cases was the salivary γ_1A-content markedly elevated despite high serum concentrations (30 to 80 per cent of total protein as determined by paper electrophoresis). In two cases, however, the γ_1A-content was slightly above the normal range. In one of these density gradient ultracentrifugation showed that the salivary γ_1A was predominantly 11S and possessed typical salivary type specificity whereas the myeloma protein was 7S. The salivas of both patients with 11S myeloma proteins had normal γ_1A-concentrations.

DISCUSSION

There are several possible explanations for the selective occurrence of γ_1A in body fluids: (*a*) Simple filtration of serum proteins with subsequent reabsorption of γ_2 and most of the other proteins with the exception of γ_1A. This mechanism although unlikely cannot be excluded with presently available information. Such a mechanism would be analogous to the filtration which occurs at the renal glomerulus with subsequent reabsorption of much of the filtrate by the renal tubules. In order to directly demonstrate reabsorption it would be necessary to obtain fluid (by micropuncture) from various segments of the ductules which drain the glandular acini. (*b*) Transudation or secretion of serum proteins with selective degradation by proteolytic enzymes present in these fluids, the γ_1A being more resistant to proteolysis than the other proteins. This mechanism can be excluded only in the case of parotid saliva. Previous work indicates that the parotid fluid lacks proteolytic activity (27). Addition of 7S γ_2 to parotid fluid with subsequent incubation did not result in significant degradation of this protein. Moreover, no difference could be demonstrated in the susceptibility of γ_1A as compared to γ_2 to proteolysis by pepsin and papain. (*c*) Active transport of γ_1A from serum to fluid. (*d*) Local synthesis of γ_1A by the corresponding gland. These latter two mechanisms are the most likely and are discussed in some detail below.

Since the vast majority of the γ_1A in serum is 7S and the γ_1A in these secretions is present largely as 11S or higher polymers it is obvious that the serum 7S γ_1A is not secreted unaltered. The failure of the γ_1A-myeloma proteins and of the I^{131}-labeled γ_1A to appear in saliva in significant amounts is also against simple transport of serum γ_1A. There are three possible mechanisms of secretion which are consistent with our experimental findings. It is known that there are small amounts of polymeric γ_1A present in normal serum (26). Moreover, Kunkel and Rockey (28) have shown that the blood group isohemagglutinins are in part γ_1A of intermediate (approximately 11S) size. It is possible therefore that there is selective transport of this minor polymeric form of serum γ_1A by the glandular epithelium. Contrary to this, however, is

the finding that the 11S salivary and colostral γ_1A are immunologically specific and did not show identity with any of three γ_1A-myeloma polymers (also approximately 11S) or with an intermediate sedimenting γ_1A-isohemagglutinin from serum. The serum γ_1A-polymers unlike the salivary and colostral 11S γ_1A dissociate on treatment with reducing agents. Moreover, the appearance of higher polymers of γ_1A with anti-B activity in saliva when only intermediate sedimenting antibody is present in serum (Text-fig. 10) indicates the ability of the salivary gland to secrete a γ_1A differing from that in serum. The second possibility is that the glandular epithelium alters serum 7S γ_1A, perhaps by adding a piece which results in its polymerization and/or transport. This piece presumably would be the immunologically specific determinant which was detected in the salivary and colostral γ_1A in these studies. This mechanism seems unlikely in view of the lack of evidence of transport of labeled γ_1A. However, it can not be excluded since the objection can be raised that the γ_1A-preparation used was not obtained from a normal serum and also may conceivably have been slightly altered by the iodination procedure. The third possibility is the local synthesis of a specific γ_1A by cells present in the interstitium of the gland. The autoradiographic studies demonstrate the local synthesis of γ_1A by normal parotid tissue. Neither 7S or γ_1M were found in significant amounts. This confirms the studies of Hochwald *et al.* (29) who have reported that both salivary and mammary tissue are able to incorporate C^{14}-labeled amino acids into the γ_1A *in vitro* as demonstrated by immunoelectrophoresis radioautography. The presence of striking fluorescence in the interstitial cells of the salivary gland in our studies is also consistent with the local synthesis hypothesis although the amounts of protein produced and the cell types involved have not been definitely identified. The number of interstitial cells showing fluorescence is small and it is possible that local synthesis contributes only a small fraction of the total salivary γ_1A. In spite of the demonstration of local synthesis the question still remains however whether the glands synthesize an immunologically specific polymer or a serum type of γ_1A which is subsequently modified by the glandular epithelial cell. This latter mechanism is suggested by the finding of fluorescent staining restricted to the acinar and ductule epithelial cells using the anti-11S γ_1A-antiserum which had been absorbed with normal human serum.

Agglutinin activity against several microorganisms and blood group isohemagglutinins have been found in normal saliva and in pathological salivas we have demonstrated rheumatoid factor activity and antinuclear factors (30). However, because an antibody is found in saliva does not necessarily mean that it is of the γ_1A-type. Small amounts of the other immunoglobulins are often present and may be responsible for the observed activities. In the case of the isohemagglutinins however, the antibody activity is clearly of the γ_1A-type as demonstrated by: (*a*) the concentration of activity in homogeneous

preparations of γ_1A, (*b*) the similar distribution of the antibody activity and γ_1A-concentration in the intermediate portions of a density gradient and (*c*) by the inhibition studies using specific antiserum against the various types of immunoglobulins. In addition to the isohemagglutinins a number of serum antibodies have been reported to be of the γ_1A-type including *Brucella* and diphtheria (8), thyroglobulin (31), insulin (32), antinuclear factors (33) and skin-sensitizing antibodies to allergens such as ragweed (34, 35). Recent studies have reported γ_1A anti viral activity in nasal fluid (2) and antibodies of γ_1A-type to ragweed pollen in saliva and nasal secretions (36, 3).

In view of the relatively high concentrations of γ_1A in certain external secretions and its known antibody activity against microorganisms and allergens it is possible that the γ_1A in these fluids serves a defense function. In the two healthy volunteers whose serum and saliva lacked γ_1A, the γ_1A was replaced by both 7S γ_2 and particularly γ_1M. Additional studies are required however before any definite role can be assigned to the γ_1A normally present in these fluids.

SUMMARY

The γ_1A present in saliva and colostrum exists largely in the form of higher polymers, the major component of which has a sedimentation coefficient of 11S. The 11S γ_1A in these fluids differs from the polymers found in normal and myeloma sera both immunologically and by the fact that their sedimentation coefficients are unaffected by disulfide bond reduction in the absence of urea. However, like other γ-globulins the 11S γ_1A molecules consist of multiple polypeptide chains linked by disulfide bonds.

Local synthesis of γ_1A in the salivary gland has been shown by fluorescent and autoradiographic studies, although the fraction of the total salivary γ_1A which is derived from local production is uncertain. No evidence of transport of intravenously administered I^{131}-labeled 7S γ_1A from serum to saliva was obtained.

Immunological specificity has been demonstrated in the salivary and colostral γ_1A. Whether that portion of the γ_1A which is immunologically specific is a piece incorporated during the local synthesis of γ_1A in the gland or is added by the epithelial cell in the process of transport remains to be determined.

Antibody activity (isohemagglutinins) have been demonstrated in saliva and colostrum and have been shown to be of the γ_1A-type. In both of these fluids activity is associated primarily with γ_1A-polymers of 11S and 18S sizes.

There appears to be an immunological system which is characteristic of certain external secretions. Its properties including the local production of a distinctive type of antibody separate it from the "systemic" system responsible for the production of circulating antibody. This system may play a significant role in the body's defense mechanisms against allergens and microorganisms.

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EXPLANATION OF PLATE 2

FIGS. 1 *a* to 1 *f*. Fluorescent studies of human parotid tissue.

FIG. 1 *a*. Parotid gland section stained directly with fluorescein-conjugated antiserum to serum γ_1A-globulin. This is an area of gland adjacent to the collecting ducts. There is specific fluorescence of the cytoplasm of interstitial cells, some of which look like plasma cells. × 250.

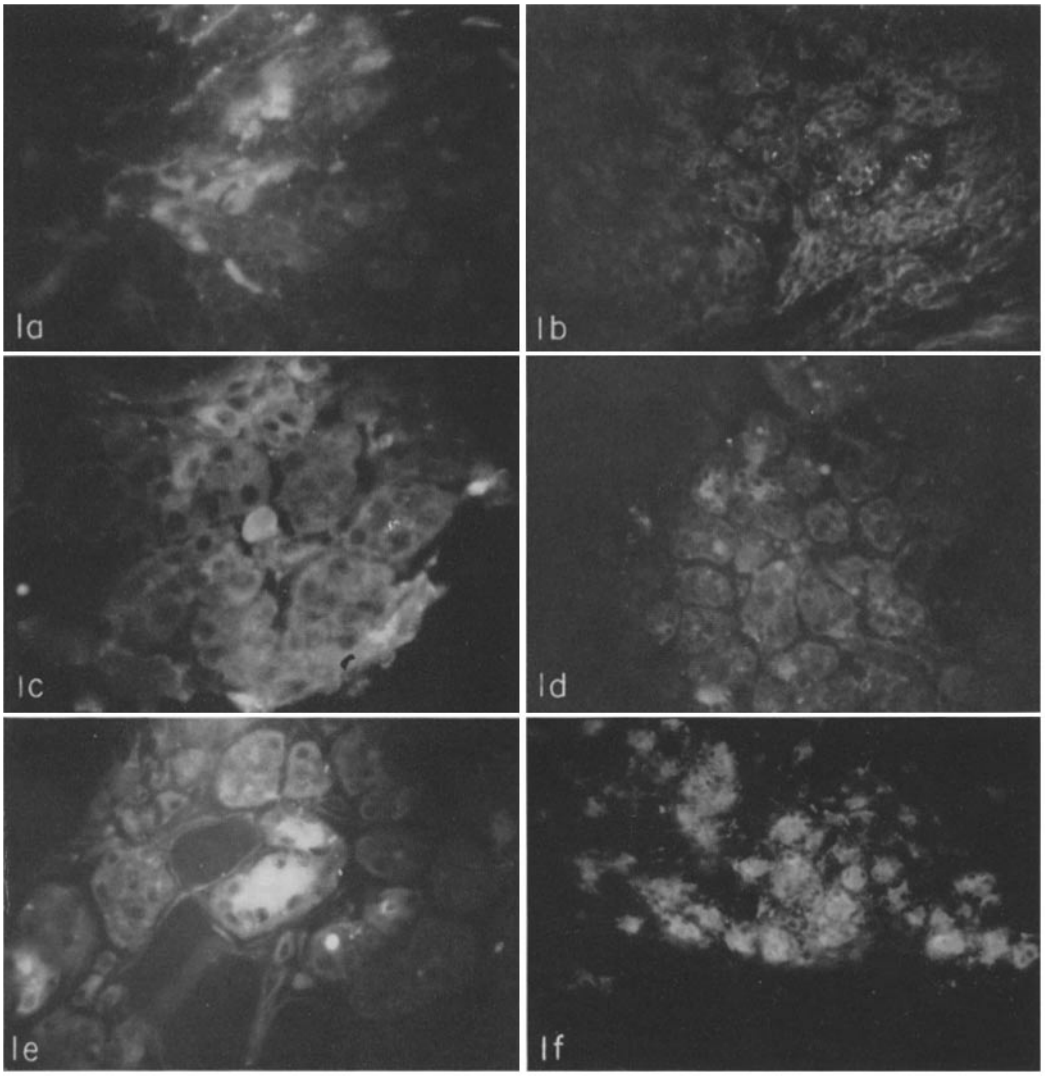
FIG. 1 *b*. Adjacent section of parotid gland in the same area stained with the same fluorescein-conjugated antiserum that had been absorbed with isolated serum γ_1A-globulin. The specific fluorescence of interstitial cells is abolished. × 250.

FIG. 1 *c*. Parotid gland section stained with fluorescein-conjugated antiserum to salivary γ_1A-globulin. The cytoplasm of interstitial cells show specific fluorescence. This section is an area distant from the collecting ducts and the acinar cells in this region generally do not stain. × 450.

FIG. 1 *d*. Same area of gland as in Fig. 1 *c*, stained with antiserum to salivary γ_1A-globulin absorbed with isolated serum γ_1A-globulin. The fluorescence of interstitial cells is abolished. × 250.

FIG. 1 *e*. Parotid gland stained with fluorescein-conjugated antiserum to salivary γ_1A-globulin absorbed with isolated serum γ_1A-globulin. There is specific fluorescence of material in the lumen of some glandular acini. No fluorescence of interstitial cells is present. × 450.

FIG. 1 *f*. Area of parotid gland adjacent to collecting ducts stained with antiserum to salivary γ_1A-globulin absorbed with isolated serum γ_1A. There is widespread fluorescence of the cytoplasm of acinar cells in this region. This is the region of the parotid gland where salivary 11S γ_1A-globulin appears to be most abundant. × 250.



(Tomasi *et al.*: Characteristics of immune system)