Journal of Clinical Investigation
Vol. 42, No. 10, 1963

THE SELECTIVE OCCURRENCE OF γ_1A GLOBULINS IN CERTAIN BODY FLUIDS *

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(Submitted for publication March 16, 1963; accepted June 13, 1963)

It is generally recognized that there are three antigenically distinct types of serum γ globulin, commonly designated γ_2 (7 S γ), γ_1A (B_2A), and γ_1M (B_2M or 19 S γ globulin). The γ_2 and γ_1A globulins have similar molecular weights (approximately 160,000), but different electrophoretic mobilities, carbohydrate content, and antigenic properties. The γ_1M, on the other hand, has a mobility and carbohydrate content similar to those of γ_1A, but a molecular weight of approximately one million. It is well established that the γ_2 and γ_1M fractions contain antibody activity and that certain antigens give rise to one or both types of antibodies. Although to our knowledge the existence of a γ_1A antibody has not been proven, much circumstantial evidence suggests that certain antibodies are γ_1A (1, 2).

During investigations of the chemical and immunological properties of salivary proteins, most of the γ globulin in parotid fluid was found to be closely related to or identical with serum γ_1A. Other external secretions such as colostrum and urine were also found to contain significant amounts of γ_1A. The results of the qualitative and quantitative immunological studies of the γ globulins in saliva and colostrum suggest that secretion is highly selective. As a possible explanation for these results, it is suggested that γ_1A contains a specific (transport) site that is responsible for its secretion into these biological fluids.

METHODS

Collection of samples. Parotid fluid was collected by a Curby parotid cap from healthy volunteers. Flow was stimulated with flavored Chiclet. Samples of 50 to 100 ml were collected and concentrated either by lyophilization or negative pressure dialysis to approximately 1 ml. Colostrum was collected with a standard breast pump at the end of postpartum day 1, 2, or 3. Cord serum was obtained at the time of delivery from the placental end of the cord before the placenta was delivered. Twenty-four-hour samples of urine were collected from healthy adult males. The urine samples were concentrated in the cold by negative-pressure dialysis with $\frac{8}{32}$-inch (flat width) Visking tubing. The dialysate was subsequently placed in 2-inch Visking tubing and concentrated 100-fold by pervaporation at room temperature.

Ultracentrifugation. Analytical ultracentrifugation was carried out in a Spinco model E ultracentrifuge at 52,640 rpm and 20° C. Viscosity measurements were used to correct the observed s rate to $s_{20,w}$. Infinite dilution values were calculated from plots of s rate against concentration. Protein concentrations were measured by the method of Folin-Ciocalteu or by the biuret reaction (3). Density gradient ultracentrifugation with a 10 to 40% sucrose gradient was performed as previously described (4). All gradients were removed from the bottom by collection of drops through a needle hole in the plastic centrifuge tube.

Chromatography of the parotid fluid was carried out on DEAE cellulose; 1 ml of concentrated saliva with a protein concentration of 15 to 25 mg per ml was applied to columns 1×25 cm^2. A continuous gradient of increasing ionic strength and decreasing pH was accomplished with an initial phosphate buffer of pH 7.6, ionic strength 0.01, and final buffer of pH 4.6, ionic strength 0.30.

Isolation of fast and slow fragments from papain-treated γ globulin. Serum was separated by zone electrophoresis on starch, the γ globulin portion was chromatographed on DEAE, and the fall-through peak containing 7 S γ globulin was subjected to papain digestion as described by Porter (5). The resulting digest was separated into slow and fast fragments by starch-block electrophoresis.

Immunology. Immunoelectrophoresis was performed by the micromethod of Scheidegger (6) with 2% agar in 0.05 M barbital buffer at pH 8.2. Double diffusion in agar was performed according to the method described by Ouchterlony (7).

Quantitative precipitin reactions were performed on the density gradient fractions as follows. Three tubes of the density gradient were pooled, and 0.15 ml of the

* Work supported by U. S. Public Health Service grant AM-03986-03S1 and by the National Science Foundation, grant G19-381. Presented in part at the 9th Interim Scientific Session of the American Rheumatism Association, December 9, 1962, Richmond, Va.

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pool was mixed with 0.15 ml of the appropriate antiserum. After incubation for 1 hour at 37° C and 2 days at 4° C, the precipitates were washed twice with cold saline and dissolved in 0.1 N sodium hydroxide. The protein in the precipitate was measured by the method of Folin-Ciocalteu. In some cases where the amount of precipitate was too small for quantitative precipitin analysis, a quantitative estimate of the amounts of the various γ globulins in the gradient fractions was obtained by the following method, modified after a technique described by Heremans (8). A 0.5-ml volume of antiserum specific for the type of γ globulin to be quantitated was added to 4.5 ml of a 1% solution of agar at 50° C, mixed thoroughly, and poured into small petri dishes (i.d. 5.0 cm). Plates were stored at 4° C until ready for use. Holes were carefully made with a no. 1 cork borer, and the wells were filled to the brim with the test solutions. The plates were incubated for 24 hours at 37° C, and the diameter of the resulting ring was measured with a transparent ruler held beneath the plate. Standard curves of diameter against concentration were prepared with Cohn's fraction II [1] for γ_2, a γ_1A myeloma protein isolated from serum by starch-block electrophoresis, and a γ_1M macroglobulin isolated by starch-block electrophoresis followed by density gradient ultracentrifugation. The myeloma and macroglobulin preparations used as a standard contained less than 2% contamination by other proteins.

The quantitative analysis of the γ globulin and albumin in parotid fluid was carried out with standardized anti-γ globulin (antiserum 1b) and albumin antiserum according to the method described by Kabat and Mayer for spinal fluid (3). The standard curves were prepared with fraction II γ globulin and crystalline albumin as antigens.[2]

Several different antisera were obtained by immunizing rabbits with antigens in Freund's adjuvant. Antiserum 1b was prepared by immunization with the γ globulin separated from a normal human serum by starch-block electrophoresis. The proteins eluted from the block from the origin to the cathodal end were pooled and used as antigen. The antiserum 1b contained high titers of antibodies specific for γ_2 and γ_1M globulins and only very small amounts of antibodies specific for γ_1A. The reaction of 1b with γ_1A was therefore primarily a cross-reaction. Antiserum 5b against γ_1A was prepared with an antigen isolated from normal human serum by the zinc precipitation method of Heremans (9) followed by DEAE chromatography. This antisera contained antibodies primarily directed against γ_1A and only small amounts of γ_2-specific antibodies. The latter could be removed by absorbing either with fraction II γ globulin or with cord serum, and the resulting antiserum (6b) was specific for γ_1A. Antisaliva antiserum was made by immunizing rabbits with concentrated samples of parotid fluid. This antiserum gave a single line in the γ_1A area when reacting in immunophoresis with normal serum. It also contained a number of antibodies directed against components specific for saliva. Antiserum 462 [3] was made against a human myeloma preparation and contained antibodies against both γ_2 and γ_1A globulins.

Electrophoresis and enzyme assays. Paper electrophoresis was carried out in a Spinco Durrum-type hanging-strip cell in barbital buffer at pH 8.6, 0.05 ionic strength. Twelve- to 24-μl samples of concentrated saliva were applied to the paper. The location of the amylase was determined by running paper strips in duplicate, one being strained in the usual manner with bromphenol blue, the other being placed wet on a trough containing agar into which unhydrolyzed starch had been incorporated. After incubation for 5 minutes at room temperature, the strip was removed, the trough flooded with a potassium iodide solution containing iodine, and the position of the amylase activity noted as an area of decoloration resulting from the depolymerization of the starch. The position of the lysozyme activity was measured by cutting the strips into 0.5-cm fractions and eluting with saline. The lysozyme activity was assayed by the method of Smalelis and Hartsell (10).

RESULTS

Electrophoresis and ultracentrifugation of parotid fluid. The results of the paper electrophoresis of a concentrated parotid fluid are shown in Figure 1. The location of the lysozyme and amylase activity is indicated by the vertical and horizontal bars, respectively. The ultracentrifugal pattern of concentrated parotid fluid is illustrated in Figure 2. A small amount of material with an s rate greater than 11 S is seen in the first frame in Figure 2 (peak 1). Peak 2 has an $s_{20,w}$ of 11.0 S, and peak 3 an $s_{20,w}$ of 4.4 S. In some concentrated samples, a fourth component with an $s_{20,w}$ of 1.8 S was also seen. Evident on the centrifuge plate is a significant amount of light material that does not sediment beyond the meniscus during the period of centrifugation (4 hours). Similar values for the s rates of the salivary components were obtained in 6 samples of normal parotid fluid. No 7 S component is present in the sample shown in Figure 2. Since the concentration of protein in this particular sample is 28 mg per ml and 2.0% of the total protein is γ globulin, approximately 0.6 mg per ml of γ globulin is present. This concentration of a homogeneous protein should be visible on the centrifuge plate as a small but definite peak. It is possible, especially in view of the density gradient data presented in

[1] Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

[2] Gamma globulin and albumin obtained from Pentex Co., Kankakee, Ill.

[3] Kindly supplied by Dr. H. G. Kunkel, New York, N. Y.

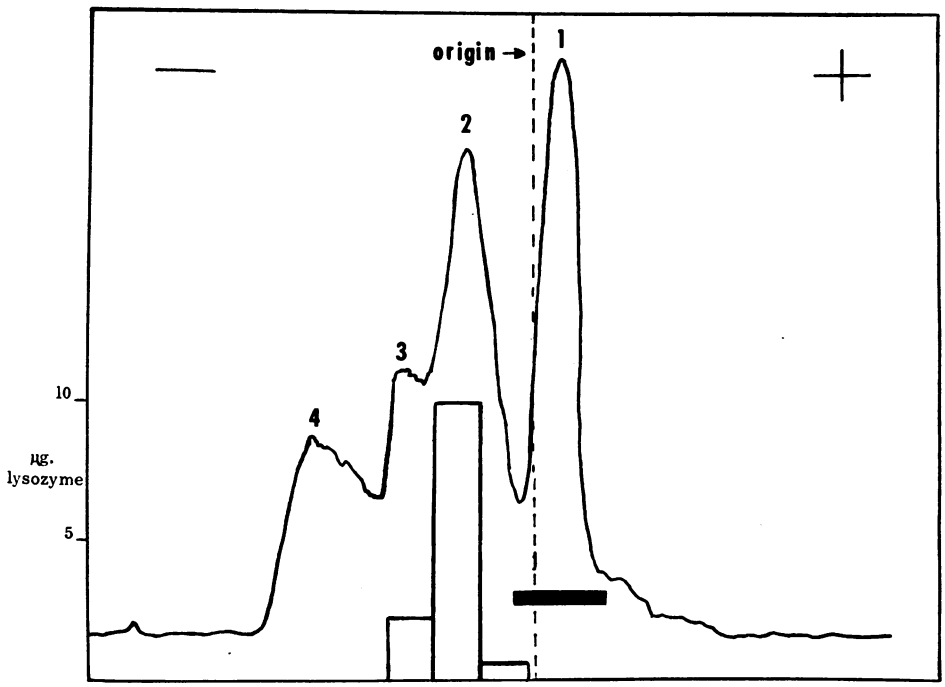

FIG. 1. PAPER ELECTROPHORESIS OF PAROTID SALIVA: DENSITOMETER TRACING OF PAPER STRIPS STAINED WITH AMIDO BLACK. Vertical bars indicate location of lysozyme activity quantitated from turbidity measurements (see text) on the ordinate. Horizontal solid bar represents location of the amylase activity.

a later section, that peak 2 (11 S) represents the salivary γ globulin.

Immunology. The protein concentration of twelve normal parotid fluids averaged 166 mg per 100 ml (range, 106 to 207 mg per 100 ml) as measured by the biuret reaction. The Folin-Ciocalteu reaction gave consistently lower values for total protein, for an average ratio of biuret: Folin-Ciocalteu values of 1.52. The quantitative precipitin analysis with antiserum 1b of the parotid fluids from three normal persons indicates that unconcentrated parotid saliva contains approximately 1.5 mg per 100 ml of γ globulin and 1 mg per 100 ml of albumin. By the plate method, the various

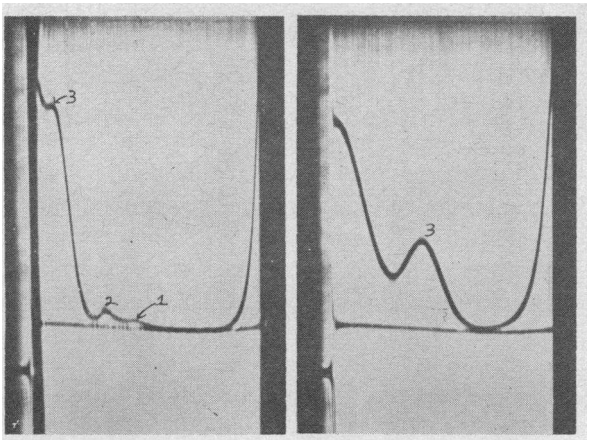

FIG. 2. ULTRACENTRIFUGE PATTERN OF PAROTID SALIVA AT A CONCENTRATION OF 28 MG PER ML. Photograph on the left was taken 40 minutes, that on the right, 120 minutes after full speed was reached. Sedimentation is from left to right. Peak 2 has an $s_{20,w}$ of 11 S and peak 3, of 4.4 S. Peak 2 may represent the salivary γ globulin (see text).

TABLE I

*Concentrations of various immunoglobulins in normal human parotid saliva**

Subject	Total protein	γ_1A	γ_1M	γ_2
	mg/100 ml		*mg/100 ml*	
T.T.	120	2.96	0	0
W.C.	180	1.92	0	0
L.T.	182	3.08	0	0
T.B.T.	111	2.06	0	0
A.C.	149	3.71	1.94	0
A.A.	194	2.78	.97	0

* Total protein of unconcentrated parotid saliva was measured by the biuret reaction. Immunoglobulins were quantitated by the plate technique (see Methods).

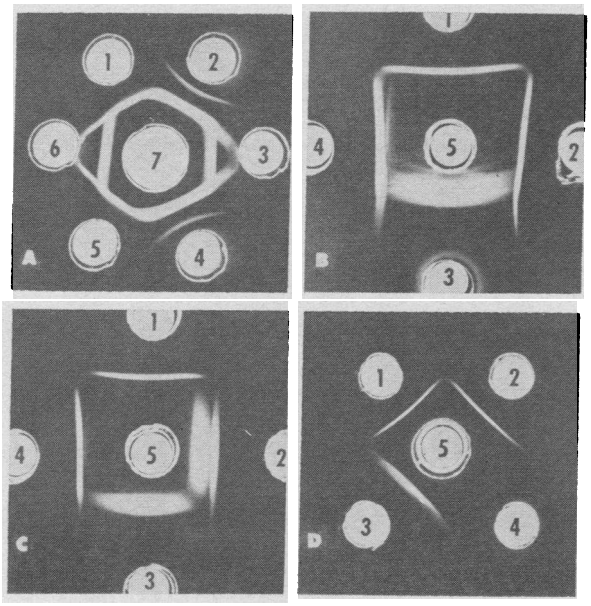

FIG. 3. OUCHTERLONY PATTERNS SHOWING γ_1A NATURE OF COLOSTRAL AND SALIVARY γ GLOBULIN. A. 1,5 = Cohn fraction II γ globulin, 2,4 = human serum T.T. and W.C., 3,6 = parotid saliva T.T. and W.C., and 7 = antiserum against human γ globulin (1b). B. 1 = parotid saliva T.T., 2 = parotid saliva D.C., 3 = human cord serum, 4 = human colostrum, and 5 = antiserum against serum γ_1A (5b). C. 1 = parotid saliva S.Z., 2 = human sera S.Z., 3 = human cord serum, 4 = human colostrum, and 5 = antiserum against serum γ_1A (5b). D. 1 = parotid saliva, 2 = urine, 3 = colostrum, 4 = cord serum, and 5 = antiserum against serum γ_1A absorbed with cord serum (6b).

types of immunoglobulins could be separately quantitated. As noted in Table I, normal saliva contained no detectable γ_2 or γ_1M, with two exceptions where small amounts of γ_1M were present. By this technique, it was possible to detect about 0.05 mg per ml of each of the immunoglobulins.

Figure 3A demonstrates that the major component of parotid γ globulin is antigenically deficient in relation to both the individual's own serum γ globulin and also to Cohn fraction II. The antiserum 1b used in Figure 3A contains high titers of antibodies against γ_2 and also antibodies specific for 19 S γ globulin, but negligible amounts of specific anti-γ_1A antibodies. All of the eight normal parotid salivas studied showed this pattern.

Since the fragments derived from serum γ globulin by papain digestion are also antigenically deficient relative to untreated γ globulin, the relationship between these fragments and parotid γ globulin was investigated. The parotid γ globulin showed immunological cross-reaction with the slow fragment but not with the fast fragment of the papain digest. Since the slow fragment contains the portion of the γ globulin molecule responsible for the cross-reaction between 7 S and γ_1A, as well as with γ_1M and myeloma proteins (11), experiments were carried out to elucidate any relationship between serum γ_1A and parotid γ globulin. On immunoelectrophoresis, parotid γ globulin migrated in a position consistent with that of γ_1A (Figure 4). As illustrated in Figure 5, an antisaliva antiserum gave a single line against normal serum on immunoelectrophoresis, and the precipitin arc is typical in form and location of serum γ_1A.

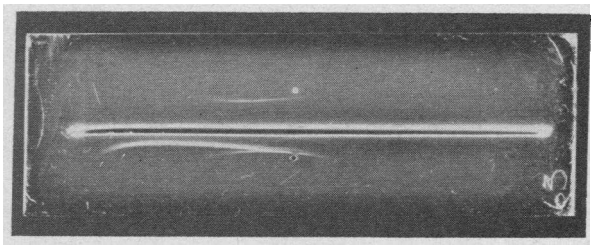

FIG. 4. IMMUNOELECTROPHORESIS OF PAROTID SALIVA (UPPER WELL) AND SERUM (LOWER WELL). Trough contains antiserum 462, which has antibodies against both γ_1A and γ_2. Precipitin arc with saliva is typical of γ_1A.

The immunological relationship between human colostrum and saliva is shown in Figure 3B. Parotid fluid gave a precipitin reaction showing identity with colostrum, and both saliva and colostrum gave a spur with cord serum. The antiserum 5b used in this figure contained antibodies that cross-react with γ_2 and are responsible for the in-

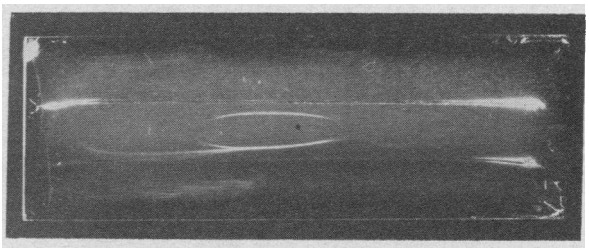

FIG. 5. IMMUNOELECTROPHORESIS OF NORMAL HUMAN SERUM (CENTER WELL), ANTISERUM AGAINST WHOLE PAROTID SALIVA (UPPER TROUGH), AND ANTISERUM 462, WHICH REACTS WITH BOTH γ_2 AND γ_1A (LOWER TROUGH). Precipitin arc with the antisaliva antiserum in the upper part of the figure corresponds with that given by serum γ_1A.

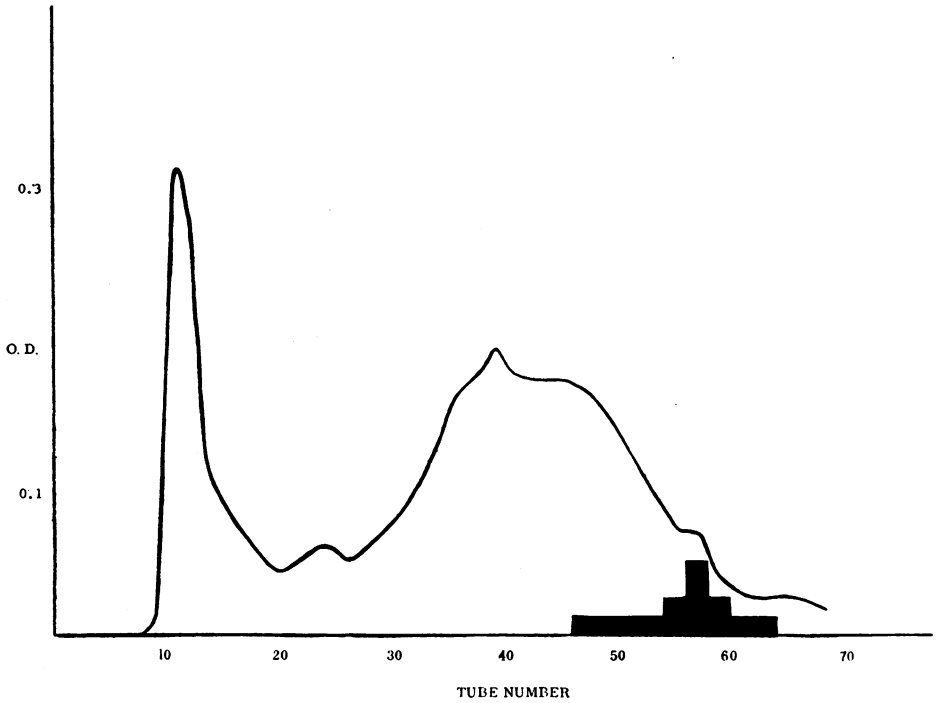

FIG. 6. DEAE CHROMATOGRAM OF NORMAL HUMAN PAROTID SALIVA SHOWING DISTRIBUTION OF SALIVARY γ GLOBULIN (SOLID BARS) IN THE LATE PORTIONS OF CHROMATOGRAM. A similar distribution is shown by serum γ_1A. Continuous phosphate gradient: initial buffer of pH 7.6, ionic strength 0.01; final buffer of pH 4.6, ionic strength 0.30.

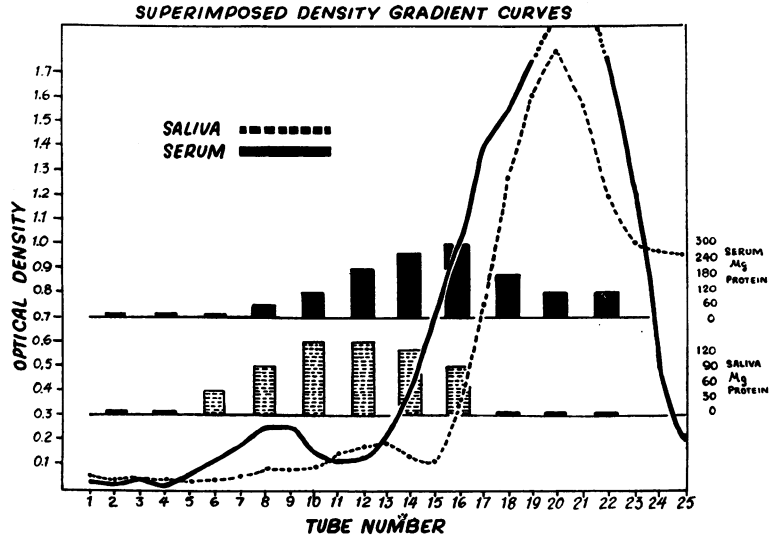

FIG. 7. DENSITY GRADIENT ULTRACENTRIFUGATION OF NORMAL HUMAN SERUM AND PAROTID SALIVA. On the left ordinate is protein concentration expressed in units of optical density. Anti-γ globulin antiserum (1b) was added to pooled density fractions of serum (solid bars) and saliva (stippled bars). Micrograms of precipitate are on the right ordinate. Salivary γ globulin migrates somewhat faster than serum 7 S γ globulin.

teraction with cord serum. It also contains large amounts of antibodies directed against the antigenically specific portion of the γ_1A molecule, and it is these antibodies that, on interaction with the γ_1A of saliva and colostrum, result in the spur formation between these fluids and the cord serum. As shown in Figure 3C, this same antiserum gives two lines with normal human serum: an inner line due to the reaction with γ_2, showing identity with the cord serum, and an outer line due to γ_1A, showing identity with the salivary and colostral bands. If antiserum 5b is made specific for γ_1A by absorption with cord serum (or Cohn fraction II), thus removing the cross-reacting antibodies, the identity of salivary, colostral, and urinary γ_1A can be clearly demonstrated (Figure 3D).

Chromatography. Parotid fluid γ globulin is eluted in the late portions of a DEAE chromatogram (Figure 6). The position of the γ globulin was detected with an antiserum to serum γ_1A (5b) and was semiquantitated from the Ouchterlony patterns. No γ_2 or γ_1M could be detected in any of the column fractions, although the concentrated saliva showed a definite but faint γ_1M line. The distribution of the parotid γ in the late portions of the chromatogram is similar to that of serum γ_1A (12).

Molecular size of the γ_1A. Evidence of the size of the γ_1A was obtained by density gradient ultracentrifugation. Figure 7 shows a comparison of the density gradient patterns of saliva and adult serum. The quantitative precipitin data obtained on the fractions from the density gradient with an antiserum to whole human γ globulin (1b) indicate that the γ globulin is of high molecular weight, with an s rate in the 7 to 11 S range. The position of the peak of salivary γ in Figure 7, between that of serum 7 S and 19 S, suggests an intermediate s (11 S) for parotid γ_1A. Ten density gradient experiments were performed on five different normal parotid salivas, with results essentially similar to those shown in Figure 7. The

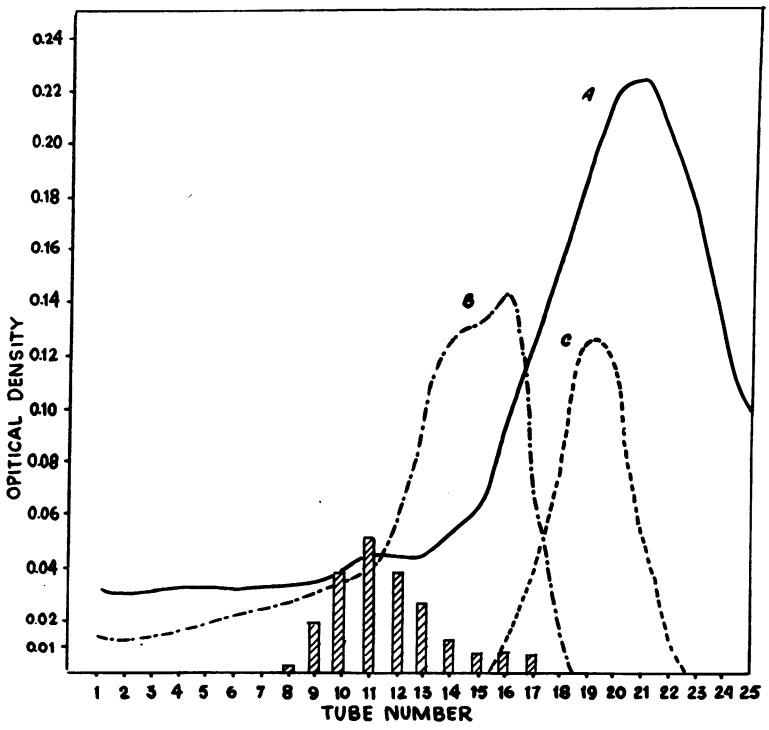

FIG. 8. DENSITY GRADIENT ULTRACENTRIFUGATION OF NORMAL URINE (CURVE A). Curve B represents the location of Cohn fraction II, and curve C, the papain fragments of fraction II that have been added to the urine. Bar graph shows the position of the urinary γ_1A primarily in the 7 to 11 S region of the gradient.

density gradient pattern of human colostrum obtained on postpartum day 2 also showed γ_1A to be distributed primarily in the intermediate fractions (between 7 S and 19 S) of the gradient.

Normal concentrated urine showed a density gradient pattern very similar to that of parotid saliva. The γ_1A in urine has a distribution in the density gradient most consistent with an s rate in the 7 to 11 S range (Figure 8). In order to localize more accurately the γ globulins in urine, 7 S γ globulin (mol wt about 160,000) and the papain-split products of γ globulin (mol wt about 50,000) were added to urine samples. The location of Cohn fraction II and papain fragments was determined by subtracting the protein concentrations of the tubes containing urine alone from those containing urine plus fraction II and urine plus papain fragments. The location of γ_1A relative to these is shown in Figure 8. Gradients on urine samples from four normal persons were similar in both the form of the gradients and the distributions of γ_1A. No attempt was made in the present study to quantitate the amounts of γ_1A relative to the total urinary γ globulins.

DISCUSSION

Previous studies using electrophoretic and immunological techniques have identified many of the serum proteins in human saliva (13, 14). It is not clear, however, whether the serum components in saliva are a result of transudation, secretion, or whether the salivary gland is capable of the synthesis of plasma proteins. The results of the quantitative analysis indicate that the γ globulin : albumin ratio of saliva is six to eight times higher than that of serum. This fact, together with the finding that the γ globulin is primarily of the γ_1A type, would suggest that factors in addition to simple transudation from serum operate in the excretion of serum proteins in saliva. There is no evidence available regarding the ability of the salivary gland to synthesize γ globulin, so that the possibility of local production by the parotid gland cannot be excluded. If salivary γ globulin is derived from serum, then transport must be highly selective, since the γ_2 globulin which is of similar size is secreted in small amounts, if at all. One could postulate that the high γ globulin : albumin ratio in saliva is due to the small size of the γ globulin molecules analogous to the small species of γ globulins found in human urine. Judged, however, from the position of the γ globulin line in gel diffusion and especially the density gradient studies, there is no evidence of smaller fragments of γ globulin in parotid fluid.

A number of studies have indicated that human colostrum contains large amounts of γ globulin and possesses a variety of antimicrobial activities (15). Detailed studies of the immunological characteristics of colostral proteins have been carried out by Hanson (16). These studies are in general agreement with ours, but Hanson's more detailed immunological analysis has demonstrated the complexity of the antigenic structure of the immunologic globulins of milk and has revealed the presence of small amounts of other types of γ globulins in addition to γ_1A.

The difference in antibody distribution between adult serum, cord serum, and colostrum might be explained on the basis of the differential permeation of 7 S and 19 S antibodies. Colostrum contains 19 S γ globulin, as we as well as others (16) have shown, whereas cord serum has little, if any. The absence of certain antibodies from cord serum, however, could also be explained by the antibody's being γ_1A rather than 19 S. This may be the case with skin-sensitizing antibody (17).

Analysis of the density gradient fractions with a specific anti-γ_1A antiserum indicated that urinary γ_1A is entirely of the 7 to 11 S type. It seems likely from our own studies, as well as those of others (18), however, that 7 S γ_2 globulin is present in normal human urine in addition to the smaller fragments previously described (19, 20). An important question not yet fully answered is whether the ratio of γ_2 to γ_1A in urine is the same as that in serum.

The results of the density gradient experiments suggest the possibility that the γ_1A of these fluids is of larger size, perhaps 11 S, as compared with the 7 S γ_2 of serum. These results are consistent with those of Rockey and Kunkel (17), who found sulfhydryl-sensitive, intermediate sized isoagglutinins and skin-sensitizing antibody by the density gradient technique. Although the evidence presented is suggestive, further work is certainly necessary to establish the molecular size of the γ_1A globulins in these fluids.

We know of no reports that have isolated and

identified an antibody as a γ_1A globulin. Since both colostrum and saliva, however, contain antimicrobial antibodies and their γ globulins are predominantly γ_1A, presumptive evidence exists for γ_1A antibodies. Recent studies suggest that isoagglutinins are present in some salivas and that in certain pathological conditions, abnormal proteins such as antinuclear antibodies and rheumatoid factors are excreted (21). The appearance of an antibody in parotid fluid, although suggestive, is not proof that the antibody is of the γ_1A type, since small amounts of 7 S and γ_1M proteins below the level of detection by the methods above could be responsible for the antibody activity.

It may be the function of the γ_1A globulins as part of the body defense mechanism to furnish antimicrobial and perhaps other antibodies to the external secretions. This could be accomplished either by local synthesis, or by the presence of a specific chemical site that allows the selective transport of γ_1A from serum. The γ_1A content of other body fluids will be of interest. Studies now in progress (22) indicate that a similarly high γ_1A : γ_2 ratio is found in human tears, bile, and small intestinal secretions. Perhaps the predominant γ globulin in external secretions is γ_1A rather than γ_2. Further work is needed, however, before any conclusions can be drawn. Especially important is careful quantitation and a consideration of the possibility of a differential susceptibility of the various types of immunoglobulins to the proteolytic enzymes present in these fluids.

Finally, although the evidence presented here suggests that parotid γ globulin is identical with serum γ_1A, the possibility that it is a unique type of γ globulin closely related to, but not identical with, serum γ_1A has not been excluded. Moreover, since the γ_1A proteins, as well as other serum γ globulins, have been found to occur in two major types on the basis of their antigenic characteristics (23), further studies will be necessary to define the antigenic type or types of the γ globulins in these fluids.

SUMMARY

Normal human parotid saliva contains large amounts of γ globulin relative to other serum proteins and has a γ globulin : albumin ratio approximately six times greater than that of serum. Most of the γ globulin in parotid saliva is closely related to, if not identical with, serum γ_1A. Human colostrum and normal urine were found to contain significant amounts of γ_1A immunologically identical with the γ globulin in parotid saliva. The γ_1A in colostrum, saliva, and urine have sedimentation rates in the 7 to 11 S range as demonstrated by density gradient and immunological analysis. The possibility that the selective secretion of γ globulin in these fluids depends on the presence of a specific transport site on the γ_1A molecule is discussed.

ACKNOWLEDGMENTS

The author is indebted to Dr. H. G. Kunkel for his continuing interest and advice and to Dr. Ethan Sims for help in concentrating the urine samples.

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