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In vitro Protein Synthesis by Human Salivary Glands

I. SYNTHESIS OF SALIVARY IgA AND SERUM PROTEINS

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Summary. Protein synthesis by human salivary glands was studied *in vitro* by incorporation of ^{14}C-labelled amino acids and autoradiographs of double diffusion tests and immunoelectrophoresis of culture fluids.

Submandibular and parotid glands synthesized five serum proteins: one α_1-globulin, one β_1-globulin and three immunoglobulins, IgG, IgA and IgM. IgG and IgM, synthesized in small amounts, corresponded immunologically to serum IgG and IgM.

IgA, synthesized in large amounts, possessed antigenic determinants supplementary to serum IgA. There was also a synthesis of the free transport piece. Synthesis of serum IgA was never detected, alone or simultaneously with salivary IgA. These facts indicate a transport piece synthesis greater than local salivary IgA synthesis.

INTRODUCTION

Previous studies demonstrated that human saliva contains some proteins immunologically identical to the serum proteins: albumin, haptoglobin, transferrin, caeruloplasmin, IgG and IgM (Gabl, 1959; Ellison, Mashimo and Mandel 1960; Gabl and Wachter, 1961; Stoffer, Kraus and Holmes, 1962). Their origin has not been elucidated and some doubt exists whether they are transuded from plasma or synthesized locally in the salivary glands.

Human saliva contains a large amount of salivary IgA, an immunoglobulin which has antigenic determinants specific to serum IgA and antigenic determinants specific to saliva (Tomasi and Zigelbaum, 1963; Tomasi, Tan, Solomon and Prendergast, 1965). It has been found that the latter are on a piece which can be isolated by the reduction and alkylation of salivary IgA (South, Cooper, Wollheim, Hong and Good, 1966a). This piece was called transport piece. Some studies suggest that salivary IgA is the result of a binding of serum IgA (coming from plasma and/or from local synthesis by plasmocytes) with the transport piece synthesized by acinar and ductule epithelial cells (Tomasi *et al.*, 1965). Probably two or three molecules of serum IgA combine with one molecule of transport piece to form the salivary IgA with a sedimentation coefficient of 11S (Hong, Pollara and Good, 1966; Cebra and Robbins, 1966). Nothing is known about the relationship between the synthesis of the transport piece and that of serum IgA. Some doubt exists also whether the serum IgA is transuded from plasma or synthesized in the salivary glands.

The present experiments were undertaken to study the serum protein synthesis by human submandibular and parotid glands in tissue culture.

This *in vitro* method was selected because contamination by the plasma or transudation of some components from the serum cannot occur, and one can determine without doubt which serum proteins are really synthesized by salivary tissues.

The synthesis of imunoglobulins and salivary IgA was closely examined. A synthesis of IgM, IgG and of large amounts of salivary IgA was observed. It was demonstrated that the transport piece and serum IgA were synthesized locally. Free transport piece as well as salivary IgA was always found in the culture medium demonstrating that transport piece synthesis exceeded salivary IgA synthesis.

MATERIALS AND METHODS

Human salivary glands

Fragments of human parotid and submandibular glands were obtained in the course of surgery for tuberculosis or tumor in the region of these glands. A portion of each fragment was fixed in formol saline and examined histologically after hematoxylin–eosin staining. No lesions were observed in the fragments used for tissue cultures.

Saliva

Saliva collected from normal humans after stimulation with chewing-gum was dialysed against distilled water for 48 hours, lyophilized and pooled. For immunological analysis the powder was dissolved in 0·9 per cent saline in the proportion of 40 mg of powder per millilitre of solvent which corresponds to a normal saliva concentrated twenty-fold.

Preparation of salivary IgA

A pool of lyophilized saliva was dissolved in distilled water in the proportion of 60 mg powder/ml water. After overnight dialysis against phosphate buffer 0·01 M, pH 7·4, the material was applied to a column of DEAE-cellulose, as described by Tomasi *et al.* (1965), using a ratio of 60 mg of lyophilized material per gram of DEAE-cullulose. Columns of $50 \times 2{\cdot}4$ cm were used and the chromatography was performed using a stepwise gradient of three buffers: 0·01 M, pH 7·4; 0·1 M, pH 6·2; and 0·3 M, pH 4·8. The material eluted with 0·1 M, pH 6·2, buffer was dialysed, lyophilized and dissolved in distilled water (60 mg of powder per millilitre of water). After overnight dialysis against phosphate buffer 0·005 M, pH 5·0, the material was passed on a column of CM-cellulose as described by Masson, Carbonara and Heremans (1965), using the same column length and ratio of material per gram of cellulose as for the DEAE chromatography. Elution commenced with the 0·005 M phosphate buffer, pH 5·0, continuing at constant pH with a concentration gradient rising to molarity 0·3. The fraction eluted during this gradient was dialysed against distilled water and lyophilized. The powder was dissolved in phosphate buffered saline, pH 7·4, and passed through a column of Sephadex G-200 equilibrated with phosphate buffered saline (volume of sample 2 ml, column $80 \times 2{\cdot}4$ cm, flow rate 13 ml/hr). Three peaks were obtained. The first peak was passed a second time on Sephadex G-200, dialysed and concentrated. Analysis of this material by double diffusion in agar and immunoelectrophoresis using a 1 per cent protein solution, showed only one precipitation line with rabbit antisera to human IgA, human serum and saliva. Analytical ultracentrifugation, using a 0·8 per cent protein solution, showed one large and symmetrical peak with a sedimentation coefficient of 11S plus a very small 18S peak corresponding probably to aggregates. This material was considered pure salivary IgA.

Reduced and alkylated salivary IgA

Obtained as described by South *et al.* (1966a). Salivary IgA prepared as described above was incubated with β-mercaptoethanol (final concentration 0·2 M) in 0·3 M Tris–HCl buffer, pH 8·2, at room temperature for 1 hour. Iodoacetamide (final concentration 0·2 M) was added. The mixture was left at 4° for 1 hour then dialysed against 0·15 M sodium borate buffer, pH 8·0, at 4° for 24 hours.

Saliva from a patient without IgA

Obtained from an adult whose serum did not contain any IgA as tested by double diffusion in agar, immunoelectrophoresis and ring test with specific rabbit anti-IgA serum.

Human gastric juice

Collected by gastric intubation following histamine stimulation. After centrifugation at 3000 ***g*** for 15 minutes and dialysis against distilled water for 48 hours, it was lyophilized and pooled.

Submandibular gland extract

Obtained from fragments ground 10 minutes in 0·15 M sodium phosphate buffer, pH 7·4, at 4° in a Silverson mixer (Silverson, London, England). After centrifugation at 3000 ***g*** for 20 minutes, the supernatant was dialysed against distilled water and lyophilized.

Antisera

These were obtained by immunization of rabbits of various strains, weighing 2·5–3·5 kg. The antigen solution in saline was mixed with an equal part of complete Freund's adjuvant (Difco, Detroit, Michigan). The emulsion was injected into the hind footpads the 1st week and then into four sites in the musculature once a week. From the 3rd week on circulating antibodies were revealed by double diffusion in agar. When the antibody titre was high enough, the rabbits were bled out (usually 4–7 weeks after the beginning of the immunization). The amount of injected protein was the same every week namely, for saliva—10 mg; for submandibular extract—4 mg; for salivary IgA—500 μg; for gastric juice—10 mg; and for human serum—30 mg. Rabbit antisera specific for α_1-antitrypsin, Gc-factor, haptoglobin and α_1-acid glycoprotein were obtained from Behringwerke AG (Marburg, Germany), through the kindness of Dr G. Schwick. Horse anti-human serum was obtained from the Pasteur Institute, Paris. Mono-specific sheep antisera to human IgA, IgM and IgG were obtained from Dr T. Webb WHO Reference Centre, Lausanne, Switzerland. Each of these showed only one precipitation line with normal human serum.

In some studies, antisera to saliva, submandibular extract, or salivary IgA were absorbed with a pool of normal human sera. After absorption they were no longer able to precipitate any serum constituent.

Immunoelectrophoresis

The micromethod of Scheidegger (1955) was followed with slight modifications, using the LKB apparatus 6800 (LKB products, Stockholm, Sweden).

Double diffusion in agar

The micromethod on microscope slides was used with 2 per cent agar in 0·025 M barbital buffer, pH 8·2.

Tissue cultures and analysis of culture fluids

The technique as described by Hochwald, Thorbecke and Asofsky (1961) was used. One hundred to 200 mg of tissue in fragments of about 1 mm^3 were cultured in roller tubes with 2 ml of medium. This medium consisted of Hanks's balanced salt solution, an amino acid mixture prepared according to Neuman and McCoy (1958) without lysine and isoleucine, 0·3 per cent glucose, 0·5 per cent ovalbumin (ovalbumin 5 × crystallized, Pentex, Kankakee, Illinois), a vitamin mixture according to Eagle (1955), 1 μg/ml of hydrocortisone hydrogensuccinate, 50 μg/ml of crystalline insulin and 121 μg/ml of crystalline sodium penicillin G. One microcurie per millilitre of [^{14}C]L-isoleucine and 1 μc/ml of [^{14}C]L-lysine (1200–1270 μc/mg, The Radiochemical Centre, Amersham, England) were added and the pH was adjusted to 7·4 with sodium bicarbonate.

After cultivation for 48 hours at 37° with rotation, the culture fluids were dialysed against 0·01 M sodium phosphate buffer, pH 7·2, and lyophilized. The powder was put in 0·15 ml of distilled water and the solution analysed by means of autoradiography of Ouchterlony and immunoelectrophoresis plates. They were added to various carriers to obtain good precipitation lines. The antigen wells were first filled with the carriers and later three times with concentrated culture fluids. Various antisera were used. After double diffusion, the slides were washed with saline, dried, stained with Amido Black and placed in contact with photographic film (Kodak professional Royal Pan 400 ASA) for 14 days at room temperature. The film was developed with DK 60 a (Kodak) for 4 minutes and fixed with Metafix (Kodak).

The autoradiographs were compared with stained slides. When a component was produced in large amounts *in vitro*, but did not exist in the cold carrier, it could precipitate with the specific antiserum. In such a case, the stained slide of the carrier plus the culture fluid showed an additional precipitation line not seen with the carrier alone; usually this line was blurred and located near the antigen well.

The intensity of the autoradiographic lines was classified from 0 = negative; ± = just visible; 1 = visible to 3 = very dark. The final evaluation included at least two immunoelectrophoretic analyses with various carriers and antisera.

RESULTS

ANALYSIS OF CULTURE FLUIDS

Nineteen submandibular glands and eight parotid glands were studied. Five labelled serum proteins were found in the culture fluids: one α_1-globulin, one β_1-globulin and three immunoglobulins (IgG, IgA and IgM) (Table 1).

α_1-Globulin

This protein was synthesized by all the cultures. The labelling of the precipitin line in radioautographs was always intense. The line spread from the antigen well to the α_1-region, sometimes showing a double curve. It was revealed by anti-human serum and unabsorbed antisera to saliva, submandibular extract and gastric juice. Specific antisera proved that it was not α_1-antitrypsin, α_1-acid glycoprotein, Gc-factor nor haptoglobin.

β_1-Globulin

Twelve of nineteen culture fluids from submandibular glands and six of eight from parotids showed a β_1-globulin line. The labelling was light and the line visible only with horse anti-human serum. It did not correspond to transferrin. This protein has not been further identified (Fig. 1A).

TABLE 1

In vitro FORMATION OF SERUM PROTEINS BY SUBMANDIBULAR AND PAROTID GLANDS

Tissue	Culture No.	Serum protein labelling*				
		α_1-Globulin	β_1-Globulin	IgA	IgM	IgG
Submandibular glands	2	3	1	2	0	1
	9	3	1	3	±	1
	10	3	0	2	0	±
	15	3	0	3	0	1
	19	3	1	2	±	1
	21	3	0	2	±	1
	23	3	0	2	±	1
	26	3	0	2	±	1
	34	3	1	2	±	1
	36	3	±	3	1	1
	46	3	0	3	0	1
	49	3	0	3	1	±
	37	3	1	2	0	1
	57	3	1	2	0	1
	60	3	1	2	0	1
	63	3	±	2	±	1
	69	3	1	2	±	1
	82	3	1	2	±	1
	99	3	1	2	±	1
Parotid glands	43	3	1	3	+	1
	47	3	0	3	0	0
	51	3	±	3	0	0
	53	3	1	2	±	1
	66	3	1	2	±	1
	72	3	1	1	0	1
	85	3	0	2	0	1
	98	3	1	2	±	1

* The serum protein labelling is estimated according to the intensity of autoradiographic lines from 0 (no labelling) to 3 (very strong labelling).

IgM

There was synthesis of IgM in twelve of nineteen submandibular glands and in four of eight parotids studied. The labelling was always light often at the limit of visibility (± to 1). This line was visible only with anti-human serum and anti IgM (Fig. 1C).

IgG

All the culture fluids from submandibular glands and six culture fluids from the eight parotids had a labelled IgG line (Fig. 1A). The line was always fine and its intensity did not exceed 1. Horse anti-human serum, sheep anti-IgG, rabbit anti-human serum, anti-submandibular gland extract and anti-saliva revealed the line. Following absorption with human serum the last two antisera no longer revealed the line. This proved that IgG in saliva or in submandibular gland has no antigenic determinant supplementary to serum IgG and that the labelled IgG in the culture fluid is identical to serum IgG.

IgA

A labelled IgA line was found in all culture fluids. The intensity of labelling was always strong or very strong. The line was visible with anti-human serum, anti-saliva, anti-submandibular extract, anti-serum IgA and anti-salivary IgA (Fig. 1B). The antisera to submandibular extract, saliva and salivary IgA still gave this line despite absorption with normal human serum. Therefore, unlike IgG, IgA in saliva or submandibular gland has antigenic determinants supplementary to serum IgA. This peculiarity led to the study of labelled IgA in culture fluids in greater detail, namely to examine separately the synthesis of the transport piece and the synthesis of IgA.

For this purpose five different carriers were used and immunologically characterized:

Normal human serum showing one precipitation line with anti-serum IgA and anti-salivary IgA, but no line with anti-salivary IgA absorbed with normal human serum. This carrier contains molecules of IgA that have only serum antigenic determinants.

Normal saliva showing two precipitation lines in double diffusion in agar with anti-salivary IgA and one precipitation line with anti-serum IgA and anti-salivary IgA absorbed with normal human serum. Comparison on the same slide with salivary IgA, serum IgA, and reduced and alkylated salivary IgA showed that one precipitation line revealed by anti-salivary IgA corresponded to salivary IgA and the other to unbound transport piece. Therefore the pool of normal saliva used as carrier contained both molecules of free transport piece and molecules of salivary IgA.

The same was also demonstrated in seven of ten salivas obtained from adults. Three had only salivary IgA.

Saliva from a patient without IgA showed one precipitation line with anti-salivary IgA or with anti-salivary IgA absorbed with normal human serum but no line with anti-serum IgA. This carrier contained only molecules of free transport piece.

Salivary IgA showed one precipitation line with anti-salivary IgA, anti-salivary IgA absorbed with normal human serum and anti-serum IgA. This carrier consisted of molecules having antigenic determinants specific to serum IgA and antigenic determinants specific to transport piece.

Reduced and alkylated salivary IgA showed two precipitation lines with anti-salivary IgA and one line with both anti-serum IgA and anti-salivary IgA absorbed with normal human serum. Comparison on the same slide with serum IgA, salivary IgA and saliva from a patient without IgA, showed that one precipitation line produced by anti-salivary IgA corresponded to serum IgA, the other to the unbound transport piece. Thus this carrier contained molecules of free transport piece with only salivary determinants and molecules of serum IgA with only serum determinants.

The same culture fluid was added to these five carriers and double diffusion tests were made using three different antisera to develop the precipitation lines: anti-salivary IgA absorbed with normal human serum revealing only determinants of the transport piece, anti-salivary IgA revealing determinants of transport piece and of serum IgA, anti-serum IgA revealing determinants of the serum IgA only.

With anti-salivary IgA (Fig. 2A), the autoradiographs showed two labelled lines with all the carriers, one superimposed on the salivary IgA precipitation line, the second on the line given by transport piece.

With anti-salivary IgA absorbed with human serum (Fig. 2B), whatever the carrier autoradiographs showed one labelled line which corresponded exactly to the stained precipitation line of the transport piece.

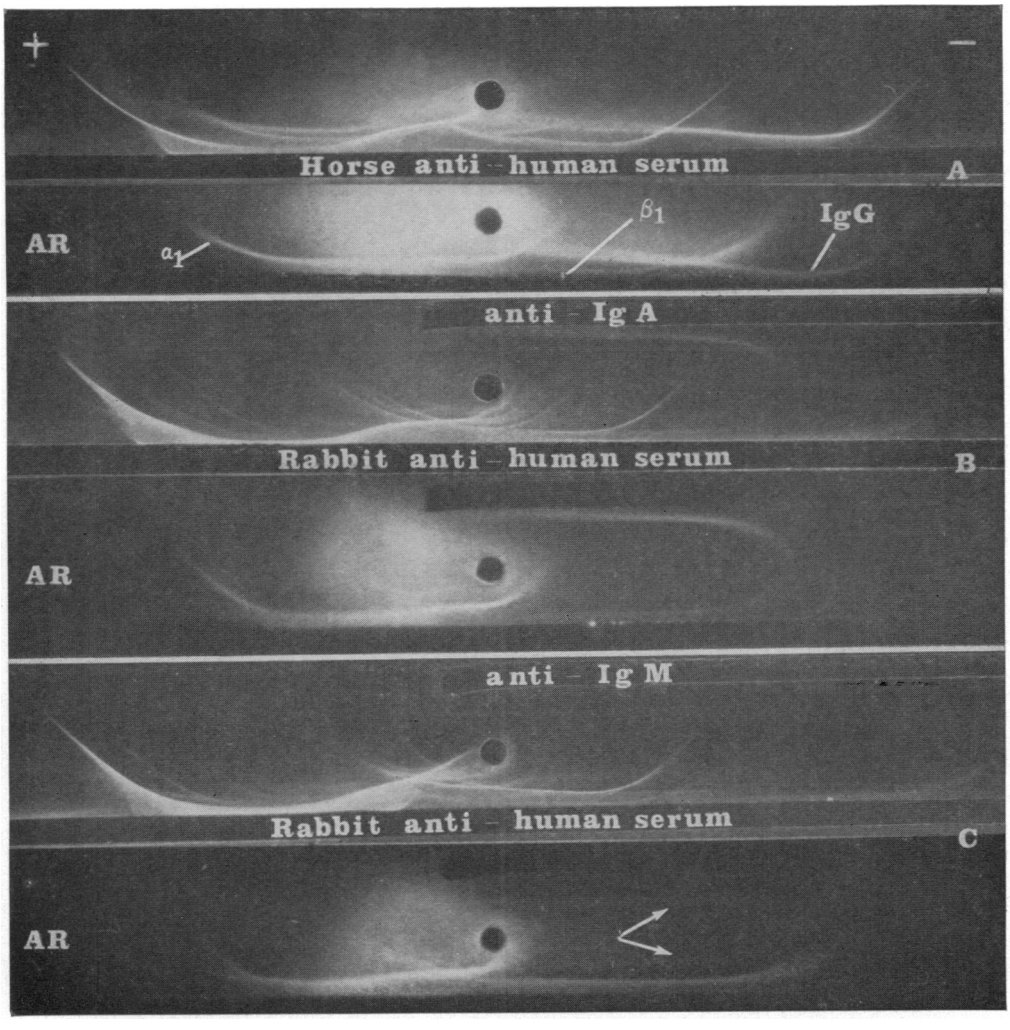

FIG. 1. Autoradiograph (AR) of immunoelectrophoretic patterns made with culture fluids from human submandibular glands. Normal human serum as carrier in all antigen wells. The corresponding stained pattern is above each autoradiograph. A, Carrier developed with anti-human serum; B, carrier developed with anti-serum IgA and anti-human serum; C, carrier developed with anti-IgM and anti-human serum. The arrows indicate the IgM line.

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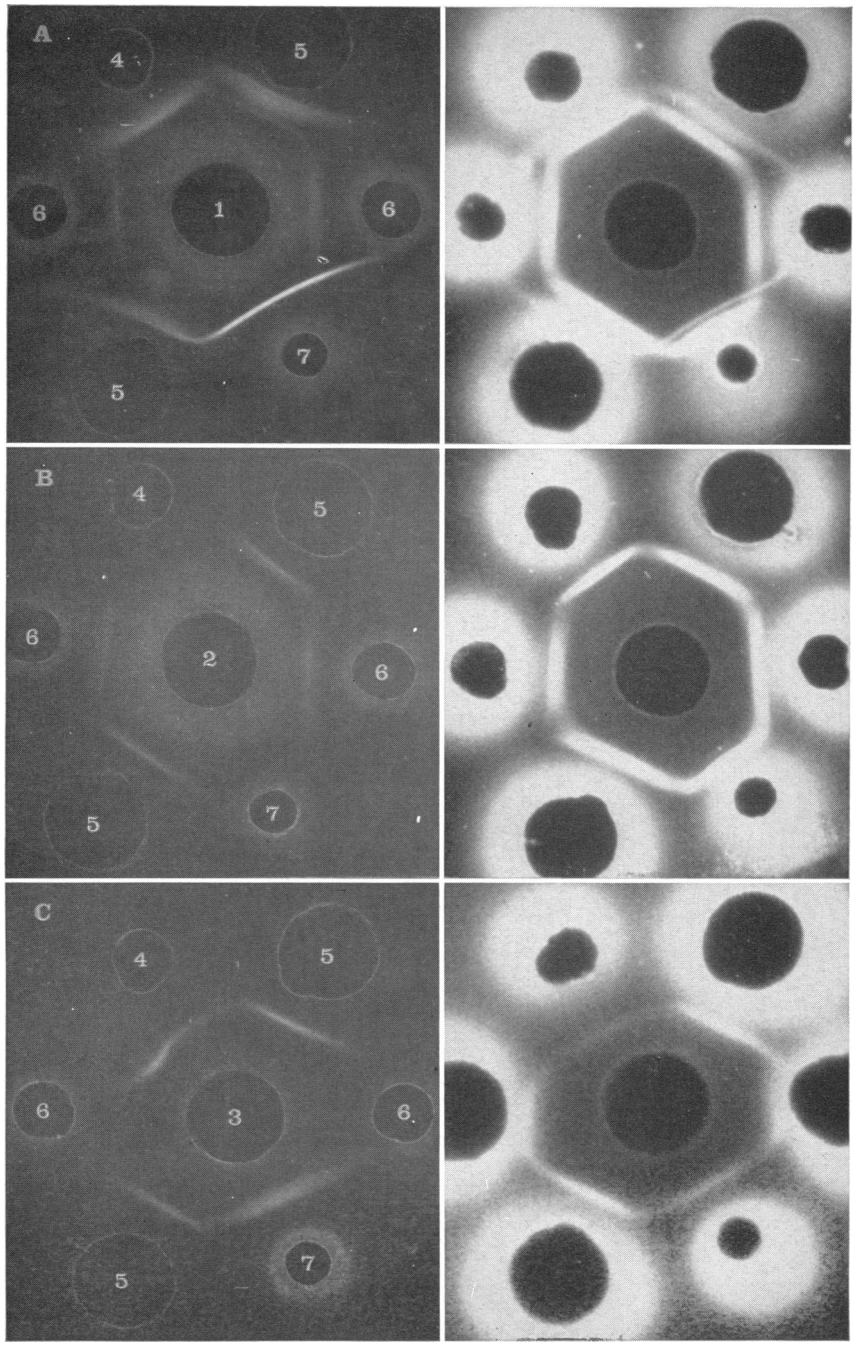

FIG. 2. Autoradiographs (on the right) of double diffusion tests in agar. The stained patterns with the various carriers alone are on the left. The antisera in the central wells are: anti-salivary IgA (1), anti-salivary IgA absorbed with normal human serum (2), anti-serum IgA (3). The carriers in the peripheral wells are: reduced and alkylated salivary IgA (4), normal saliva (5), saliva from a patient without IgA (6), normal human serum (7). The same culture fluid from a submandibular gland was added to all the antigen wells.

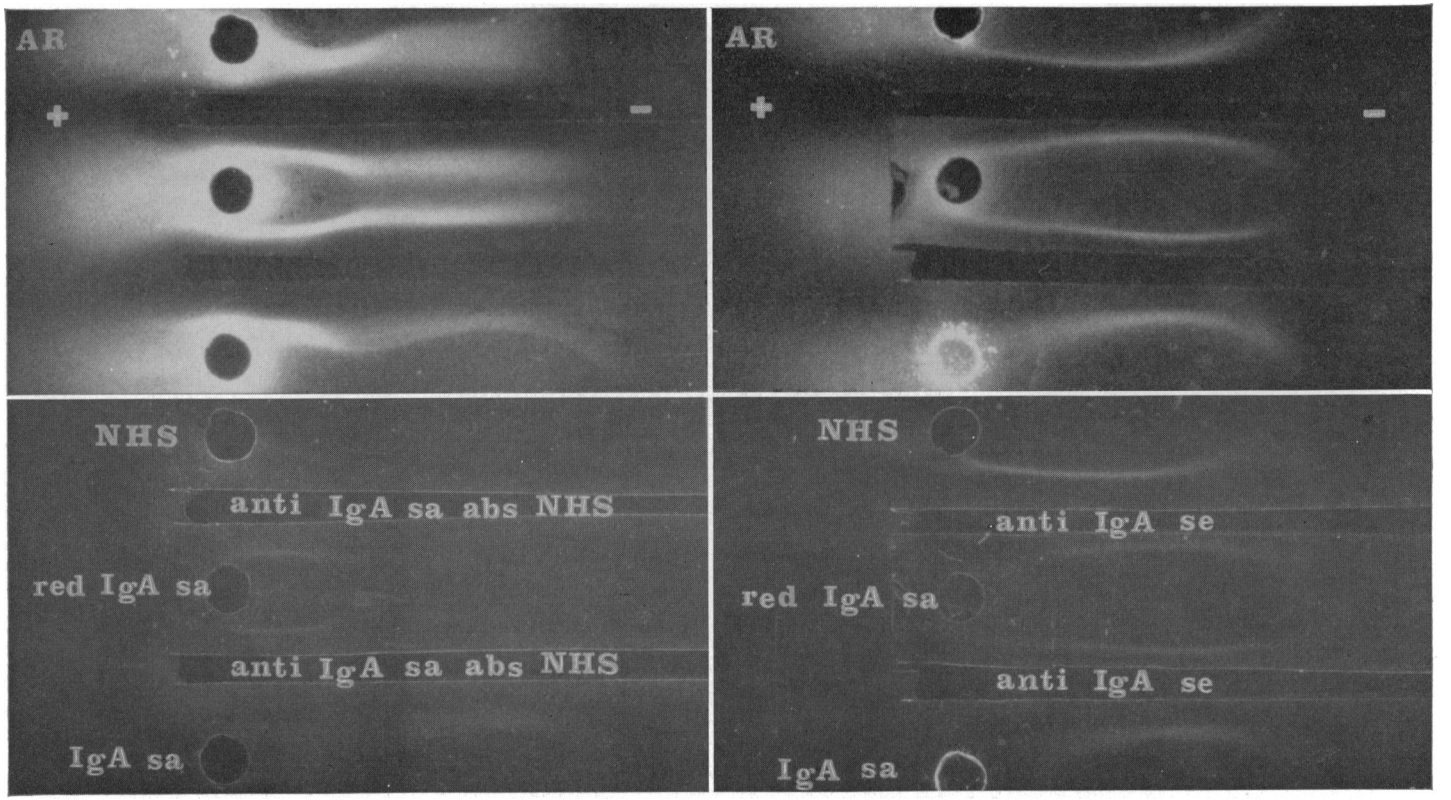

FIG. 3. Autoradiographs (AR) of immunoelectrophoretic patterns made with a culture fluid from a human submandibular gland. The corresponding stained slide is shown beneath each autoradiograph. Three different carriers are in the antigen well: normal human serum (NHS), reduced and alkylated salivary IgA (red IgA sa), salivary IgA (IgA sa). On the left the lines are developed with anti-salivary IgA absorbed with normal human serum (anti-IgA sa abs NHS), on the right with anti-serum IgA (anti-IgA se).

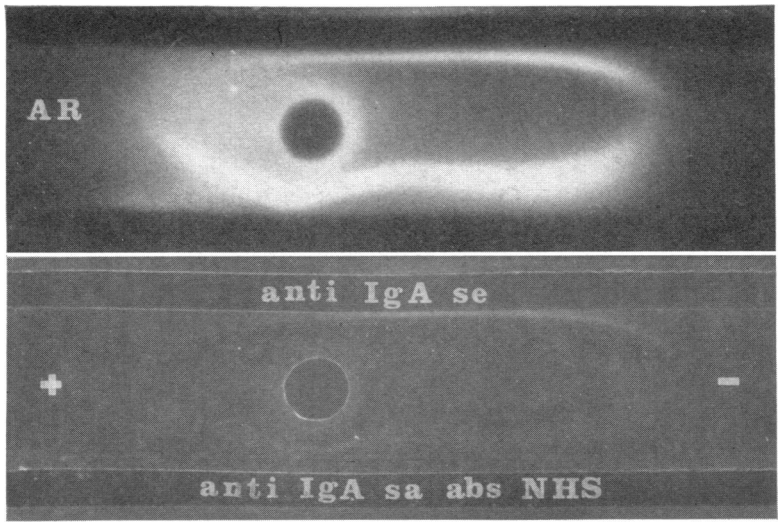

FIG. 4. Autoradiograph (AR) of immunoelectrophoretic pattern (at the bottom) made with a culture fluid from a submandibular gland. Normal human serum as carrier in antigen well. The lines are developed with anti-serum IgA (anti IgA se) on top and anti-salivary IgA absorbed with normal human serum (anti IgA sa abs NHS) beneath.

With anti-serum IgA (Fig. 2C), whatever the carrier autoradiographs showed one labelled line which corresponded exactly to the stained precipitation line of serum and salivary IgA.

These results showed that there is labelling of one line corresponding to the transport piece, and one line corresponding to serum IgA or to salivary IgA. Using the Ouchterlony technique it was difficult to deduce the type of the labelled IgA. If the antibodies against transport piece determinants were absorbed completely in the first precipitation line which corresponds to the transport piece, the second line would correspond to serum IgA; if not, the second line could include precipitation of salivary IgA. Serum and salivary IgA can be precipitated in immunoelectrophoresis without interference from the free transport piece owing to their slower electrophoretic mobility. This technique was, therefore, used to study some of the culture fluids.

The same culture fluid was added to three different carriers (normal human serum, salivary IgA, reduced and alkylated salivary IgA) and, after electrophoresis, the precipitation lines were developed in one slide with anti-salivary IgA absorbed with human serum, in the other with anti-serum IgA. Autoradiographs of the first slide showed a double-curved line with all the carriers (Fig. 3 left). One curve of β-mobility corresponded exactly to the stained precipitation line of the transport piece, the other of γ mobility corresponded to the stained precipitation line of salivary IgA. Autoradiography of the second slide showed one line corresponding exactly to the stained precipitation line of serum IgA (Fig. 3 right).

The difference between the antigenic determinants in these labelled lines was further shown with the following system. Culture fluids were added to human serum as carrier and developed on the same slide with anti-serum IgA, and anti-salivary IgA that had been absorbed with normal human serum (Fig. 4). Autoradiography of this slide showed two labelled lines. Both antisera revealed a line of γ mobility corresponding to the precipitation line of the carrier. The second line was of β-mobility. It was revealed only by anti-salivary IgA absorbed with human serum and did not correspond to the stained precipitation line.

DISCUSSION

Incorporation of labelled amino acids into proteins in tissue cultures has been assumed to represent *in vitro* synthesis of these proteins (Hochwald *et al.*, 1961; Van Furth, Schuit and Hijmans, 1966). Culture fluids from salivary glands without any carrier gave immunoelectrophoretic precipitation lines corresponding to the autoradiographic lines. Therefore labelling was due to *de novo* formation of proteins able to react in agar with the corresponding antiserum.

It has also been demonstrated that autoradiography of immunoelectrophoretic slides gives semi-quantitative information (Asofsky and Thorbecke, 1961). Experiments by Van Furth (1966) with monkey IgG have shown that there is a relationship between the quantity of IgG synthesized and the intensity of the radioactive line.

Our results have shown that an α_1-globulin, a β_1-globulin, and IgG, IgM and IgA are synthesized by human salivary glands. The other serum proteins found in saliva such as albumin, transferrin, haptoglobin and caeruloplasmin probably came from the serum by transudation, since they were not found to be synthesized locally.

The α_1-globulin synthesized in the above system corresponded to a serum protein which

must be present in gastric juice since it was revealed also by anti-serum against gastric juice. We have no information about its function.

The IgG and IgM synthesized *in vitro* were of serum type not possessing antigenic determinants specific to saliva whereas the synthesized IgA was of salivary type possessing determinants supplementary to serum IgA and specific to saliva. Synthesis of IgA but without characterization of its type was also demonstrated by Hochwald, Jacobson and Thorbecke (1964) in monkey and human salivary glands.

The synthesis of free transport piece was demonstrated. This transport piece was precipitated by anti-salivary IgA but not by anti-serum IgA. It was of β mobility. The presence of free transport piece was not a result of dissociation of synthesized salivary IgA, for if this had occurred unbound serum IgA would be present. This was not observed in these experiments. It must, therefore, be concluded that an excess of transport piece over salivary IgA was produced in the *in vitro* system. The free transport piece was found in pooled adult saliva as well as in seven of ten individual adult salivas, therefore a similar disproportionality probably exists in the synthesis of transport piece *in vivo*. In three of ten salivas, salivary IgA without the free transport piece was found, in agreement with South *et al.* (1966a). Saliva may contain the unbound transport piece alone [infantile saliva (South, Cooper and Wollheim, 1966b) our adult patient without IgA in the serum], the transport piece plus salivary IgA (as in the majority of adult and infantile salivas), or salivary IgA alone (saliva of some adults). Serum IgA alone or serum IgA plus salivary IgA was never found in culture fluids or saliva.

The binding mechanism between the transport piece and serum IgA has not been elucidated. However, it must be specific, since the piece, although present in excess, was bound only to IgA never to the other immunoglobulins or proteins in the saliva.

IgA synthesis in the human salivary glands seemed to be five to ten times greater than IgG synthesis, as judged by autoradiographic line intensity according to Van Furth's criteria (1966). Local synthesis of IgM occurred only occasionally and was always slight. The relative concentrations of immunoglobulins synthesized by salivary tissues thus seem to be strikingly different from those found in serum, but parallel to the concentrations of the immunoglobulins in saliva (Chordiker and Tomasi, 1963). This suggests that salivary immunoglobulins result uniquely from local synthesis. Tomasi *et al.* (1965) and Haworth and Dilling (1966) transfused patients with IgA which did not appear in the saliva. This indicates also a local synthesis of salivary immunoglobulins.

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