

A STUDY OF IMMUNE MECHANISMS IN THE
MOUTH IN RELATION TO ORAL DISEASE

by

FRANCES JANE McKEAN, B.Sc. (Hons)

DEPARTMENT OF ORAL MEDICINE AND PATHOLOGY
UNIVERSITY OF GLASGOW DENTAL SCHOOL

THESIS PRESENTED TO THE UNIVERSITY OF GLASGOW
FACULTY OF MEDICINE FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

MAY, 1973

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SUMMARY

of

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The evidence to date, from clinical, epidemiological and laboratory investigations, underlines the causal role of bacterial plaque in two of the most prevalent oral diseases i.e. periodontal disease and dental caries. The possibility of controlling plaque formation or of inhibiting its harmful effects on the teeth and gingivae by the production of salivary and/or tissue antibodies was investigated.

Saliva is a more difficult and variable substance with which to work than serum, and although extensive research has been carried out on the nature of the salivary immune system, with especial reference to Secretory IgA, gingival fluid and gingival tissue immune reactions, there seems to be little correlation of the methodology, the results and their interpretation.

A number of related problems in connection with saliva and gingivae and the possible roles of their components in relation to oral disease were investigated in both humans and small laboratory animals.

Little data exists on the increase in salivary immunoglobulin levels following the concentration of saliva by volume reduction. Lyophilisation, ultrafiltration and polyacrylamide gel were compared as to their efficiency in concentrating stimulated, mixed, human saliva, the total protein and individual immunoglobulins being determined and compared. Reliability and feasibility of method were

evaluated using solutions of human gamma globulin and chymotrypsinogen. It was found that neither total protein nor individual immunoglobulin concentration increased linearly with reduction in volume, therefore volume reduction alone cannot be taken as a reliable indication of concentration of immunoglobulins or total protein in saliva.

Although Secretory IgA is known to differ from serum IgA in that it contains a secretory piece, it has always been assumed that salivary albumin is identical to plasma albumin. The results reported in this thesis suggest the contrary. Immunoelectrophoresis has confirmed the presence of an albumin-like component in mixed saliva. However, although a precipitin arc occurs in the albumin region of mixed saliva against rabbit anti-mixed saliva serum, the arc shows a reaction of non-identity with the plasma albumin: anti-plasma albumin arc; this arc is still present after adsorption of mixed saliva with anti-human plasma albumin. Thus it appears that the mixed saliva:anti-mixed saliva arc in the albumin region does not result from plasma-like albumin. Double-diffusion results in a reaction of identity between salivary and plasma albumins, suggesting antigenic similarities between them; this does not exclude the possibility of a secretory piece. Furthermore, after intravenous injection of human plasma albumin into rabbits, the human albumin appearing in the saliva seems to be a single entity. These results suggest the existence of both a serum-type and salivary-type albumin in saliva, with at least one identical antigenic component, the concentration of the serum-type being related to the degree of periodontal

disease and therefore tissue destruction.

A comparison of salivary and serum IgA levels of patients presenting with different periodontal diseases, with a clinically healthy control group, closely matched for age, sex, medical histories and caries rates was made. Little regard has been taken of the amount of caries or systemic disease in previous similar studies, with subsequent difficulty in relating the results purely to the type of periodontal disease under investigation (and vice-versa). Classification by longevity of the inflammatory lesions resulted in more significant differences between the male salivas and the controls, than the females. On sub-division, trends to definite differences between health and specific diseases appeared. Significant differences in salivary values were recorded in the acute grouping with Acute Ulcerative Gingivitis, Herpes plus Acute Ulcerative Gingivitis, Aphthous Ulceration (in agreement with LEHNER, 1969,b) and acute Herpes plus chronic Gingivitis; serum levels differed in 'convalescent' groups of Acute Ulcerative Gingivitis, chronic Gingivitis and Rheumatoid patients. All values were calculated on the basis of the World Health Organization standards to enable correlation with any future results reported from other laboratories. The study discusses the value of immunoglobulin measurements and emphasises the need for maximum control and correlation of all variables apart from the disease under study to obtain fully meaningful results.

Implantation of cariogenic bacteria in the mouths of rabbits fed a cariogenic diet resulted not in caries, but in gingivitis with a marked inflammatory cell infiltration.

No salivary antibody was detected to these organisms (which adhered to the oral tissues) not normally present in the rabbit mouth. These results were compared to those of humans, by estimating if salivary antibodies were present in control and diseased mouths to an organism implicated in the pathogenesis of periodontal disease; the control group have a higher level than the latter. The role of salivary antibodies compared to tissue immune mechanisms is discussed in relation to these results.

The ultimate investigation on local immunization outlined the difficulties of producing sufficient quantities of salivary immunoglobulin to overcome accumulated bacteria and their products; also the strict control necessary over type and quantity of antigen, route and timing of injections. The highest response was obtained by local immunization after primary systemic challenge. 'Passive' immunization resulted in the appearance in the saliva of the immunogen within a short time (confirming and extending the work of SCHEIN and TUNG, 1962), the amount produced varying with the molecular weight, implying there may only be secretion into the saliva of molecules below a certain molecular weight, in health. Further investigations into local immunization appear warranted.

The possible interrelationships of salivary and tissue immune mechanisms and their relevance to, and roles in, oral disease are discussed, with reference to the results obtained throughout the investigations.

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CHAPTER 1.

INTRODUCTION.

Saliva, as defined by 'The Concise Oxford Dictionary' is a "colourless liquid given by mixed secretions of salivary and mucous glands discharged into the mouth and assisting mastication." Immunity, defined by the same source, means "being proof against contagion."

In the following thesis, it is proposed to expand the definition of saliva especially with respect to some of its protein constituents and to investigate immunity as a property of saliva, in addition to analysing to what extent the functions and properties of saliva are interrelated with other oral immune mechanisms in connection with oral diseases.

Considerable clinical, epidemiological and laboratory evidence has demonstrated the casual role of microbial plaque in both dental caries and periodontal disease (LÖE, THEILADE and JENSEN, 1965; GIBBONS et al., 1966; ALEXANDER, 1970; KELSTRUP and GIBBONS, 1970 and SHEIHAM, 1970). The possibility of inhibiting plaque formation or of altering plaque metabolism, as a means of preventing these diseases, by the production of specific antibodies in salivary secretions or in the gingival tissues, through vaccination, is thus attractive.

It was felt that successful immunization procedures could only be achieved, if adequate methods of analysis of salivary constituents were defined, and small laboratory animals used, to study the mechanisms of initiation of oral

disease.

The aims of the thesis will be to determine the immunoglobulin content of the saliva and serum of patients who presented suffering from periodontal diseases of varying severity and to compare them with that of control subjects having clinically healthy mouths (i.e. no active dental caries and a low Plaque Index value). Before this project was undertaken it was realized that basic work on the manipulation and examination of saliva (from both humans and experimental animals) was necessary before a meaningful patient survey could be carried out. This resulted in interesting observations in connection with the concentration of saliva and also with the immunochemical properties of the albumin in human saliva.

Animal experiments in relation to the production of local immunity in the oral cavity were felt to be important. Consequently, rabbits were used for experiments on the use of bacteria in inducing oral disease and for broadly-based investigations on the production of salivary and tissue antibodies in the mouth after active and passive immunization. It was felt that these results could act as a comparison and guide-line for future human studies on immunization against oral disease.

CHAPTER 2.CRITICAL REVIEW OF THE LITERATURE.

Two of the principal diseases of the mouth are periodontal disease and dental caries. Although, in some areas of dental research there is a larger volume of literature related to the latter than to the former, and this has been included in the review where relevant, the investigations reported in this thesis were principally concerned with periodontal disease. However, their application to dental caries was also considered.

THE MOUTH

A number of microbial ecosystems exist and interact within the oral environment as a whole. The tongue, teeth, hard and soft palates, gingivae and gingival crevice are colonised by, and the gingival fluid and saliva, contain, many different microorganisms, a number being common to some, or all, of these different parts of the mouth. The interaction of these organisms, the factors that affect their proliferation, production of metabolites and their capacity to induce harmful effects, the constituents of the oral tissues and fluids, are all important in influencing the reaction of the host to their presence. The general health of an individual and the capacity of his immune system, at any given time, to cope with the constant change in the oral ecosystems, governs his response to adverse changes in these systems and also his susceptibility to, and recovery from, oral disease.

SALIVA

Saliva is the substance secreted by the parotid, submandibular, sub-lingual and many minor glands, into the mouth, where it bathes the tongue, teeth and mucous membranes. Its functions, like its contents, are varied.

WHOLE SALIVA

Whole saliva is contaminated by food, desquamated epithelial cells, bacteria, gingival fluid and leukocytes emanating from the gingival crevice. Its protein content including enzymes and immunoglobulins varies with diet and length of time after eating (DAWES, 1970), flow rate (MANDEL and KHURANA, 1969), stimulation and the type of stimulation used (JENKINS, 1970) and the general oral health of the subject. (See Table 1.)

It has been shown to contain numerous ions, albumin, salivary α_1 globulin, double component, mucosal α_2 globulin, salivary α_2 globulin, lactoferrin, salivary β_1 globulin, γ globulin, preamylase, amylase, post amylase, CC-cathodic component, salivary mucins, lysozyme and blood group substances along with various growth factors (reviewed by ELLISON, 1960). STOFFER et al. (1962) have reported the presence of orosmucoid (α_1 globulin), caeruloplasmin (α_2 globulin), β_2 macroglobulin (β_2 globulin), lipoprotein (β_1 globulin), transferrin (β_1 globulin) and fibrinogen. Immunoglobulins were identified by MASSON et al. (1965) while BRANDTZAEG (1965) confirmed the presence of IgG and IgM (in very small amounts) and IgA plus secretory IgA in parotid, submandibular and whole saliva.

To identify these proteins, a number of antisera were used and at least 11 precipitin arcs were obtained on immunoelectrophoresis (ELLISON, 1960); even with unconcentrated whole saliva, precipitin bands could still be obtained. The subsequent work has resulted in varying numbers of precipitin lines against unconcentrated and concentrated whole, parotid and sub-mandibular saliva, but it must be emphasised that NO standard antiserum has been used.

Pooling of samples is often necessary to obtain sufficient amounts for analysis and care must be taken to mix only those saliva samples from subjects similar in as many respects as possible. It is not possible to obtain two or more people with exactly the same biological and immunological characteristics, so natural variation must be taken into account, along with variation in estimation of oral health and disease, when examining these saliva samples.

SALIVARY GLAND STIMULATION

Stimulation by different agents results in different flow rates, but subjects with a slow reaction to one stimulus exhibit the same reaction to others and vice-versa (JENKINS, 1970). Chewing of paraffin wax is unpleasant and causes denaturation of salivary proteins; Citric Acid may act as a chelating agent but is a good stimulant. After prolonged stimulation salivary production is reduced and the rate of constituent production can no longer parallel flow rate with concomitant dilution. MANDEL and KHURANA (1969)

TABLE 1. Variability in salivary IgA levels in the same person.

IgA level in parotid saliva (mg%).
Maximal stimulation. +

Different days.	Continuously over 2 hours. 10 ml samples collected.
1.0	1.14
1.04	0.93
1.04	1.10
1.0	1.17
1.03	0.95
0.70	0.80
0.95	0.95
0.84	Mean = 1.01

Mean = 0.95

Standard deviation = 0.12.

Standard deviation = 0.13

Unstimulated = 8.9mg%

Submaximally stimulated = 2.4mg%

+ = Maximal stimulation to salivation, produced by sour fruit candy allowed the most reproducible levels of IgA to be attained. Thus variations due to time of day, relation to meals and prolonged stimulation, were eliminated. Variability approximates the standard error of the IgA determination ($\pm 10\%$).

(From SOUTH et al., 1968)

reported a decrease in Albumin and IgA concentration on increased flow rate; MANDEL and KATZ also reported, in 1970, that pilocarpine stimulation of human saliva gave a twofold increase in all components with the exception of Ca^{++} and IgA, which varied inversely in this case. Fig.1 illustrates the decrease in salivary IgA content on increased stimulation of whole saliva.

Whole saliva is therefore a heterogeneous biochemical mixture with a wide variation of properties, depending on the environment and state of the subject at the time of collection.

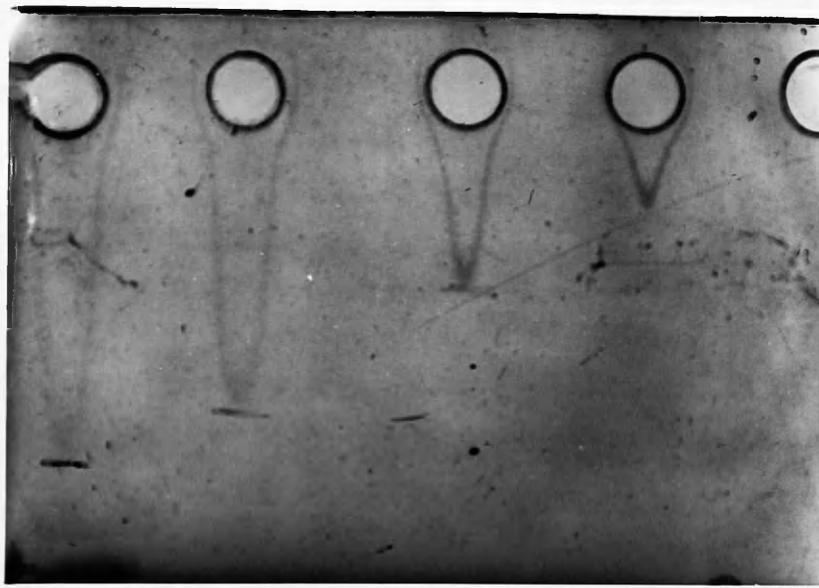
GLAND SALIVA

Saliva from cannulated ducts should be a sterile, clear fluid. Because of cannulation it is more difficult to obtain and must be stimulated to collect sufficient amounts with which to work.

Electrophoresis on cellulose acetate membranes, agar gel and polyacrylamide gel (MEYER and LAMBERTS, 1965, a,b; BEELEY, 1972) results in fewer bands compared to whole saliva. This may be because of contamination of the latter with bacterial substances, epithelial cells and crevicular fluid plus the additional secretion from the minor glands.

SALIVARY IMMUNOGLOBULINS

With the discovery that the IgG/IgA ratio in whole saliva is 1/1 (BRANDTZAEG, 1965) compared to 5/1 in serum, interest turned to the function of IgA in mucosal secretions



+ 20-----3 IgA mg%.
0.20% antiserum.

Fig.1. Decrease in IgA concentration on increased saliva stimulation, measured by the Electroimmunodiffusion technique of MERRIL et al. (1967).

i.e. IgA concentration is inversely related to flow rate.

IgA was measured in parotid saliva, stimulated by fruit gums and collected over 10 minutes.

Slide by the courtesy of IRA SHANNON, 1972.

N.B. In this, and subsequent immunoelectrophoresis figures,

+ represents the anode,

- represents the cathode.

as it was also found to be the predominant immunoglobulin in nasal, bronchial and gastro-intestinal secretions (TOURVILLE et al., 1969; JOHNSON, 1970 and ROGERS, 1970).

There is a great range of variation in the previously reported values of salivary immunoglobulins because of differences in the methods used for collection, concentration and the use of different standards for comparison. This is illustrated in Tables 2a, b, c and d, in which BRANDTZAEG et al. (1970) tabulated concentration values.

There is doubt as to the origin of salivary immunoglobulins. Many workers including ELLISON et al. (1960), STOFFER et al. (1962) and MASSON et al. (1965) referred to the demonstration of serum proteins in saliva. This implies a basic sameness in serum and salivary immunoglobulins; also that there is diffusion of immunoglobulins from the serum, through the gingival tissue into saliva.

Later studies by TOMASI and ZIEGELBAUM (1963), TOMASI et al. (1964), SOUTH et al. (1966), BRANDTZAEG, (1970) and STROBER et al., (1970) proved the existence of a secretory mechanism for IgA into the saliva, apart from the normal production of IgG and IgM. Thus although a small proportion of salivary IgA may originate from the serum, it is thought that 96% is synthesised within the salivary glands. (Reported by HURLIMANN and ZUBER, 1968; STROBER et al. 1970). BRANDTZAEG et al. (1968,a) described the selective secretion of IgM in IgA deficiency. Normally IgM (BRANDTZAEG et al. 1968; BRANDTZAEG 1971,e) and possibly IgG (STROBER et al. 1970; BRANDTZAEG, 1971,e) are selectively transmitted.

Tables 2a and b.

REPORTED VALUES OF SALIVARY IMMUNOGLOBULINS.

(after BRANDTZAEG et al., 1970)

2a

Quantitation of Immunoglobulins (mg/100 ml) in Whole Saliva and Parotid Secretion from Individuals with Normal Serum Levels

Secretion Type	Stim.	Subjects		IgA	IgM	IgG	Method	Standard for IgA	Authors
		No.	Age						
ws	no	4	adults	49.2*	2.6*	1.2*	Aggl.	serum IgA	Adinolfi et al. 1966b
ws	?	4	adults	3.0	N.D.	N.D.	SRID	?	Gabl 1966
ws	yes	30	adults	0.6**	0**	0.5**	SRID	serum IgA	Lehner et al. 1967
ws	yes	16	adults	0.7**	N.D.	N.D.	SRID	serum IgA	Lehner 1969b
ws	no	28	adults	5.95**	0.09**	0.50**	SRID	serum IgA	Rowe et al. 1968
ws	no	16	adults	16	0	0	SRID	?	Waldman et al. 1968
ws(?)	?	6	adults	4.5	0.31	1.06	SSPR	serum IgA	Salmon et al. 1969
ws	no	71	adults	1.15	0	0.83	MDD	?	LoGrippe et al. 1969
ws	no	21	adults	30.38	0.55	4.86	SRID	parotid IgA	Brandtzaeg et al. 1970
ps	yes	6	adults	2.75	0.49	0	SRID	serum IgA	Tomasi & Zigelbaum 1963
ps	?	12	adults	28	N.D.	N.D.	SRID	serum IgA	Chodirker & Tomasi 1963
ps	?	6	adults	2.8	N.D.	N.D.	SRID	?	Gabl 1966
ps	no	10	5-14 yrs.	6	N.D.	N.D.	SRID	serum IgA	Haworth & Dilling 1966
ps	yes	18	adults	9.5	0	0	EID	salivary IgA	Claman et al. 1967
ps	yes	4	adults	<6	N.D.	N.D.	SRID	nasal IgA	Douglas et al. 1967
ps	yes	20	15-20 yrs.	0.76**	N.D.	N.D.	Oudin	serum IgA	South et al. 1968b
ps	?	18	adults	6.3	N.D.	N.D.	SRID	colostrum IgA	Tomasi & Bienenstock 1968
ps	yes	9	adults	3.95	0.043**	0.036**	SRID	parotid IgA	Brandtzaeg et al. 1970

ws: whole saliva.

ps: parotid secretion.

Stim.: secretory stimulation.

Aggl.: agglutination inhibition technique.

SRID: single radial immunodiffusion.

SSPR: "sandwich" solid phase radioimmunoassay.

MDD: micro-double-diffusion.

EID: electroimmunodiffusion.

* Converted from NHS values with our factors; cf. Table I.

** Measurements made after concentration, but expressed here as the original level.

N.D.: not determined.

2b

Immunoglobulin A in Stimulated Parotid Secretions on Repeated Sampling

Subject	Date	Gland	IgA	
			Conc. (mg/100 ml)	Rate of secretion ($\mu\text{g}/\text{min}$)
P.B.	May 22, 1968	Right	1.7	20.7
	June 22, 1968	Right	3.0	29.0
		Left	2.7	41.6
B.A.	June 6, 1969	Right	4.9	17.2
	May 11, 1968	Right	2.5	20.0
	June 27, 1968	Right	6.1	50.0
		Left	5.2	37.4

Tables 2c and d.

REPORTED VALUES OF SALIVARY IMMUNOGLOBULINS.

(after BRANDTZAEG et al., 1970)

2c

2d

No. of subjects:	8		13		21	
	Normal		Periodontitis			
Oral mucosal status:	0.2 ± 0.1* (0-0.4)**		2.9 ± 1.7 (0.7-6.0)		1.9 ± 1.9 (0-6.0)	
Diagnosis	Normal		Periodontitis			
PI score	0.2 ± 0.1* (0-0.4)**		2.9 ± 1.7 (0.7-6.0)		1.9 ± 1.9 (0-6.0)	
Serum:	1473 ± 285 (1144-1870)		1195 ± 335 (910-2140)		1301 ± 339 (910-2140)	
IgG	128 ± 54 (51-222)		124 ± 57 (48-243)		126 ± 55 (48-243)	
IgM	286 ± 91 (148-410)		302 ± 160 (141-669)		296 ± 136 (141-669)	
IgA						
Whole saliva:	1.44 ± 0.90 (0.48-2.46)		6.97 ± 3.36 (0.85-12.71)		4.86 ± 3.82 (0.48-12.71)	
IgG	0.21 ± 0.19 (0-0.46)		0.76 ± 0.54 (0-1.65)		0.55 ± 0.51 (0-1.65)	
IgM	19.40 ± 5.37 (14.16-29.29)		37.14 ± 22.47 (13.21-102.24)		30.38 ± 19.77 (13.21-102.24)	
IgA						
Concentration ratios:						
Serum IgG:IgM	11.5		9.6		10.3	
Saliva IgG:IgM	6.86		9.17		8.84	
Serum IgG:IgA	5.2		4.0		4.4	
Saliva IgG:IgA	0.07		0.19		0.16	

* Mean ± standard deviation.

** Range.

PI, periodontal index (Russell 1956).

Subject	Serum				Secretion (unconcentrated)*				
	Conc. (mg/100 ml)		Conc. ratios		Conc. (mg/100 ml)		Conc. ratios		Rate of secretion IgA (µg/min)
	IgG	IgA	IgG:IgM	IgG:IgA	IgG	IgA	IgG:IgM	IgG:IgA	
P.B.	1845	67	27.5	12.5	0.027	0.023	1.70	0.016	20.7
L.F.	1144	51	22.4	5.7	0.008	0.021	6.29	0.001	29.2
S.G.	1697	137	12.4	5.2	0.000	0.021	4.58	N.D.	42.8
B.A.	1316	222	5.9	5.4	0.013	0.014	2.50	0.005	20.0
T.B.	1267	160	7.9	3.3	0.031	0.030	3.14	1.03	22.0
E.G.	1328	121	11.0	3.2	0.042	0.050	4.74	0.84	34.4
M.U.	1316	112	11.8	5.2	0.040	0.021	4.58	1.90	21.4
I.J.	1870	156	12.0	5.8	0.073	0.106	4.40	0.69	4.40
D.E.	1513	71	21.3	4.6	0.092	0.101	3.62	0.91	0.025
Mean	1477	122	12.1	5.1	0.036	0.043	3.95	0.84	0.009
S.D.	266.6	54.3	85.8		0.030	0.036	1.37		8.7

* Figures for parotid IgG and IgM were calculated from quantitative values of concentrated samples; N.D., not determined.

LACHMANN and THOMSON (1970) have demonstrated that IgA is the antibody in most normal human salivas that gives high titres of immuno-conglutinin activity, i.e. antibody to bound complement components. This may be important in intra-crevicular reactions. BRANDTZAEG et al. (1968,b) noted the 'in vivo' adsorption of IgA onto oral bacteria, and IgA was the antibody in saliva attributed by SIRISHINA (1970), to be produced against α haemolytic streptococci. The depletion of salivary IgA by adsorption to microorganisms may be a protective mechanism in shielding the oral mucous membranes and teeth from attack by bacterial products, but may also deplete the IgA pool and so lower resistance to prolonged attack.

THE SECRETORY IMMUNOGLOBULIN SYSTEM

The structure and function of the principal mucosal antibody has been the subject of a number of reviews and investigations. (BRANDTZAEG, 1968; TOMASI and BIENENSTOCK, 1968; TOURVILLE et al., 1969; BLOTH, 1970; BRANDTZAEG et al. 1970; OGRA and KARZON, 1970; TOMASI, 1970, BRANDTZAEG, 1971a,b,c,d,e and WALDMAN, 1971).

Salivary IgA was found to have a sedimentation coefficient of 11S whereas serum IgA has one of 7S. It has a molecular weight of 390,000 as it consists of 2 molecules of M.W. 170,000 bound to a Secretory Piece (S.P.) of M.W. 50,000 (TOMASI et al., 1965). The S.P. has a faster electrophoretic mobility than IgA. BRANDTZAEG (1970) and TOMASI (1970) produced work that refuted the earlier results

of HURLIMANN et al. (1969) who dismissed the idea of non-covalent as well as covalent linkage between the IgA molecules and S.P. Both linkages are now known to exist between the chains of the 7S monomeric units.

BRANDTZAEG (1968,a; 1970) stated that the increased antigenicity of IgA is due to unfolding, not to the removal of light chains, and that 'in vivo' there is an enzyme-catalyzed process producing inter-unit S-S bonds, with the non-covalent bonds being secondary. Thus secretory IgA has unique physical, chemical and antigenic properties conferred by this non-immunoglobulin glycoprotein secretory piece which appears to stabilise IgA and make it relatively resistant to proteolysis (TOMASI and BIENNENSTOCK, 1968).

Secretory IgA is unique in that the two components are synthesised in two different cells. The dimerised IgA is produced in the sub-endothelial plasma cells and the secretory component in the ductal cells (TOURVILLE et al., 1969). This confirms the mechanism proposed by TOMASI and BIENNENSTOCK (1968). They combine in the inter-cellular spaces and the bound form is secreted. Free S.P. exudes straight out and this explains its presence in patients lacking IgA, as found by HEREMANS and CRABBE (1967), SOUTH et al. (1966; 1968) and BRANDTZAEG (1968). ROSSEN et al. (1967) identified 11S IgA by immunofluorescence studies in tissues lining the oral and respiratory passages in man.

Secretory IgA is also found in trace amounts in the blood of normal individuals (THOMPSON et al. 1969; WALDMAN et al. 1970a,b and BRANDTZAEG, 1971,d). THOMPSON and ASQUITH

(1970) have stated that about 99% of healthy adults have Secretory IgA in their serum, but their report was on a small sample of people and the method of concentration of IgA was not stated. BRANDTZAEG (1971,d) detected it in 59% of normal people with an increase in the IgA myeloma group. He possibly used more sensitive methods of detection because of the hidden antigenic determinant of IgA. The mechanism of its formation is unknown but there may be a "spill-over" of free S.P. and Secretory IgA from the intercellular spaces to serum via the connective tissue ground substance. There is no apparent correlation between Secretory IgA and ordinary IgA concentrations in serum. Its level is elevated in certain conditions eg. liver disease. WALDMAN et al. (1970,b) reported high numbers of sera with Secretory IgA in enteric cases, therefore possibly the breakdown of mucous membranes allows its release into the serum; no study appears to have been made as to whether there are elevated levels in the sera of persons with severe periodontal disease.

Recently HALPERN and KOSHLAND (1970) and MESTECHY et al. (1971) have demonstrated a J chain or F component in polymeric IgA and IgM. It is synthesised along with the corresponding heavy chains by immunocytes (O'DALY and CEBRA, 1971) but its role in stabilization or transport of Secretory immunoglobulins has not yet been established.

Much of the work on Secretory IgA has been carried out by Brandtzaeg. The disadvantage of his work is that only small numbers of test subjects have been used and the secretion is reduced to a minute working volume. However, his results have, in the main, been confirmed by others and

the active transport of IgA into saliva appears generally accepted.

TAUBMAN and GENCO (1971) have suggested that many of the properties of Secretory IgA antibody could be explained (and enhanced) by a preference of this antibody to bind most of its combining sites to a single molecule possessing multiple identical antigenic determinants as opposed to cross-linking two or more such molecules. GENCO et al. (1970) reported that antibodies to Strep. mutans, which is implicated in dental caries, prevent attachment of the organisms to the tooth in the absence of complement. Secretory IgA may be important in preventing this attachment since it may bind by several of its sites to the multiple determinants of a single bacterium.

There is difficulty in producing a good antiserum to S.P.-IgA because of its low antigenicity. Only about 40% of S.P. in parotid gland becomes complexed and the non-spontaneous binding mechanism is unknown. Immunoglobulin may stimulate production of the S.P. whose function may be to stabilise the quaternary structure of the locally produced IgA dimers. Most measurements of salivary IgA have been carried out using serum IgA standards, therefore true correlation with salivary levels is difficult because diffusion takes place at a different rate due to the different molecular size and configuration. This must be taken into account in interpreting results using agar diffusion techniques.

Apart from IgA the majority of the immunoglobulins in

secretions are structurally identical to their counterparts in serum and have not yet shown to be complexed with a S.P. Their functions appear to be the same eg. IgG fixes complement; IgA does not. Elevated serum IgG levels are paralleled by a rise in salivary IgG levels (BRANDTZAEG, 1971,a). Measurement of all secretion components is difficult; TOMASI (1968) has stated that no method provides absolute values for the total protein content of a secretion, especially since preliminary concentration is often necessary.

The specific biological functions of Secretory IgA are, as yet, largely unknown, but because of its relatively high concentration in saliva, it seems reasonable to assume that it plays an important role in relation to oral disease.

CONCENTRATION OF SALIVA AND ITS CONSTITUENTS

Numerous previous studies have utilised concentrated saliva, but there is little data on the success of the methods employed (ELLISON, 1960; STOFFER et al., 1962; SIMONS et al., 1964; LEHNER et al., 1967 and STROBER et al., 1970). LEHNER (1969,b) pointed out that although the saliva he used had been concentrated twentyfold (20x) the constituent concentration might not be to the same level. SHKLAIR et al. (1969), used paraffin wax-stimulated saliva and lyophilised it to give a concentration of 50mg/ml (wt/vol) but protein concentration was not stated.

BRANDTZAEG et al. (1970) pointed out that concentration of saliva results in a loss of protein but this fact does not generally seem to have been taken into account in reporting salivary immunoglobulin levels. It is difficult to interpret

results that have been extrapolated back to concentration in the original volume when it has been assumed that reduction in volume i.e. 'concentration' results in an equivalent concentration of protein or immunoglobulin content. As the levels of IgA in both stimulated and unstimulated whole saliva have been reported to vary between 0.6 and 49.9 mg/100ml (tabulated by BRANDTZAEG et al. 1970; see table 2a) with a mean of 12.39 mg/100ml, it would seem that concentration is not linear, with different methods giving different results.

SALIVARY ALBUMIN

Salivary albumin has been reported to be passively transmitted from serum to salivary secretions (STROBER et al., 1970; BRANDTZAEG, 1971,e) and HALPERN and KOSHLAND (1970) and STROBER et al. (1970) have reported some estimations of its concentration, assuming, as is generally done, that salivary albumin is immunologically identical to serum albumin.

This seems to have resulted largely from the report by ELLISON et al. (1960) on the identity of some serum and salivary proteins. On Ouchterlony diffusion plates, a continuous band was reported between serum albumin and mixed saliva when they were diffused against anti-mixed saliva serum although the "number of reacting compounds in saliva was so great as to render resolution of the bands difficult". Also, a drawing of the immunoelectrophoretic pattern formed by the use of mixed saliva versus anti-mixed saliva serum and anti-human normal serum serum, shows a reaction of identity in the albumin region. (The same experiment is reported in

Chapter 4, Results.) Subsequent studies confirming this report have been made on salivary albumin, using anti-plasma albumin serum, not anti-salivary albumin serum (STOFFER et al. 1962; LEACH et al. 1963; MASSON et al. 1965; BRANDTZAEG, 1965 and 1971,e; MANDEL and KHURANA, 1969; STROBER et al. 1970; ZENGO et al. 1971 and OPPENHEIM and HAY, 1972).

However, the unexplained results reported by SIMONS et al. (1964) that "curiously enough" their anti-saliva serum gave no precipitin arc when allowed to diffuse against different concentrations of human plasma albumin, appears to have received little subsequent notice. There are thus discrepancies in the literature as to whether serum and salivary albumin are identical in all respects.

GINGIVAL FLUID

There is a seepage of fluid from the gingival tissues into the crevice in disease or when trauma of the tissues occurs. The fluid has been analysed by paper electrophoresis (MANN and STOFFER, 1964), agar diffusion (MANN and STOFFER, 1964; BRANDTZAEG, 1965) and immunoelectrophoresis (BRILL and BRONNESHAM, 1960; BRANDTZAEG, 1965) and the presence of a number of immunoglobulins was detected, as was lysozyme (BRANDTZAEG and MANN, 1964). Inflammation of the gingivae results in an increased flow rate (MANN, 1963) and therefore the availability of the proteins, but it may also cause dilution. The immunoglobulins present i.e. ^{IgG,} IgA and IgM appear to be in the approximate ratio of 12:4:1 as found in serum (BRANDTZAEG, 1965).

Crevicular fluid may be collected on filter paper strips

or by capillary tube (SHILLITOE and LEHNER, 1972). These authors also found high levels of complement in crevicular fluid and SHILLITOE (1972) confirmed that the IgA present is of the serum, not the secretory type. It has not yet been determined whether immunoglobulins and complement (present in much lower levels in the crevicular fluid than in serum (SHILLITOE and LEHNER, 1972)) are present in normal crevicular fluid, as all samples have been collected from periodontally diseased subjects, although OPPENHEIM (1970) suggested their presence in the clinically normal gingival crevice. Unfortunately, methods of collection cause local injury, resulting in an inflammatory exudate which appears to be coming from healthy tissues.

ORAL BACTERIA AND PLAQUE FORMATION

The normal mouth has a varied flora. The extent of colonisation of the different oral tissues such as teeth, tongue, gingivae and gingival crevice by these microorganisms, depends on the existing conditions.

Plaque is the deposit that adheres to a clean tooth on abandoning oral hygiene measures (EGELBERG, 1970). Its development is affected by the chemical nature of the environment, the number and nature of the bacteria present, their biochemical activities and the factors affecting these activities. SCHROEDER and de BOEVER (1970) have stated that plaque consists of bacteria, an intercellular matrix of microbial origin and crystallites, in mineralised plaques. Within a period of 7-14 days, it consists of 70% bacteria and 30% intercellular spaces, thus presenting a three-

dimensional picture.

Bacteria do not normally adhere to a clean surface, and accumulation is initiated by the deposition of a non-mineralised cell-free pellicle less than 1μ thick on the tooth surface. It consists of glycoprotein derived from saliva (LÖE, 1969). Bacterial 'opportunism' is probably important with streptococci forming a large part of the initial plaque due to their being the most common organism in the mouth. Plaque is consistently added to and modified, with the filamentous bacteria adhering perpendicular to the tooth surface. Many organisms that compose plaque are intermediate forms or are as yet unidentified. Some have a definite habitat on the tooth which exist and multiply in conjunction with other bacteria, forming a typical ecosystem at a given time, on the tooth surface. This continually changes, being influenced by the oral conditions during growth; see fig.2.

Saliva only appears to aid supragingival plaque formation, although conflicting reports have suggested its importance also in sub-gingival plaque accumulation, which is a slower process (EGELBERG, 1970).

PERIODONTAL DISEASE

Periodontal disease results from an accumulation of dental plaque on the dento-gingival junction. The plaque results from inadequate oral hygiene. Thus a healthy oral condition is altered to a potentially pathogenic one; the plaque and host metabolic reactions interacting to influence the process of the disease, (MACPHEE and COWLEY, 1969).

Inflammation of the periodontium is caused by physical irritation or by the damaging action of substances not normally present at this site. The degree of irritation influences the amount of inflammation and alteration in the tissue which indicates the severity of the disease.

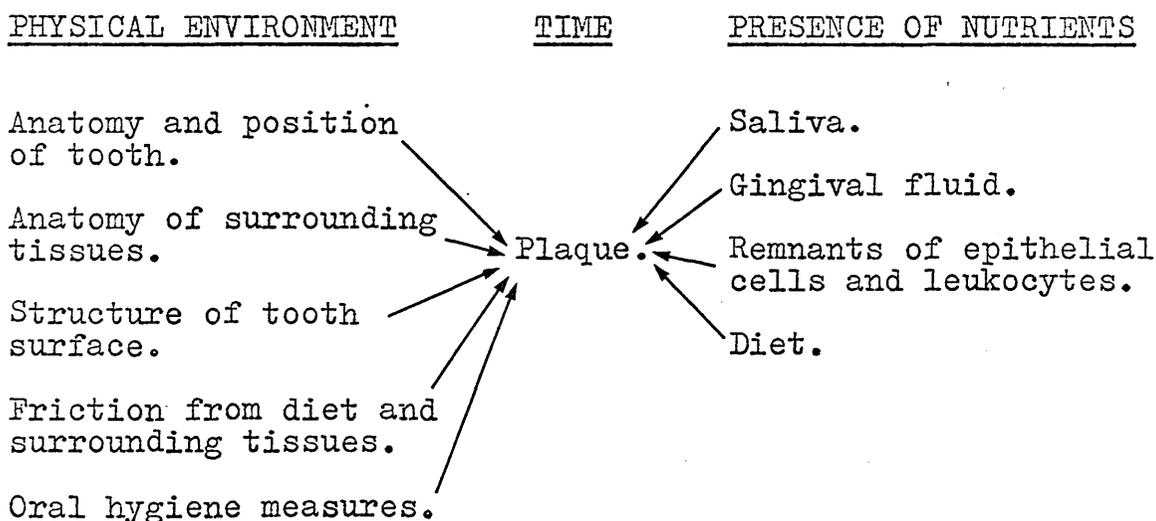


Fig.2. The oral conditions that affect plaque accumulation.

BACTERIAL PLAQUE IN RELATION TO PERIODONTAL DISEASE

The composition of bacterial plaque changes with time. RITZ (1967) studied the composition of plaque over periods of 1-9 days. He reported that during the early stages of plaque development, streptococci, neisseriae and nocardiae predominated, while at the end of the experimental period, the bulk of the organisms were streptococci, actinomyces, veillonella and corynebacteriae. These results illustrate that increasing thickness of plaque with progressively more anaerobic conditions, increases the anaerobic bacterial count. This correlates with the results of CROWLEY and ASH (1969) who reported an increase in filamentous organisms corresponding to the rate of plaque formation. These Gram negative, anaerobic organisms are often, unfortunately, resistant to antibiotics (GOLDBERG, 1970).

Difficulty is encountered in growing the organisms from plaque because of the specialised growth factors they require which are possibly produced by the interaction of metabolites from the oral flora. The microbiota also varies in different sites in the mouth and the wrong sample may be obtained for experimental cultivation of certain organisms (SOCRANSKY, 1970); difficulties therefore amount in obtaining reliable counts. However, it appears that generally, filamentous forms, spirochaetes, fusobacteria, vibrios, streptococci, odontomyces sp. and other anaerobic organisms predominate in older plaque with subsequent changes in products and the antigenic characteristics of plaque (GENCO et al., 1969).

The fact that plaque initiates gingivitis has been

established by the studies of LÖE, THEILADE and JENSEN, (1965); THEILADE et al. (1966) and LÖE and SCHIÖTT (1970). Their experiments have demonstrated that gingivitis was initiated within 9-21 days in a group of young adults with healthy gingivae who had abandoned all oral hygiene measures; the original plaque indices and gingivitis scores could be obtained with the re-institution of oral hygiene measures. Suppression of the plaque, both mechanically and chemically, maintained the plaque index at the low level necessary for a clinically healthy mouth. SAXE et al. (1967) and COURANT et al. (1968) have demonstrated in dogs what has been suggested by epidemiology in man, that plaque causes periodontal disease, as a progression from the initial gingivitis.

This concept has been corroborated by SHEIHAM (1970) in his studies on dental cleanliness and chronic periodontal disease. Those subjects with less accumulated plaque had less severe chronic periodontal disease than persons with poor dental cleanliness, as the accumulation of plaque on the teeth of people with healthy gingivae gave gingivitis which could be resolved one week after cleaning. Persons who brushed frequently, but ineffectively, were found to have more destructive periodontal disease than those with effective, but rarer, tooth-brushing. These results indicate that increase in severity results from increase in plaque and calculus and that duration of irritation is related to severity.

GENCO (1970) has proposed that any process that plays a part in periodontal disease must fulfil three criteria:

- 1) must represent a distinct immunopathologic process, the vehicle(s) for which are in the periodontal tissues or surrounding environment.
- 2) must induce the periodontal disease in experimental animals.
- 3) must be operative in naturally occurring periodontal disease.

This is a modification of Koch's Postulates and may not necessarily be applicable, especially part 2, as CARLSSON and KRASSE (1969) have shown that different organisms are cariogenic in different animals; similarly those bacteria or their products causing periodontal disease may not manifest themselves in the same way in animals.

Thus, as stated by STAHL (1970), the condition of the host, i.e. nutritional status, age, generalised infection and hormones, affects and may accelerate the initiation of, or accentuate the present inflammatory periodontal disease. These factors, plus the composition of the saliva which greatly influences microbial growth and reproduction (BURNETT, 1970) all influence the host's resistance to periodontal disease.

$$\text{i.e. Health or Disease} = \frac{\text{Virulence of parasite} \times \text{Dosage of parasite}}{\text{Resistance of host.}}$$

(BURNETT, 1970)

PATHOLOGY OF THE PERIODONTIUM IN RELATION TO PERIODONTAL DISEASE

A human tooth protrudes through the gingiva and mucous

membrane which acts as a seal between the crown and the root (MACPHEE and COWLEY, 1969). The periodontal membrane of the gingivae consists of collagen tissue, blood vessels, lymphatics and nerves. The gingival tissue (mucous membrane) adjacent to the crown is detached 1-1.5mm, resulting in the gingival crevice (see fig.3).

Mast cells are present along blood vessel walls, in the gingivae, and their action in gingivitis has been described by ZACHRISSON and SHULZ-HAUDT (1967), ZACHRISSON (1967; 1968a,b). They contain many biologically active substances which, when released into blood and surrounding tissues, act as smooth muscle stimulants and can increase membrane and vascular permeability, promoting oedema, stimulation of phagocytosis and interfere with blood clotting; i.e. they play an important role in inflammation. The human gingiva is comparatively rich in these cells and they have been found by ZACHRISSON (1967) to decrease in number as gingivitis increases in severity. Chronically inflamed gingiva was found by ZACHRISSON (1968,a) to be rich in mature and immature mast cells, the great majority of immature cells being found in the pocket area.

On inducing experimental gingivitis by abolition of oral hygiene procedures, and examination of biopsies, ZACHRISSON (1968,b) observed scant numbers of plasma cells contrary to the chronic disease condition, but a large infiltration of polymorphs was present in the sub-epithelial connective tissue. It must be noted however, that inflammatory cells (immunoblasts) are present to a small extent in clinically normal tissue (BRANDTZAEG and KRAUS, 1965; COWLEY, 1966;

CROWN

Enamel

Dentine

ROOT

Gingival tissue { Epithelium
 { Sub-epithelial
 { connective
 { tissue

Pulp

Periodontal membrane

Alveolar bone

Cement

Site of plaque accumulation and initial periodontal disease

Crevice

Blood supply

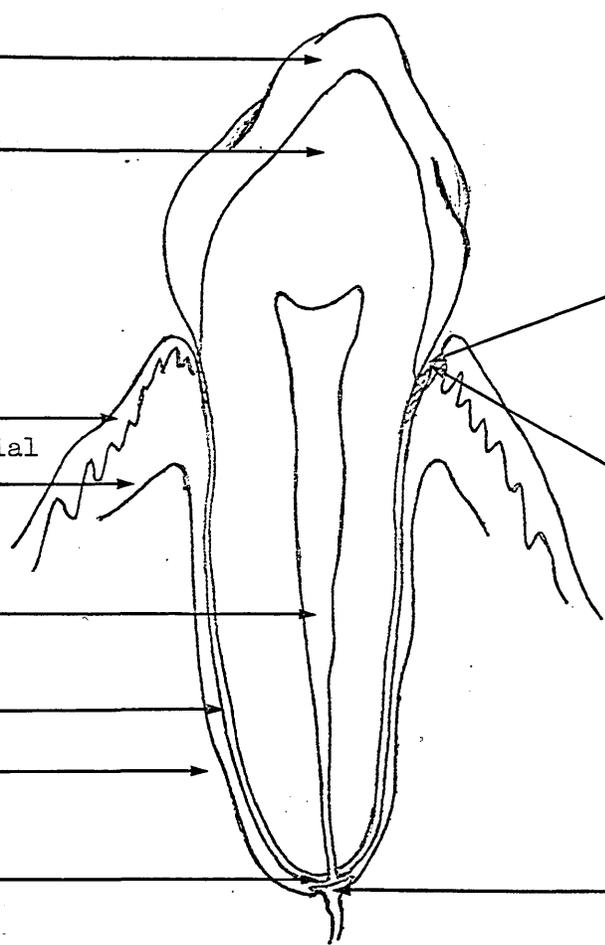


Fig.3. Diagram of the local environment of the tooth.

PORTEOUS, (1969).

There is therefore a definite change in the pathology of the periodontium in mild to chronic periodontal disease, the type of change depending on the disease incurred.

CARIES

Caries is caused by bacteria growing on the tooth surface in the presence of fermentable carbohydrate. The resulting fall in pH causes a demineralization and subsequent to this the minimal organic skeleton is removed, presumably by proteolytic enzymes, resulting in cavities in the teeth. Rate of dental caries development depends on the circumstances that affect bacterial attack, such as salivary flow rate, the intrinsic properties and composition of saliva, diet and the structure and spacing of the teeth (JENKINS, 1970).

BACTERIAL PLAQUE IN RELATION TO CARIES

Plaque formation allows local colonization by streptococci and other acidogenic bacteria such as lactobacilli, which multiply in sufficient numbers to produce the substances that cause dental caries. GUGGENHEIM (1968) found Strep. mutans in deep carious lesions.

The significance of lactobacillus counts in saliva is that they indicate the number of stagnation areas in the mouth where thick plaque has formed and sufficiently acid conditions exist to allow continued growth of lactobacilli (SIMS, 1970). If oral hygiene is improved, a healthy dietary followed and conservative treatment carried out on a carious mouth, the number of areas of stagnation fall, as do the

lactobacillus counts. People with otherwise comparable oral conditions, but statistically significant differences in mean lactobacillus counts, exhibit different caries rates. No qualitative or quantitative difference in the types of streptococci in plaque from 'caries-active' (DMF 20) and 'caries-inactive' (DMF 5 and no clinically detectable lesions for two years previously) mouths was found by ROGERS (1969).

However, as KESTENBAUM (1968) stated, increased plaque does not alone account for dental caries. Sufficient amounts of acid must be produced, therefore the type of organism in the plaque on the tooth surface, and the diet, are important.

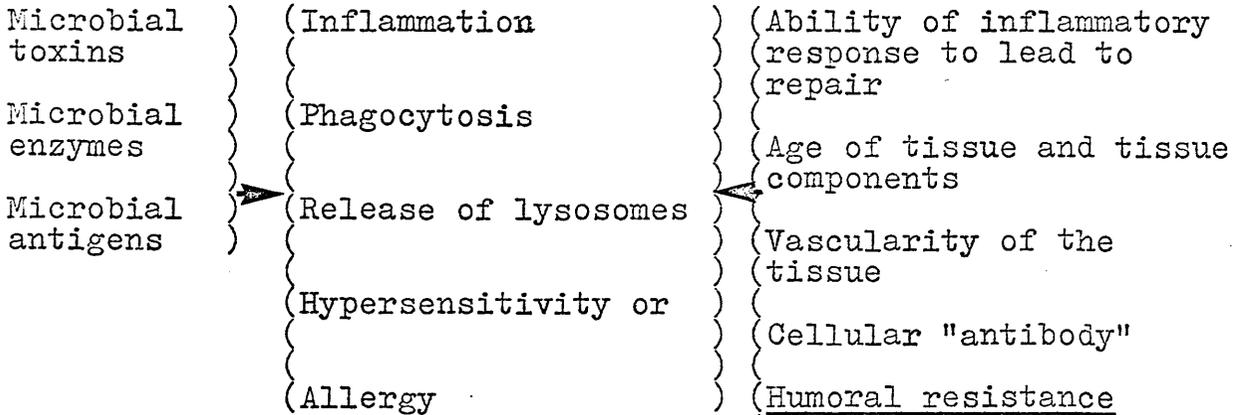
IMMUNOLOGICAL ASPECTS OF ORAL DISEASE

The interrelationship of the factors involved in oral disease is illustrated in fig.4.

It is known that on primary irritation of the gingiva there is a large infiltration of polymorphonuclear leukocytes into the crevice area (TAICHMAN et al., 1965; FREEDMAN et al., 1967; MERGENHAGEN, 1970 and TAICHMAN, 1970), which have a deleterious effect on host tissue. Subsequently, plasma cells appear, as the condition becomes chronic (BRANDTZAEG and KRAUS, 1965; PLATT et al., 1970). Therefore, tissue as well as oral fluid, immune mechanisms are implicated in oral disease.

a) Cell-mediated: Cell-mediated immunity in oral disease due to the infiltration by lymphocytes, has been reviewed by LEHNER (1972). He classified it into four groups.

I - in which lymphocyte transformation is stimulated by plaque bacterial antigens as well as by related organisms. The

INVADERSHOST RESPONSEDEFENCE OF HOSTPlaque micro-organismsTissue resistanceFACTORS THAT UPSET BALANCE

Physical irritants-
 -plaque and calculus
 -faulty restorations
 -toxic material (non-biological)

Occlusal trauma

Hormonal imbalance-
 -pregnancy

Systemic disease

Genetic defects

Nutritional disorders

Fig.4 Interrelationships of factors involved in oral disease.

(After BAHN, 1970).

functions of lymphocyte are affected by serum factors - antibodies or immune complexes. This system may be involved in the pathogenesis of periodontal disease.

II - Immunological studies of aphthous ulceration suggest there may be auto-immune damage of oral mucosa to give ulceration. This could be accounted for by immunological tolerance and cytotoxic potential of sensitized lymphocytes.

The concept of auto-immunity in relation to periodontal disease was first discussed by BRANDTZAEG and KRAUS (1965) and Secretory IgA in gastric juice has been shown to be an auto-antibody to intrinsic factor (GOLDBERG et al., 1968).

III - This involves deficiencies in the factors involved in lymphocyte sensitization and antibody formation i.e.

cytotoxicity, migration inhibition and sometimes impaired delayed hypersensitivity skin reaction to Candida albicans.

Serum IgG and IgM levels fall but salivary IgA increases and lymphocyte transformation remains intact in chronic oral hyperplastic candidosis.

IV - In the various histological changes of leukoplakia and in carcinoma where there is a significant increase in lymphocytes with autologous leukoplakic tissue. Cell-mediated studies may establish if there is impaired tumour surveillance in the leukoplakia-carcinoma relationship.

BARAM and ARNOLD (1970) have stated that there is no known feedback control mechanism to shut down a delayed hypersensitivity reaction after initiation. It is continued till all the antigen is degraded or has diffused from the oral site.

b) Humoral: Bacteria provide the antigenic stimulus which causes the irritation and this effect can be enhanced by adjuvants. As was discovered by STEWART-TULL and WHITE (1964) and WHITE (1964) it is the cell wall mucopeptide that has adjuvant properties. HOLTON and SCHWAB (1966) extended this work and demonstrated that mucopeptide from cell walls of β -haemolytic streptococci (prevalent in the mouth) can also promote the production of antibody. Therefore, the initial composition of plaque may play an important role in the promotion of an antibody response to the subsequent bacterial antigens.

RIZZO (1970) has studied antigen penetration into gingiva, using endotoxin and found that it could not penetrate unaided; however, low levels gave a local reaction and once the tissues were sensitized, very low concentrations only were needed for a secondary response. Endotoxin has been implicated also by MERGENHAGEN and SNYDERMAN (1971) in oral disease, by inducing a humoral response.

NISENGARD and BEUTNER (1970,a) have indicated that serum contains antibody to the oral flora. These antibodies must only be of importance in relation to oral disease if they are demonstrated to be produced against distinct oral pathogens and not to related commensals or organisms causing a previous systemic infection. NIDENGARD, BEUTNER and HAZEN (1968) and NISENGARD and BEUTNER (1970,b) examined the relationship of immediate hypersensitivity to periodontitis in animals and man using actinocin from actinomyces in plaque. Antibody levels and the percentage of allergic individuals increased with increased periodontal inflammation.

In 1971, BERGLUND detected the presence of immunoglobulins in gingiva from cells in the tissue, in addition to serum antibodies. PLATT et al. (1970) supported this and both authors believe that the immunoglobulins produced by these plasma cells have specificity for plaque bacteria. Immune complexes formed by immunoglobulins and bacterial antigens activate the complement system in the gingival pocket, where the fact that the fifth component is chemotactic (WARD and NEWMAN, 1969), may be important; the role of complement as a mediator of inflammation has been outlined by MERGENHAGEN (1970).

It therefore seems that a number of immunological reactions take place in the mouth on the initiation of a disease process. Salivary antibodies may be produced against cariogenic bacteria as well as those implicated in the pathogenesis of periodontal disease. In addition, DICK and TROTT (1969,a) have concluded from their investigations on rats, that traumatic inflammation increases the effects of an immune reaction induced in the oral tissues. Inflammation may thus condition those tissues so that an immune reaction, normally fulfilling its intended homeostatic function, is overactivated and becomes responsible for tissue injury.

PATIENT SURVEYS IN CONNECTION WITH ORAL DISEASE

A number of surveys have been carried out endeavouring to relate patient salivary or serum immunoglobulin levels to the degree of dental caries rather than periodontal disease. An increasing number of investigations are being undertaken to investigate with the role of cell-mediated immune responses

in periodontal disease. In any survey on humans, there is the initial and inherent difficulty of finding subjects with one OR the other disease; the fact that both caries and periodontal disease are likely to be present complicates interpretation of the results.

a) Periodontal Disease: From a search of the literature it appears that salivary immunoglobulin levels have not been greatly studied in relation to periodontal disease. SAITO et al. (1969) have reported a serum protein analysis by immunoelectrophoresis of patients with periodontal disease (classified into inflammatory, atrophic and mixed types). He reported an increase in the gamma globulin fraction concentration and a decrease in albumin, from the control group values, which contradicted the earlier results of SHANNON and GIBSON (1964) and SHANNON et al. (1966) although they agreed with those of KARSHOU et al. (1952), MATSUMURA et al. (1960) and TANIMOTO (1960). NISENGARD and BEUTNER (1970) showed that humoral antibody levels increased significantly as periodontal inflammation increase, though this might be expected, as with a breakdown of tissue, antigen may diffuse inwards more readily, to stimulate antibody production.

Serum immunoglobulin levels are of interest, but seem to have little relevance to periodontal disease, when they alone are measured, as they reflect the general state of health of the subject rather than the particular oral condition. LEHNER (1969a,b) measured serum and salivary immunoglobulin levels in subjects presenting with oral disease (excluding dental caries) in relation to controls; diagnosis was mainly

on clinical grounds. It is in this type of study that use of equivalent indices would be useful for comparison of results. He found:

(a) in acute and recurrent ulcerative gingivitis, during days 1-4, serum IgA levels decreased and IgM increased with a subsequent rise in serum IgG and IgA in the first month of onset of acute ulcerative gingivitis. These findings suggested an immune defect in the pathogenesis of AUG which might account for the high rate of recurrence.

(b) in lichen planus, serum IgG fell and IgA rose slightly, IgM levels being normal; salivary IgA showed little variation. Serum IgG and IgA rose in recurrent ulceration.

LEHNER (1967), IVANYI and LEHNER (1970, 1971a,b) and IVANYI et al. (1972) have studied lymphocyte transformation by plaque constituents from patients with periodontal disease. Their results indicated that lymphocytes from control subjects are not sensitized by those bacterial antigens which stimulate lymphocytes in subjects with gingivitis and periodontitis. These antigens are present in control subjects with normal gingivae and they stimulate lymphocytes from patients with gingivitis or mild periodontitis. Loss of reactivity in severe periodontitis may be due to the loss of feedback inhibition of cell-mediated immune responses (AXELRAD and ROWLEY, 1968) or may be due to a serum inhibitory factor (IVANYI and LEHNER, 1971,b), thought to be an antibody or antigen/antibody complex.

However, the role of salivary immunoglobulins and

cellular responses in periodontal disease, has not yet been fully elucidated.

b) Dental Caries: Apart from the studies implicating salivary immunoglobulins as an important factor in caries development, AITCHISON and CARMICHAEL (1962) reported a relationship between the ABO blood mutations and dental caries. As the study was confined only to blood grouping they state "no attempt was made to list accurately any other observations". There is therefore no indications as to how "caries-free" and "caries-rampant" were defined. A greater liability to caries in Blood Group A was reported.

GELLER and ROVELSTAD (1959) concluded that a factor related either to lysozyme or gamma globulin in parotid saliva inhibited lactobacilli and was related to the caries resistance of the individual. The DMF (Decayed, Missing, Filled, KLEIN et al. 1938) values chosen to indicate the degree of caries in the active and resistant groups were not stated. Protein measurements were by UV absorption which is inaccurate as uric acid in saliva absorbs at these wavelengths.

Another substance reported to be present in saliva is the salivary anti-bacterial factor (S.A. factor) of IWAMOTO and MATSUMURA (1966) which lyses bacteria but is not lysozyme. GREEN (1966a,b) also reported this. It was thought that the bacteriolysin in caries-immune saliva was an antibody, with genetically controlled production. This bacteriolysin has since been suggested to be IgA, but as salivary IgA does not fix complement and complement is necessary for bacteriolysis, it appears to be unlikely. It may lyse bacteria in the presence of complement and lysozyme as secretory IgA in human

colostrum is reported to do (ADINOLFI et al. 1965) but this might solely be the action of lysozyme. WILSON and GREEN (1963) have also reported on differences in salivary gland function between "caries-immune" and "caries-active" persons which suggested that genetic differences in these groups caused differences in immunoglobulin production. GEDDES (1972) failed to demonstrate this bacteriolysin, perhaps because it was thought to be bound by susceptible bacteria and not released till after cell lysis, its presence may be the result of, rather than the cause of low lactobacillus counts, as there are too few organisms in "caries-free" saliva to remove it completely.

In 1962, KRAUS and SIRISINHA reported no statistical difference in gamma globulin levels in parotid saliva depending on the carious state of the individual; their experimental criteria were not clearly defined. LEHNER et al. (1967) found significantly higher serum IgA titres, and lower IgA levels and relatively lower clearance rates in the saliva of "caries-prone" individuals compared to relatively "caries-resistant". Their experimental criteria were clearly defined (although "caries-free" DMF values were high) and the control and experimental groups were matched for age and sex. These results implicated the role of salivary IgA in the control of caries-immunity.

SHKLAIR et al. (1969) found no significant differences in IgA content of whole and parotid saliva of "caries-free" and "caries-active" subjects matched for age and sex. The former had no decay or a DMF of 0 and the latter had extensive decay with at least 7 open lesions. IgA in "caries-free" gave

increased leukocyte opsonisation of cariogenic streptococci 'in vitro' and they concluded that this resulted from their being more non-specific, and the possibility of an immunoglobulin causing caries-immunity was mentioned but not pursued. This was an extension of the work of FRIEDMAN and TONZETICH (1968) who investigated the relationship of oral leukocytes to caries incidence, evaluated on the basis of leukocyte enumeration, disintegration and glucose metabolism. The leukocytes did not significantly enhance rate of glucose oxidation or whole saliva or salivary fractions.

In 1968, KENNEDY et al. reported on a controlled trial to examine serum antibody levels to cariogenic streptococci in humans. They differentiated between "caries-free" and "caries-active" by means of very rigid criteria and included a questionnaire on the subjects' oral case history; there was no significant difference in this between the two groups. Only a slight difference in antibody between "caries-free" and "caries-rampant" was found and the protective effect of serum antibody only suggested by the data. The resistant subjects must have been previously exposed to the organisms because of their existing high circulating antibody titre; oral and other streptococci also have cross-reacting antigens, which might result in these serum titres.

ZENGO et al. (1970, 1971) studied saliva in human caries resistance; parotid and sub-mandibular saliva were used from radiographically and clinically confirmed "caries-resistant" adults (no cavities or fillings) and an equal number of susceptible individuals (15 or more DMF). There was a statistically significant increase in IgA and albumin levels

of sub-mandibular saliva of the resistant persons; otherwise there was no difference and their results throw doubt on the sole use of parotid saliva in the study of immunity to dental caries in humans, increased levels of IgA in whole saliva perhaps coming from the sub-mandibular glands.

c) Summary: Due to differences in controls and experimental methods, evaluation and comparison of results is difficult. No account of the involvement of caries appears to have been made in the study of periodontal disease, and vice versa. Patient surveys are only of use if the limits of the study are clearly defined.

Not all aspects of study on human periodontal disease and dental caries can be initially carried out on human subjects, eg. immunization, therefore some use must be made of laboratory animals.

ANIMAL SALIVARY STUDIES

CEBRA and ROBBINS (1966) studied the rabbit secretory system with reference to colostral IgA. Further studies by GENCO and TAUBMAN (1969) have indicated that IgA, IgG and IgM are all present in rabbit saliva, with IgA predominating, although by DEAE analysis, IgG appeared first in the secretion, in relation to animal age.

VAEREMAN and HEREMANS (1969) have shown that IgA is the predominant immunoglobulin in canine milk, saliva, tears, hepatic bile and intestinal fluid, whilst BISTANY in his work with TOMASI (1970) has reported that rat IgA is the equivalent of Human IgA, though a secretory piece could not be identified. The method of saliva collection and concentration value were

not stated and the system of measurement may not have been sufficiently sensitive to detect secretory piece, therefore its existence cannot be discounted.

Hamster immunoglobulins have been studied by BIENENSTOCK and BLOCH (1970). They found IgM and IgA in serum, based on their physio-chemical characteristics. On studying the immunoglobulins of the Syrian golden hamster secretions, BIENENSTOCK (1970) could identify IgA in colostrum and saliva (it being the only detectable immunoglobulin in the saliva) and in urine. The salivary IgA had no detectable antibody activity, but this was probably due to its being a local secretion and the animals were only immunized parenterally. IgA antibody activity was detected in the serum.

BOWEN (1968; 1969a,b) has experimented with monkeys, but there has been little reported examination of monkey saliva. IgA was demonstrable by acrylamide gel electrophoresis (BOWEN and MANNING, 1970).

Measurements have mostly been made of serum antibody levels in the study of caries and periodontal disease with animals, and little attention has been paid to salivary antibody levels. There is of course, the difficulty of raising antiserum to the animal salivary immunoglobulins, once they are isolated in a pure form.

PERIODONTAL DISEASE IN ANIMALS

Both rats and dogs have been used in experimental induction of this disease. The organism necessary is not so well defined as with cariogenic streptococci but a filamentous species eg. *Actinomyces* sp. has been reported by

KEYES and JORDAN (1964) to be necessary, along with other types of organisms from the subgingival plaque of affected animals, for induction in hamsters. A high carbohydrate diet is also necessary.

In 1966, ROVIN et al. initiated periodontal disease in rats by introducing irritation in the form of silk sutures between the teeth of both gnotobiotic and conventional rats. These plus the microorganisms caused the disease and illustrated the essential role the latter play, for only on their retention was the disease initiated. Polymorphs were found to be present in the gingival tissue, but may be a normal component of these tissues. A similar experiment was conducted by RANNEY and ZANDER (1970) using sensitized squirrel monkeys; comparable results, in that both local irritation and microorganisms are necessary to produce periodontal inflammation, were obtained.

In experimental periodontal disease, inflammation must be present, otherwise it differs from that of man. The mongolian gerbil appears to develop an accumulation of plaque leading to an inflammatory periodontal disease similar to the human type; the pathology of its periodontal tissues are also similar to that of the human, although the coronal anatomy is different (MOSKOW et al. 1968). The disadvantage is that its immunoglobulins have not been studied like those of the other rodents so far experimented with.

DENTAL CARIES IN ANIMALS

It is agreed that to induce dental caries, a cariogenic flora must be present and a caries-inducing diet fed, whether

the animals are conventional, have their indigenous flora depressed by the administration of antibiotics, or are gnotobiotic (reviewed by KEYES, 1968). Certain animal strains are more susceptible than others, but the challenge organism must be successfully implanted for meaningful results.

GIBBONS et al. (1966) discovered that human cariogenic streptococci are similar to rodent strains, several of which synthesise large quantities of capsule-like carbohydrate from sucrose. Non-cariogenic strains form less, therefore capsule formation was implicated as being important in the aetiology of dental caries. KELSTRUP and GIBBONS (1970) have further studied a cariogenic strain isolated from human dental plaque that synthesises large amounts of gelatinous plaque deposits, in gnotobiotic rats fed a sucrose-containing diet. Alveolar bone loss occurred along with dental caries, the site of which depended on the type of diet fed.

BLACKMORE and GREEN (1970) have emphasised that gnotobiotic rats are of more use in the study of animal caries, because only a short experimental period is necessary to produce fissure caries when the rats are mono-contaminated with a cariogenic strain; their use also leads to a reduction in experimental variation.

BOWEN (1969,a) found that monkeys when implanted with cariogenic streptococci, had the highest population of these organisms in the animals with the most lesions. Similarly, those with fewest lesions had the lower number of organisms. In between, correlation of caries and cariogenic streptococci was not found. However, he confirmed earlier demonstrations

by BOWEN (1965a,b), COHEN and BOWEN (1966) and BOWEN (1968) that monkeys are useful experimental models; caries could be induced rapidly without the need for relative gnotobiosis and their results strengthened the hypothesis that dental caries may be a transmissible disease in man.

Hamster caries has been discussed by KRASSE and CARLSSON (1970); their results on the caries-inducing effects of numerous strains of streptococci illustrated the limitations of the animal caries models when studying the role of various microorganisms in human caries and emphasised that the terms "cariogenic" and "non-cariogenic" organisms should only be used with reference to specific animal experiments to avoid confusion.

BACTERIAL IMPLANTATION IN ANIMALS

A cariogenic diet is a prerequisite for studies on bacterial implantation in dental caries (KEYES, 1964; KRASSE, 1965; SHARAWY and SOCRANSKY, 1967; KEYES, 1968; BAHN et al. 1969; BOWEN, 1969a,b; BLACKMORE and GREEN, 1970; GUGGENHEIM et al. 1970; KELSTRUP and GIBBONS, 1970; KRASSE and CARLSSON, 1970; TANZER et al. 1970; GAFFAR et al. 1970, 1971, and HAMMOND, 1971) and periodontal disease (KEYES and JORDAN, 1964; JORDAN et al. 1965; GIBBONS et al. 1966; SHARAWY and SOCRANSKY, 1967; SOCRANSKY et al. 1970, and KELSTRUP and GIBBONS, 1970).

KRASSE (1965) studied the diet necessary for implantation of cariogenic streptococci in hamsters, concluding that a standard powdered caries test diet was necessary for implantation and recovery of the organisms. Different

supplements enhance the growth of different organisms eg. BOWEN (1969,b) favoured a high sucrose diet for the streptococci he implanted in monkeys, whereas KRASSE (1965) favoured either lactose or sucrose for the hamster diet. It is also an aid to recovery of the implanted bacteria, when a particular characteristic for identification is employed such as the resistance to Erythromycin exhibited by Strep. mutans OMZ 176E, used by GUGGENHEIM et al. (1970) in their rat studies.

ANIMAL IMMUNIZATION

NASH et al. (1969) immunized germfree C₃H mice (3-4 months old) with Ferritin, by different routes. Orally stimulated mice produced IgA antibodies mainly, by local stimulation of the gut, whereas intra-peritoneal and sub-cutaneous injections resulted in IgG apart from a little IgM. Repeated injections increased immunoglobulin but not necessarily specific antibody levels i.e. more non-specific antibody is produced on repeated immunization. FAHEY and COOPER (1970a,b) attempted oral immunization of Swiss-Albino mice in experimental Salmonellosis. Intra-peritoneal immunization with temperature-sensitive mutants of Sal. enteritidis (TSF-19) gave protection against oral challenge of Sal. enteritidis; oral immunization also gave protection. Therefore local immunization produced secretory antibody at sites, other than injection sites, in sufficient quantity to protect against lethal doses of related organisms. This may well work for other antigens.

DOLEZEL and BIENENSTOCK (1971) examined the cellular and serum antibody response to oral and parenteral immunization of hamsters with Bovine Serum Albumin (B.S.A.). Their results suggested that the route of immunization and antigen distribution influences the commitment to IgA antibody synthesis; also extraintestinal IgA synthesis may result from intestinal tissue dissemination.

On immunizing rabbits with Strep. sanguis, CARLSSON and KRASSE (1968) found that sucrose intake lowered the tendency for plaque formation due to leakage of serum antibodies into the mouth via the gingival pocket fluid. BEGLUND et al. (1969) measured the immune response to bacterial somatic antigen administered to New Zealand White rabbits via the oral mucosa. Low doses of antigen given intra-mucosally produced antibody-forming cells in local lymph nodes. The cellular infiltrate corresponded with areas of antibody activity; control sections were negative. Larger doses also gave a systemic response; intra-venous injections gave only a systemic response. They concluded that relatively large amounts of antigen, locally injected, gave a greater cellular immune response than intra-venous injection; smaller doses were more effective in producing this response if administered intra-venously.

GENCO and TAUBMAN (1969) executed a number of experiments in relation to stimulation of local secretory antibody by local immunization. They pointed out that the antigen used is important as anti-ferritin antibody was not detectable in rabbit colostrum after local immunization (CEBRA and ROBBINS, 1966). They elicited a local secretory IgA response to

DNP-bovine gamma globulin injected in the mammary gland; injection in footpads did not produce secretory IgA in colostrum. They carried out further experiments in 1971, by immunizing the mammary glands of rabbits with Group A streptococcal carbohydrate, which resulted in specific IgA antibody in the colostrum.

BALL and TROTT (1971) measured humoral antibody after inter-gingival injections of B.S.A. and E. coli endotoxin^{in rabbits.} Only serum antibody levels were measured and there were no controls. As expected, the initial IgM response was followed by IgG (time varied with antigen), and repeated injections gave a plateau of immunoglobulin levels (cf. NASH et al. 1969).

From the evidence cited above, it may be concluded that local antibody production can be stimulated by the injection of a suitable antigen (of correct dose) in a suitable site, though time and type of responses vary with the antigen, site, and species of animal.

ANIMAL IMMUNIZATION IN RELATION TO ORAL DISEASE

There appears to be no report of attempted immunization against periodontal disease, in the literature. There have been a number of attempts at immunization against caries in laboratory animals.

SWEENEY et al. (1966) gave fortnightly sub-cutaneous injections of gamma globulin to caries-susceptible rats (Harvard strain). The gamma globulin was from caries-resistant Harvard rats, but it was ineffective in inducing caries resistance, and this indicated that it is a property that may not be conferred from one rat to another by passive

immunization.

WAGNER (1967) reported that gnotobiotic rats which had been fed a cariogenic diet and immunized against Strep. faecalis showed decreased caries activity when compared with non-immunized controls. It was suggested that this might be due to the protective effect of antibodies secreted into the saliva and in his thesis conclusion (1966) he stated that control of dental caries may be possible through immunization.

GUGGENHEIM et al. (1969) used Osborne-Mendel rats, some of which had the indigenous flora initially depressed to find the effect of immunization against a cariogenic Strep. mutans OMZ 176 (the ones with the depressed flora were infected with the Erythromycin resistant strain OMZ 176E) or glucosyl transferases, on plaque formation and dental caries. The vaccinated rats (injected in the tail vein) gave precipitating antibodies against the streptococci, but antibodies could not be detected against the enzymes. Six sera, chosen at random, had complement-fixing antibodies. The animals kept in relative gnotobiosis had less plaque and lower caries incidence, but it could be concluded that intravenous immunization does not alter the caries activity of rats with indigenous cariogenic flora; there was smooth surface caries in all. Salivary antibodies were not studied.

NRI weanling rats were immunized intra-peritoneally by BAHN (1969) with an extract of the culture fluid of the cariogenic streptococcus strain FA-1, which contained dextranucrase activity. The rats were prepared for oral microbial challenge with antibiotics. The immunized rats had significantly less caries than the non-immunized, 112 days

after challenge. TANZER et al. (1970) showed an inability to immunologically protect Osborne-Mendel rats against smooth-surface caries on immunization sub-cutaneously with Strep. mutans (Streptomycin-resistant) even though high humoral antibody titres were detected. NAKANO and SAITO (1970) pointed out that if experimental mice were already infected there would be an altered response to the immunizing agent if different from the infecting agent.

GAFFAR et al. (1970) have worked with weanling golden hamsters (NIDR strain). All hamsters in the experiment were partially gnotobiotic and they and the controls were fed a Mitchell's cariogenic diet. Those animals immunized with formalin-killed streptococcus plus Freund's Complete Adjuvant and inoculated with the same organism had a 68% reduction in caries compared to the non-immunized inoculated group which had a 55% reduction in caries compared to the non-inoculated, conventional hamsters. The inoculation site was not stated. In 1971, experiments on intra-peritoneal immunization of weanling golden hamsters (NIH strain) with dextransucrase and inoculation with Strep. mutans did not result in a reduction in caries compared to the non-immunized group. Both groups were fed the cariogenic diet and had their flora depressed before inoculation. Serum and salivary antibodies were produced that inhibited the 'in vitro' production of the polysaccharide by the enzyme, therefore antibodies may affect the catalytic site of the enzyme.

BOWEN (1969,b) used monkeys for the induction of rampant caries because the carious lesions produced in these animals is more similar to that of humans than those produced

in other experimental animals. He (1969,a) infected young monkeys with Strep. ingbritt; they were partially gnotobiotic due to Erythromycin in their drinking water for 12 months. Other, older monkeys were infected with a dextran-producing streptococcus and both groups were fed a diet containing 15% protein and 18% sucrose. Caries took longer to develop in the older monkeys (both fissure and smooth surface caries developed) but caries could still be produced rapidly without resorting to partial gnotobiosis. He also found that after 18 months, those monkeys that had been vaccinated prior to infection with a known streptococcus, showed 80% less carious activity than the unprotected monkeys. Though only 6 monkeys were used and no salivary antibody titres were recorded, it may be concluded that the reduction in caries might be due to immunization in this case.

It appears, therefore, that in carefully controlled conditions, the local oral immune response may be stimulated into antibody production. The role of the antibodies and tissue responses is still speculative as is the true success of the immunization procedures employed.

CONCLUSION FROM LITERATURE REVIEW AND SUMMARY OF AIMS OF PRESENT THESIS

The long term aim of the present research was to investigate the feasibility and effectiveness of immunization as a preventive measure against oral disease.

Keeping in mind the information gleaned from the survey of the literature, a survey of the immunoglobulin levels in

patients suffering from periodontal disease and an investigation into the production of local immunity in the mouth using small laboratory animals were undertaken. Preliminary work was carried out in order that the results of the main investigations might be more meaningful, and as a result, the report of the experimental work may be classified into the five sections stated below.

- a) The effects of different methods of concentration on immunoglobulin and total protein levels in stimulated, mixed, human saliva.
- b) Immunological studies on human salivary albumin.
- c) An investigation into the salivary and serum IgA levels of patients suffering from different types of periodontal disease.
- d) The effect of implantation of cariogenic streptococci in the rabbit mouth.
- e) Studies on the local immunization of rabbits.

CHAPTER 3.THE EFFECTS OF DIFFERENT METHODS OF
CONCENTRATION ON IMMUNOGLOBULIN AND
TOTAL PROTEIN LEVELS IN STIMULATED,
MIXED, HUMAN SALIVA.INTRODUCTION

The comparison of the results of immunoglobulin estimations from different laboratories is now generally realized to be unsatisfactory (ROBBINS, 1968). Difficulty occurs in salivary immunoglobulin measurements because of the small quantities of these substances in the secretion. Therefore equivalent methods of collection, concentration and estimation compared to a common standard must be used for a satisfactory comparison.

As little is known concerning the induction of antibodies in saliva it was felt that data on concentration of saliva was an essential prerequisite to the experimental programme. Collection of saliva from experimental animals involved stimulation by intra-peritoneal injection of pilocarpine hydrochloride with the concomitant disadvantage of dilution (MANDEL and KHURANA, 1969); this was necessary to collect sufficient amounts for analysis. To obtain basic data therefore, stimulated, mixed, human saliva was selected for the experiment as it can readily be obtained in sufficient quantities to allow adequate experimental design.

Several problems arise in attempting to obtain a homogeneous sample, due to differences in the salivary glands of the people from whom the saliva was collected, the time lag between eating and saliva collection, the individual degree of stimulation necessary to produce a satisfactory

flow rate to collect a sufficient sample within a reasonable time and the oral health of the individual. The latter two factors are important as with intense stimulation, the flow rate increases markedly but the protein and immunoglobulin content decrease (MANDEL and KHURANA, 1969), and if the gingival tissues are inflamed, immunoglobulins from the crevicular fluid leak into the saliva (BRANDTZAEG, 1966). Intense stimulation over a period of time longer than 30 minutes, 'tires' the glands (JENKINS, 1970). Pooling samples should, however, have evened out these differences.

MATERIALS AND METHODS

Collection of human saliva-

Mixed human saliva was stimulated with 5% Citric Acid and the secretion collected over ice. It was stored at 4°C until sufficient had been collected and was then pooled and centrifuged at 15,000 rpm (27,000g) for 40 minutes at 4°C to remove microorganisms and epithelial cells. The supernatant fraction to be used was stored at 4°C and that not for immediate use was frozen at -20°C.

Dialysis of saliva-

Dialysis tubing (3cm in diameter) was washed overnight in distilled water. The saliva supernatant was then dialysed against several changes of distilled water at 4°C for 24 hours.

Concentration of saliva-

A minimum number of 12 aliquots of saliva were concen-

trated by each method specified under the different headings.

a) Lyophilisation: 2x100ml, 1x50ml and 1x10ml amounts of the dialysed saliva supernatant were placed in lyophiliser flasks and shelled in a mixture of dry ice and acetone. The saliva was then evaporated to dryness under vacuum and reconstituted with distilled water to aliquots of 1ml, apart from the contents of one flask which were reconstituted to the original volume of 100ml.

b) Ultrafiltration: 2x100ml, 1x50ml and 1x10ml amounts of saliva supernatant were placed in an Amicon cell (Scientific Systems division, Amicon Corporation, 21 Hartwell Avenue, Lexington, Massachusetts 02173, USA) with a UM-20membrane and concentrated to 1ml under a positive Nitrogen pressure of 40-50psi at 4°C; as much saliva as possible was recovered from the membrane by washing with minimal amounts of distilled water. The membrane was thoroughly washed with distilled water between the concentration of different saliva samples. One 100ml amount reduced to 1ml, was reconstituted to the original volume of 100ml.

c) Polyacrylamide gel: The appropriate amounts of Lyophogel (Gelman Instrument Co., packed by Hawksley and Sons, 12 Peter Road, Lancing, Sussex), calculated by determining the volume of fluid to be removed and dividing by five to give the weight needed, were placed in 1x100ml, 1x50ml and 1x10ml aliquots of saliva supernatant, and after 16 hours at 4°C the resulting 1ml aliquot in each case, was transferred to a sterile serum bottle.

Controls of Kabi Gamma globulin (Kabi Pharmaceuticals Ltd., Bilton House, Uxbridge Road, Ealing, London, W.5.) and

Chymotrypsinogen (Miles-Seravac (Pty.) Ltd., Moneyrow Green, Holy-port, Maidenhead, Berks., England) were also concentrated as described in a) and b).

Reconstitution of Lyophilised saliva in Disodium Ethylenediamine tetra-acetate, (EDTA) (DAWES, 1963)-

- a) 0.1g EDTA was added per 100ml of saliva before dialysis and lyophilisation.
- b) 0.1g EDTA was added per 100ml of saliva after dialysis and before lyophilisation.
- c) The saliva was reconstituted in 0.1M Veronal Buffer, pH 8.6, containing 3g or 10g of EDTA/litre.

Quantitation of protein-

This was carried out by the Lowry method (LOWRY et al. 1951) using Chymotrypsinogen in the appropriate range of dilutions, as standard. The average of a minimum of two values for each estimation was made; those results outwith 4% of the average, were repeated.

Quantitation of immunoglobulin-

The technique of Radial Immunodiffusion (SRID) of MANCINI, CARBONARA and HEREMANS (1965) was used to measure the IgA and IgG in the saliva and also to measure the IgG in the standard Kabi Gamma globulin solution; the diameter of the precipitin ring being related to the antigen concentration used.

2g Oxoid 'Ionagar' no.2 (Oxoid Ltd., Southwark Bridge Road, London, S.E.1) plus 0.1% Thiomersal was dissolved in Michaelis diethyl barbiturate buffer, prepared by adding

14.8ml of 0.1N HCl to 100ml of 0.1M Barbitone Sodium, with the pH adjusted to 8.6 with 0.1N NaOH or 0.1N HCl. 0.15ml of the appropriate Hyland antiserum (Baxter Laboratories Ltd., Thetford, Norfolk, England) (tested to check the appropriate heavy chain specificity by Immuno-electrophoresis against normal human serum) i.e. goat anti-human serum IgA (α chain) or goat anti-human serum IgG (γ chain) which had been brought to 48°C in a water bath was added to 3ml of molten agar (cooled to 50°C) and the mixture flooded over a clean glass slide. Both 1mm and 2mm wells were punched in the solidified agar. Sensitivity of the 1mm wells was found to be poor although they required less antigen, due to difficulty in filling the wells and reading the results accurately. A wider range of correlating results over more than one estimation was obtained from 2mm wells. A mean of at least two readings was taken for each sample using a calibrated eye piece, after enhancing the rings by the addition of 1% Tannic acid to the plates. Those values that were outwith a 3% limit of the mean were repeated. Hyland Immunoglobulin standards were used to obtain a standard curve and these were later calibrated against the MRC Immunoglobulin standards (World Health Organization International Laboratory for Biological Standards, Division of Biological Standards, National Institute for Medical Research, Mill Hill, London, N.W.7). The standard curves were obtained by plotting diameter of the precipitin ring in mm against concentration of the reference immunoglobulin in mg/100ml on semi-logarithmic graph paper. The lowest value of standard immunoglobulin that could be measured with a deviation in result no greater than 3% was 0.5mg/100ml. Lower concentrations increased the

inaccuracy of the measurements.

Immuno-electrophoresis-

This was carried out, using Shandon equipment, by the method described in the manufacturer's manuals (Shandon Scientific Company Ltd., 65 Pound Lane, Willesden, London, N.W.10). The electrophoresis gel consisted of 1% Ionagar in 0.025M Barbitone/Acetate Buffer, pH 8.6, incorporating 1% Sodium Azide as a preservative. The wells punched in the agar with the standard punch were filled with the sample and a few grains of Bromophenol blue (G.T. Gurr Ltd., Coronation Road, Cresswick Industrial Estate, High Wycombe, Bucks., England) were added to one well to monitor the speed of electrophoresis. After electrophoresis (using 0.1M Barbitone/Acetate Buffer, pH 8.6 plus 5ml 5% Thymol in Isopropanol/litre as preservative in the tank) at 10V/cm for 1.0-1.5 hours, the troughs were filled with antiserum (Hyland) and diffusion allowed to take place in a moist atmosphere, at room temperature, for 48 hours. Cooled, molten electrophoresis gel was poured over the plate to prevent distortion of the troughs before the slides were dried at 37°C and stained by triple protein stain (CROWLE, 1961) and mounted in Harlicot Synthetic Resin (Kodak Ltd., Kirkby, Liverpool, England).

RESULTS

Estimation of immunoglobulin levels and total protein in mixed saliva:

Centrifugation caused a drop in protein levels as determined by the Lowry technique, from 70 to 67 mg/100ml,

probably due to the fact that salivary immunoglobulins adsorb to the surface of oral bacteria (BRANDTZAEG et al. 1968). There was a further drop to 52 mg/100ml on dialysis, probably due to a further adsorption to the dialysis membrane.

On measuring total protein concentration, it was noted (fig.5) that ultrafiltration and lyophilisation give similar increases in concentration up to 50x reduction in volume, the former being slightly more effective, until 100x decrease. At this final reduction in volume, lyophilisation appeared to be more effective, perhaps due to fine particles of undissolved protein being included in the assay. Lyophogel was ineffective in concentrating the proteins in the salivary supernatant but did effectively reduce the volume.

Measurement of IgA in saliva by quantitative immunodiffusion gave the results shown in fig.6. Lyophogel did not concentrate this immunoglobulin. Ultrafiltration concentrated it to a greater extent than lyophilisation at 100x reduction in volume.

Gamma (γ) globulin estimations-

In conjunction with saliva concentration, γ globulin solutions of known concentration (5 mg/100ml physiological saline) were reduced in volume by the same methods. This was carried out to determine whether a protein solution, chemically less complex than saliva, would concentrate in a linear manner and to give an indication whether other constituents of saliva might affect the concentration of immunoglobulins.

As can be seen in fig.7 ultrafiltration was most

effective, giving greater than 50x increase at the 100x decrease in volume. Lyophogel was not effective.

Fig.8 shows a similar result with the quantitative immunodiffusion.

Determination of degree of denaturation-

None of the methods concentrated as effectively as had been anticipated and to determine whether lyophilisation and ultrafiltration actually denatured the proteins in saliva, solutions of chymotrypsinogen and γ globulin were reduced in volume 100x and reconstituted to their original volumes, as was the saliva supernatant. On full reconstitution after lyophilisation, no protein was lost, as shown in fig.9, but on reconstitution to a smaller volume and extrapolation back to the original volume, the protein values fell. Similar results were obtained by ultrafiltration. These results were also paralleled using SRID for measuring IgA and IgG in the concentrated and reconstituted saliva supernatant and the solution of γ globulin.

The addition of EDTA to the supernatant either before or after dialysis, before lyophilisation or ultrafiltration or reconstitution of the freeze-dried material in Veronal buffer containing EDTA, did not result in increased concentration. Addition of EDTA after dialysis but before concentration gave the best reconstitution value, being the same as that obtained without addition of EDTA whereas the other results were lower.

Final analyses-

Final concentration values for the samples analysed and quantitated are shown in Tables 3 and 4.

Figs. 10a and b demonstrate the normal electrophoretic mobility of serum proteins including IgA and IgG. After saliva concentration, IgG was checked by immunoelectrophoresis to determine whether it had altered properties (fig.11). Apart from a slight increase in intensity of the precipitin band, neither concentration by lyophilisation nor ultrafiltration appeared to alter the electrophoretic mobility of immunoglobulins.

Figs. 12a, b and c illustrate the proteins detected in normal, concentrated human saliva (concentrated by either lyophilisation or ultrafiltration) by the immunodiffusion technique.

Fig.5. Concentration of human, mixed saliva supernatant (sup) by Lyophilisation, Ultrafiltration and Polyacrylamide gel; measurement of total protein by Lowry.

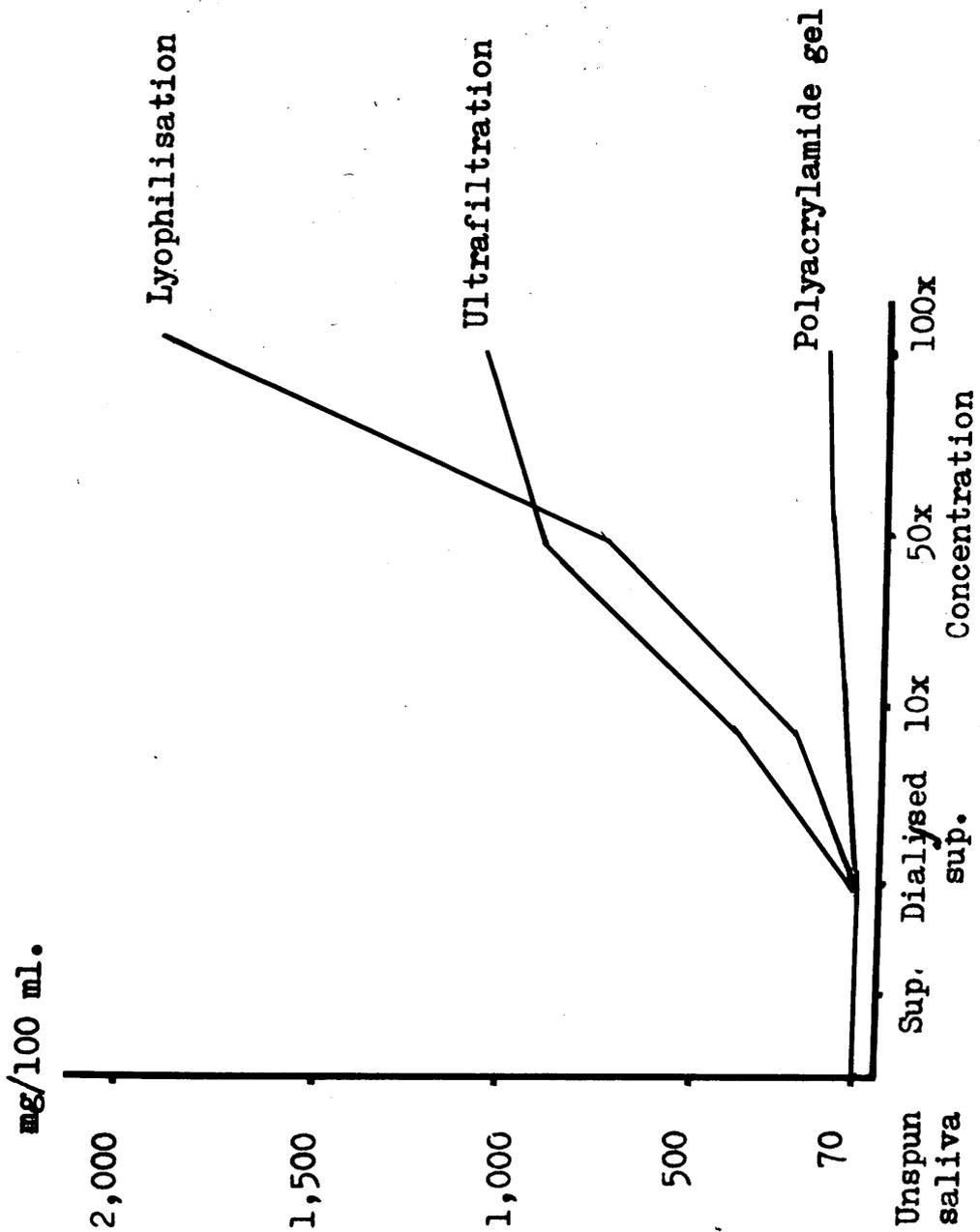


Fig.6. Concentration of human, mixed saliva supernatant (sup) by Lyophilisation, Ultrafiltration and Polyacrylamide gel; measurement of IgA by SRID.

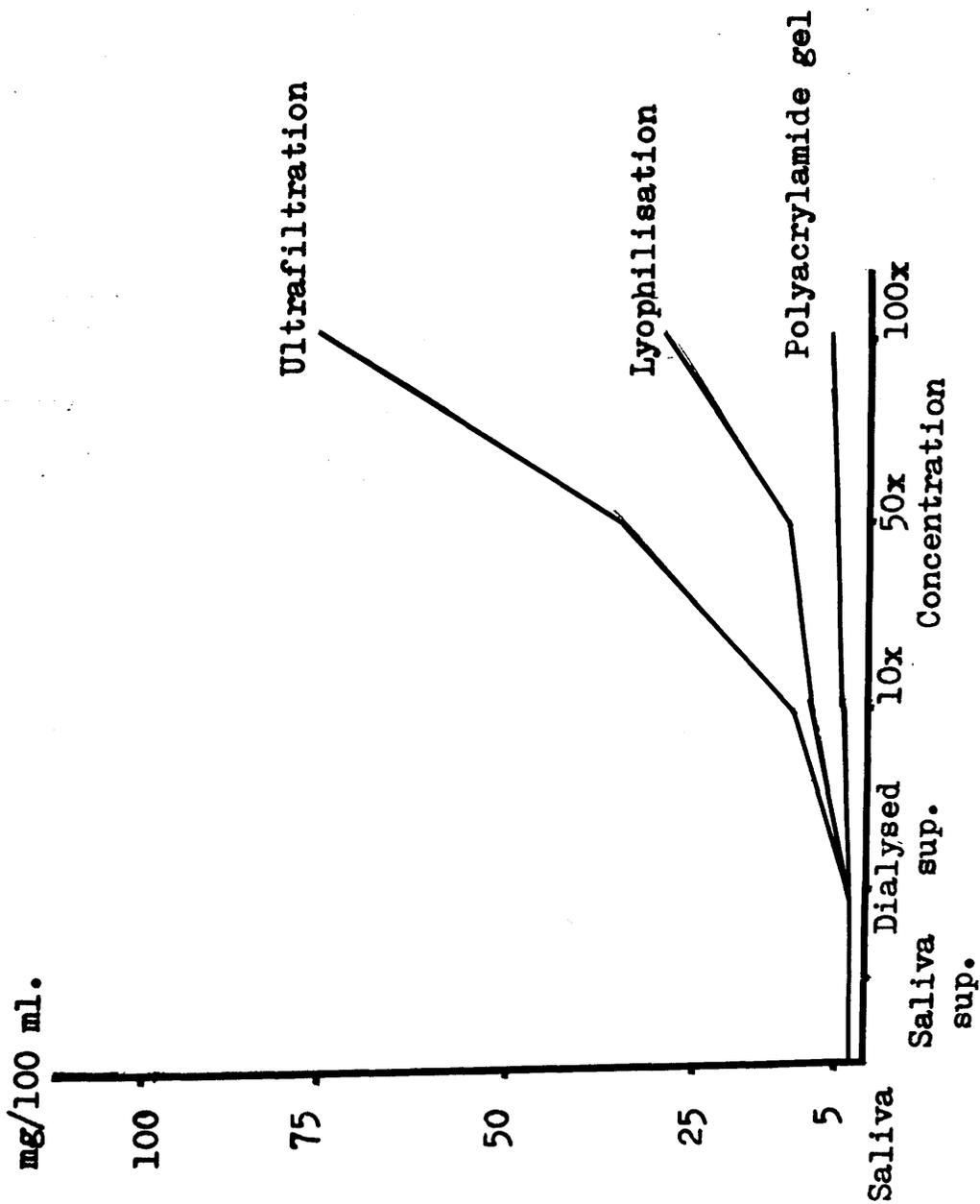


Fig.7. Concentration of Gamma globulin (γ G) by Lyophilisation, Ultrafiltration and Polyacrylamide gel; measurement of γ G by Lowry.

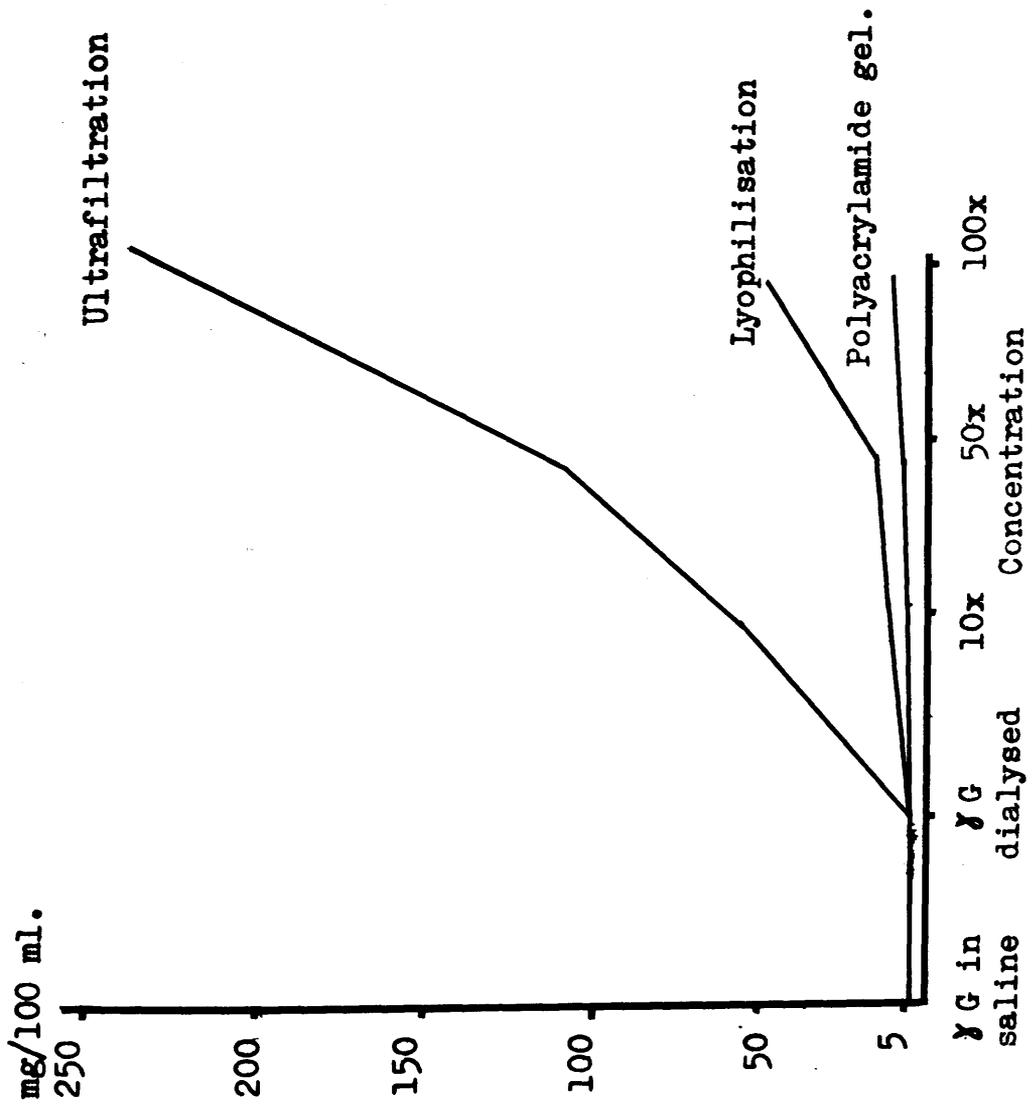


Fig.8. Concentration of Gamma globulin (γ G) by Lyophilisation, Ultrafiltration and Polyacrylamide gel; measurement of IgG by SRID.

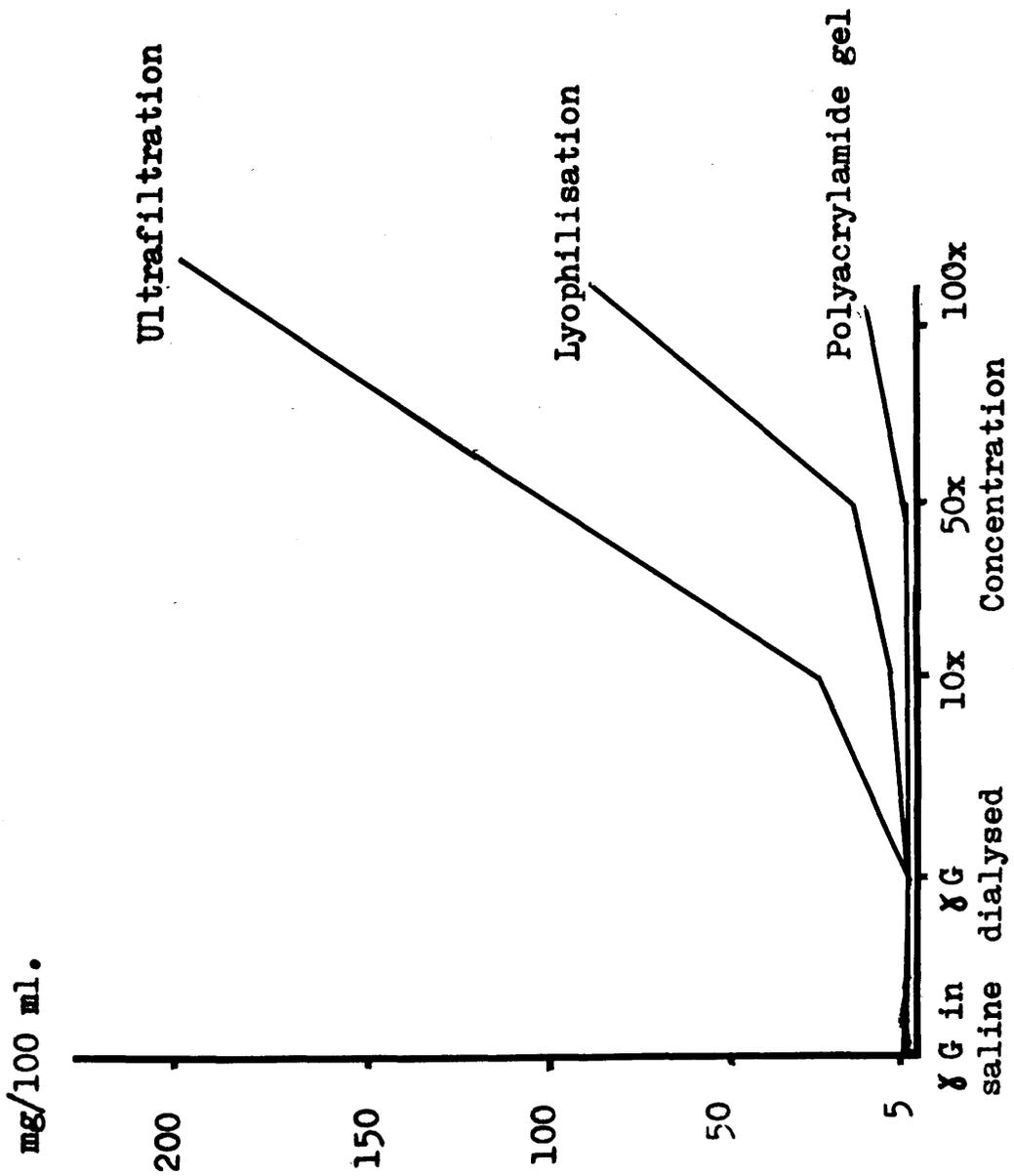


Fig.9. Protein loss on Lyophilisation. Measurement of total protein in the three samples by Lowry after Lyophilisation and partial reconstitution (concentration) and full reconstitution to the original volume.

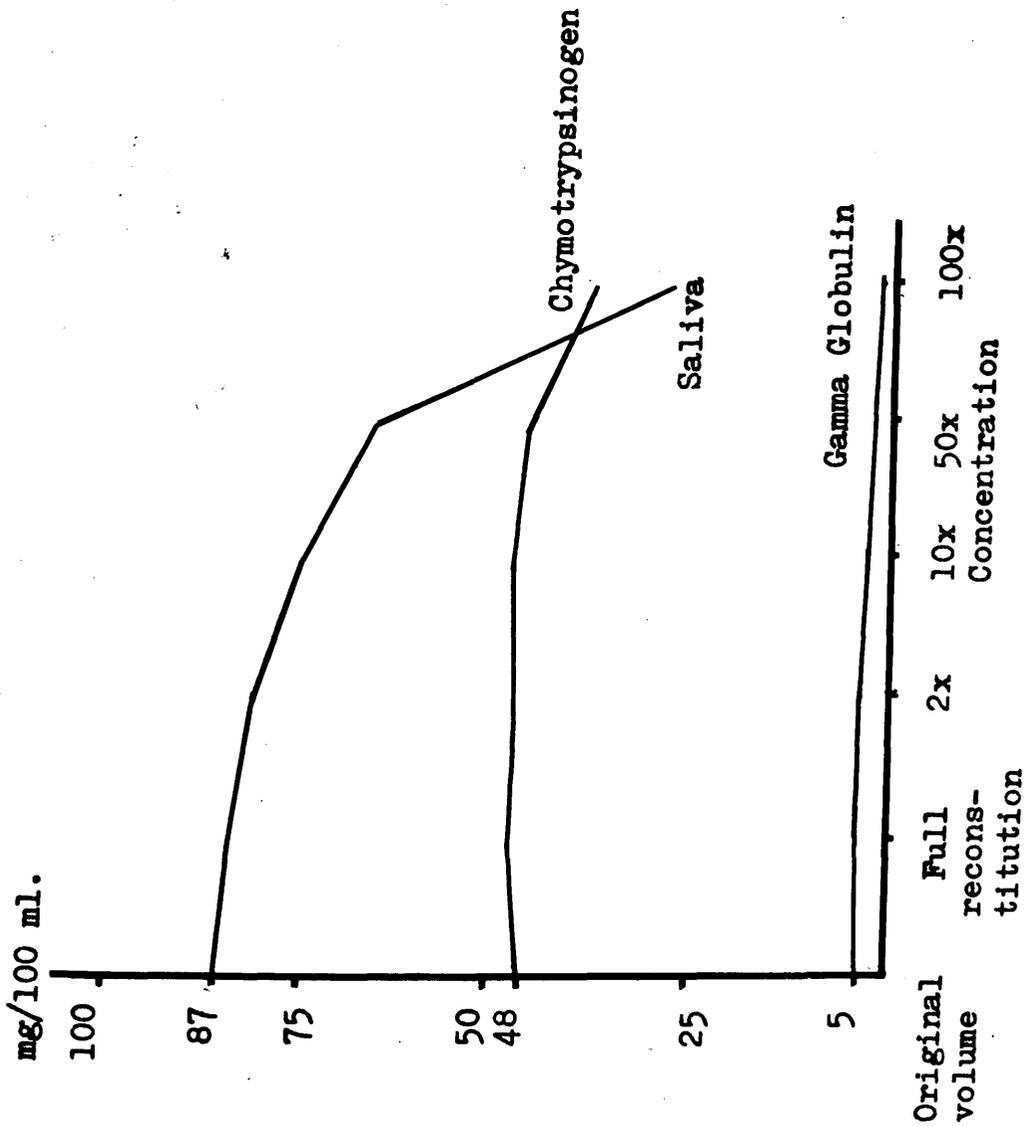


TABLE 3. TOTAL PROTEIN CONCENTRATION - LOWRY ESTIMATION

	LYOPHILISATION			ULTRAFILTRATION		
Reduction in volume	10x	50x	100x	10x	50x	100x
Chymotrypsinogen	6x	20x	57x	6x	14x	26x
Gamma globulin	6x	20x	38x	10x	26x	52x
Saliva	5.5x	14.5x	36x	7.5x	16x	30x

CONCENTRATION VALUES

TABLE 4. IMMUNOGLOBULIN CONCENTRATION - SRID

	LYOPHILISATION			ULTRAFILTRATION		
Reduction in volume	10x	50x	100x	10x	50x	100x
IgG in Kabi Gamma globulin	2.5x	9x	19x	7.5x	27x	50x
Salivary IgA	2.5x	3.5x	6x	3x	8x	16x
Salivary IgG	3x	7x	17x	5x	15x	30x

CONCENTRATION VALUES

Fig.10a.

Normal immunoelectrophoretic pattern of human serum.

All the wells contain normal human serum.

- | | |
|---------------------------|----------------------------|
| 1. Goat anti-human serum; | 2. Rabbit anti-human serum |
| 3. Goat anti-IgA; | 4. Goat anti-IgG |
| 5. Goat anti-IgM; | 6. Goat anti- α_2 |
| | macroglobulin (x2) |

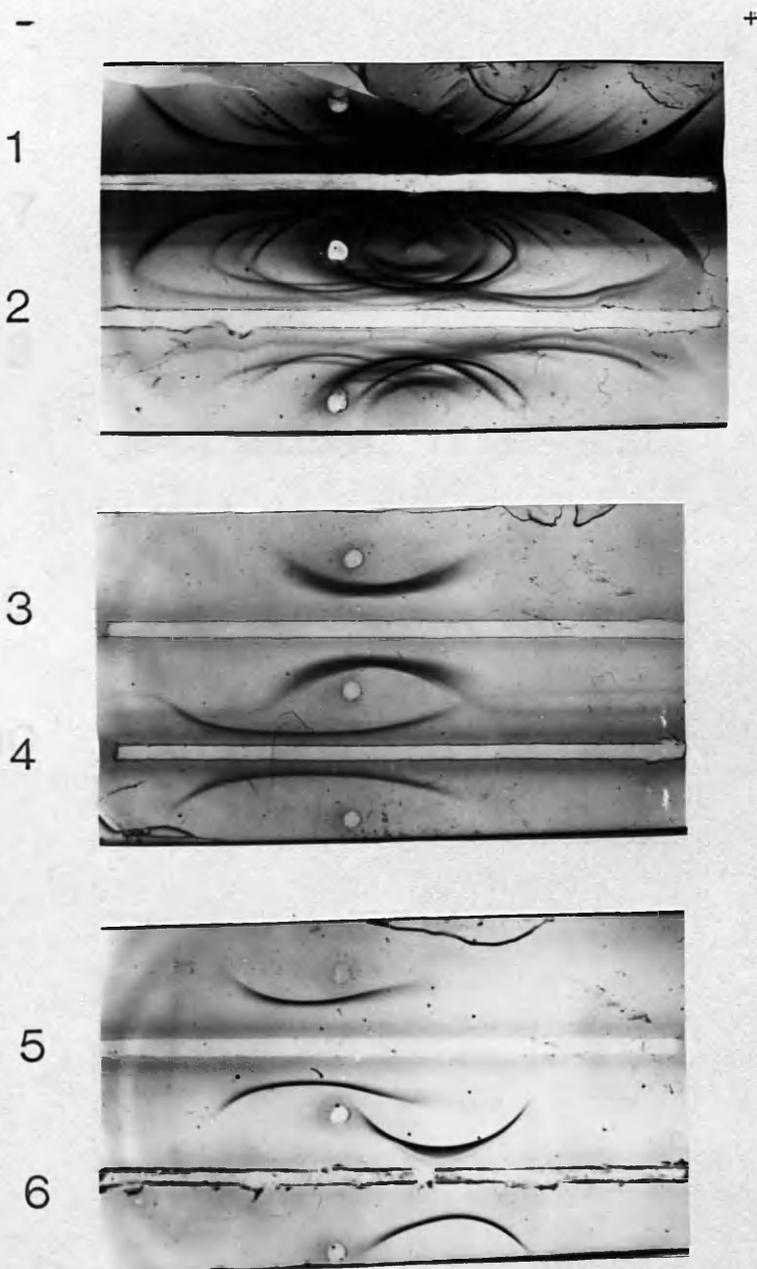


Fig.10b.

Normal immunoelectrophoretic pattern of human serum (cont.)

7. Goat anti-plasma albumin; 8. Goat anti-complement
 9. Goat anti-caerulo- 10. Goat anti-transferrin
 plasmin;

Antiserum 2 is Wellcome; 1 and 3-10 are Hyland (x2)

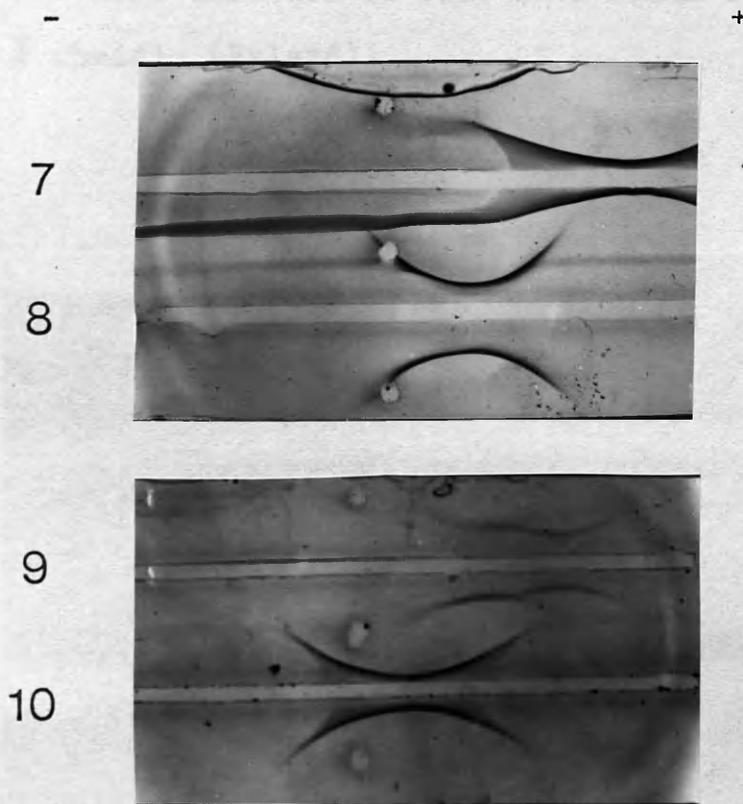


Fig.12a.

Immunodiffusion patterns of human saliva. x4.

1. Horse anti-human serum; 2. Goat anti-human serum
 3. Goat anti-IgA; 4. Goat anti-IgM
 5. Goat anti-IgG; 6. Rabbit anti-IgG
 7. Human, mixed saliva, reduced in volume 10x by
 lyophilisation.

(All antiserum is Hyland, 1:10 dilution).

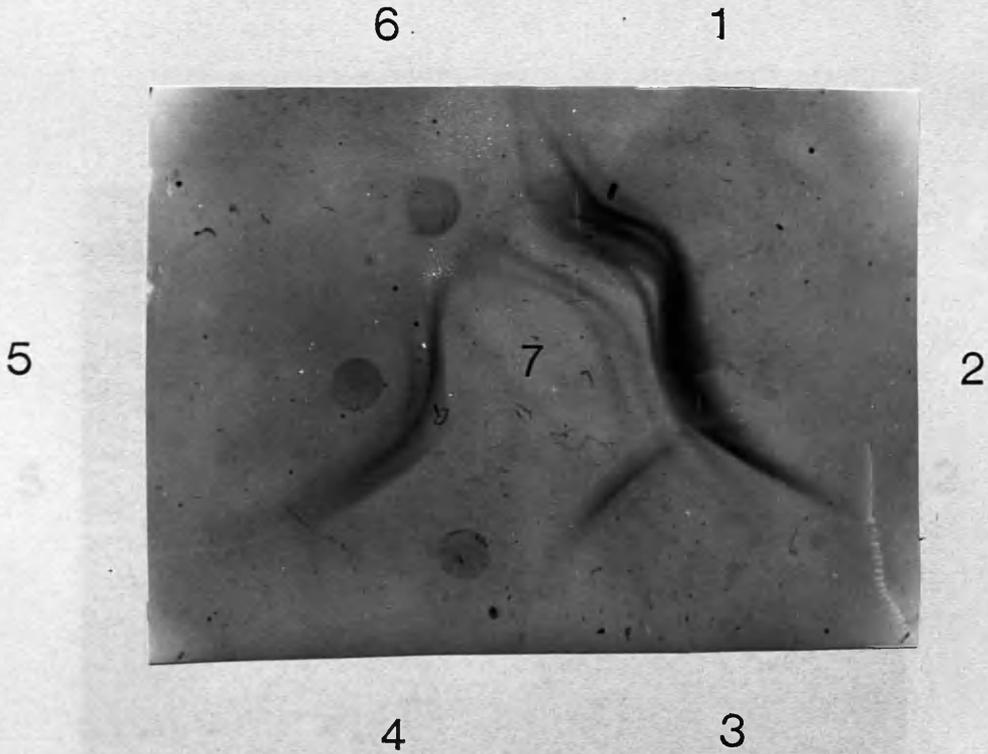


Fig.12b.

Immunodiffusion patterns of human saliva. x4.

- | | |
|---|---------------------------|
| 1. Goat anti- α_2
macroglobulin; | 2. Goat anti-transferrin |
| 3. Goat anti-caerulo-
plasmin; | 4. Goat anti-rabbit serum |
| 5. Winthrop anti-human
serum; | 6. Nordic horse anti-IgA |
| 7. Human, mixed saliva, reduced in volume 10x by
lyophilisation. | |

(Antiserum 1-4 is Hyland, 1:10 dilution.)

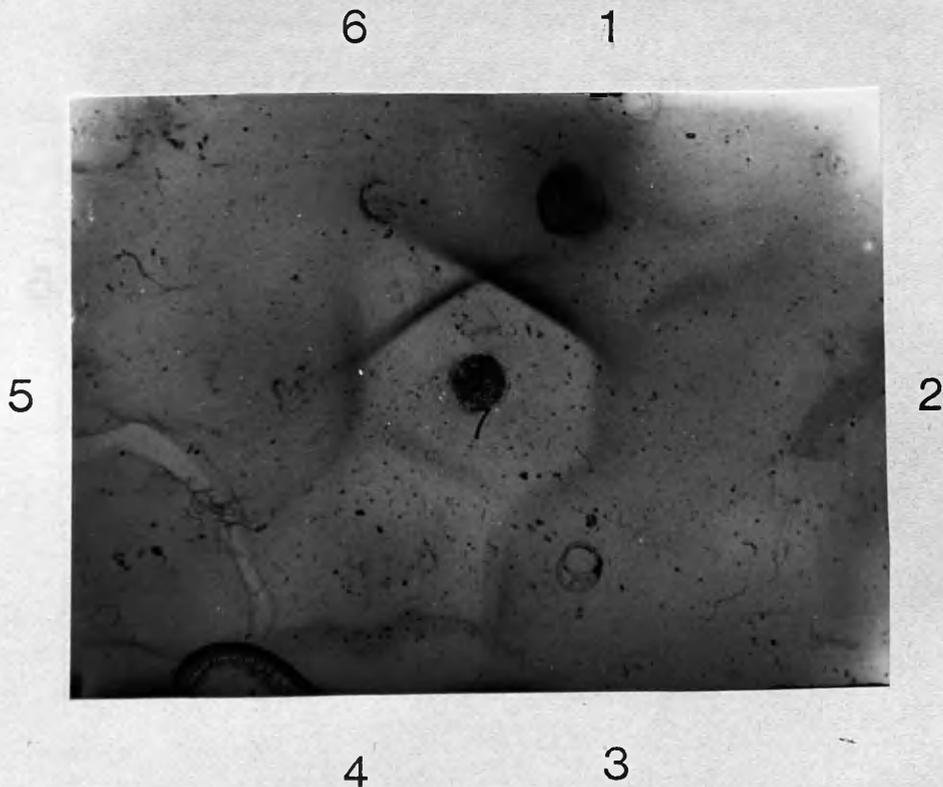
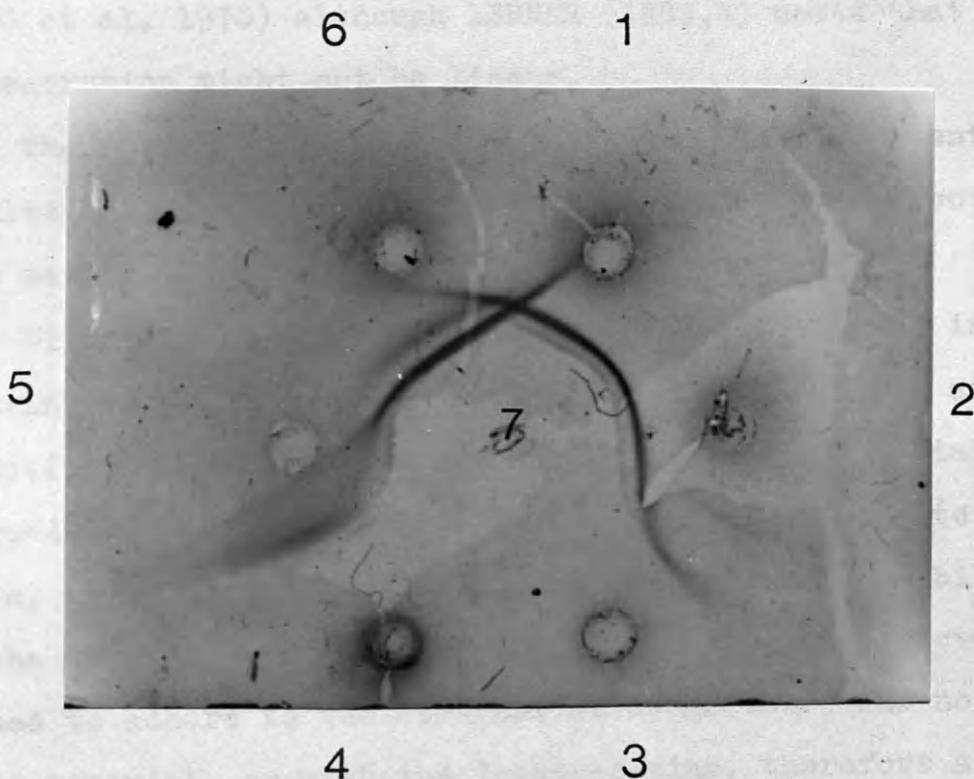


Fig. 12c.

Immunodiffusion patterns of human saliva. x4.

- | | | |
|--------------------------|------------------------|-----------|
| 1. Goat anti-IgA (1:10); | 2. Goat anti-IgA (1:3) | } Hyland. |
| 3. Goat anti-IgM (1:10); | 4. Goat anti-IgM (1:3) | |
| 5. Goat anti-IgG (1:10); | 6. Goat anti-IgG (1:3) | |
7. Human, mixed saliva, reduced in volume 10x by ultrafiltration.



DISCUSSION

The purpose of this series of experiments was to evaluate several possible methods of saliva concentration and, if possible, to develop a reproducible, effective method. An additional objective was to ascertain if salivary protein and immunoglobulin concentrated in a linear manner following volume reduction. A number of previous workers have apparently assumed a parallel increase in concentration of the protein with volume reduction (TOMASI and BIENENSTOCK, 1968; MANDEL and KHURANA, 1969; BRANDTZAEG et al. 1970 and ZENGO et al. 1970) although LEHNER (1969,b) noted that concentration might not be linear.

The actual methods of concentration gave different results and highlighted different problems with respect to each method used.

With lyophilisation, difficulty was encountered in removing all the dried material from the flask wall, and in reduction to a small volume, not all the dried material could be re-dissolved, even after dialysing before drying to remove salts, probably due to a change in the relative solubility of the proteins. With ultrafiltration, the glyco-proteins seemed to adhere to the membrane although there was no actual solid material, as with the freeze-drying, therefore a large proportion of the total protein was presumably lost due to its being left behind on removal of the concentrate. Repeated washing with distilled water and reconstitution in both cases increased protein uptake, but the degree of concentration (by volume) was inevitably reduced. Repeated concentration of the same sample was very time-consuming,

especially if a large number of samples were to be concentrated.

Lyophogel was discarded as a useful method of concentration, because although the volume was quickly and accurately reduced, the protein and immunoglobulin constituents of the saliva appeared to be adsorbed giving no effective increase in concentration.

Although an increase in protein concentration equivalent to the volume reduction was not obtained, the constituents did not appear to be denatured, as full reconstitution gave total protein values almost identical to the original levels. It was therefore concluded that with both methods of concentration, the decrease in volume probably affected the solubility of the proteins.

Both methods showed a progressive loss of salivary protein on volume reduction, the loss being much greater with the whole saliva than a protein solution such as gamma globulin. Concentration of immunoglobulins in saliva therefore appears to be complicated by other salivary components. This was supported by the fact that IgG added to saliva which was then concentrated and measured by SRID, did not concentrate at the same rate as an IgG solution alone, concentrated and measured by the same methods. The change in solubility of the immunoglobulins may have affected their ability to react in quantitative immunodiffusion and so may partly explain the low results using this method of quantitation. It was noted that the chymotrypsinogen did not concentrate satisfactorily by ultrafiltration because it has a molecular weight of approximately 25,000 and the membrane used, filtered

substances of 20,000 molecular weight or less. Fragments of the protein molecules therefore, passed through and an effective concentration value could not be expected.

A reliable reference standard for quantitation of salivary IgA would seem desirable (serum IgA was used in the above experiments and calibrated to the WHO standard for this immunoglobulin, as was also the IgG) as a portion of it differs from serum IgA by the addition of a Secretory Piece (TOMASI et al. 1965) and this affects its mobility in agar. Different authors (cited by BRANDTZAEG et al. 1970) have used different forms of IgA as a standard and this also makes comparison of results of the measurement of IgA, the predominant salivary immunoglobulin and therefore presumably the most important one in combating oral disease, difficult.

Apart from the observations of LEHNER (1969,b), previously published work on the concentration of salivary proteins appears to depend on reduction of volume as being directly proportional to concentration of immunoglobulins; as is shown above, reduction in volume is not an indication of salivary immunoglobulin concentration, therefore care must be taken in interpreting results stated as "concentration" value and not "reduction in volume" value. Neither of the above methods was found to give a very satisfactory concentration with increasing reduction in volume. However, it is unlikely that a 100x volume reduction will be essential for the measurement of all but those substances present only in trace amounts. Smaller volume reductions give relatively better protein concentrations and concentrate the immunoglobulins to easily detectable amounts.

CONCLUSIONS

The choice of method of saliva concentration must therefore depend on the substance under examination and its original concentration. Extrapolation back to the original concentration must be avoided as:

- a) concentration of individual immunoglobulins does not parallel either total protein concentration or volume reduction, and
- b) neither total protein nor individual immunoglobulins concentrate linearly.

CHAPTER 4.IMMUNOLOGICAL STUDIES ON HUMAN SALIVARY ALBUMIN.INTRODUCTION

Further investigations into the nature of salivary albumin seemed warranted because of some anomalous results that were observed by the author, on routine electrophoretic analysis of human saliva against anti-human serum and anti-mixed saliva preparations. They emphasised the disparity in the reports of earlier investigations, including that of ELLISON et al. (1960) and SIMONS et al. (1964), which is outlined in Chapter 2, pages 17 and 18.

Although no direct correlation has been previously reported between serum and/or salivary albumin and oral diseases, this work is included because of the relevance of the conclusions that may be drawn from it, to the studies on local immunization (Chapter 7).

MATERIALS AND METHODSCollection, dialysis and concentration of human saliva-

The saliva used in this series of experiments was collected and concentrated in the manner described in the previous chapter (with the exception of polyacrylamide gel concentration).

Quantitation of albumin and IgA in saliva-

The albumin and IgA contents of the saliva preparations were determined by SRID, as described in Chapter 3, p53. The antiserum used was Hyland rabbit anti-human plasma albumin serum and Hyland goat anti-human IgA.

Adsorption of saliva with anti-human plasma albumin serum-

A mixture containing 1 volume of mixed saliva (protein concentration 35mg/ml), 1 volume of 0.85% (w/v) saline and 2 volumes of rabbit anti-human plasma albumin serum (Hyland) was incubated in a water bath at 45°C for 3 hours. After centrifugation at 13,000rpm (12,000g) for 1 hour at 4°C, the supernatant was removed and stored at -20°C.

Preparation of rabbit antiserum to concentrated, human, mixed saliva-

(i) 0.5ml of concentrated saliva (protein concentration, 10mg/ml) together with 0.5ml 0.85% (w/v) saline emulsified in 1ml Freund's Complete Adjuvant, was injected intramuscularly into each of 4 sites in male Californian rabbits once a week for 5 weeks. Serum was collected 1 week after the last injection of saliva and was termed rabbit antiserum 02-70.

(ii) This was prepared by Dr. J.A. Beeley, Departments of Oral Biology and Biochemistry, University of Glasgow. The method used was as follows: 1ml of an emulsion containing equal volumes of concentrated saliva (protein concentration, 20mg/ml) and Freund's Complete Adjuvant was injected intramuscularly into each hind leg, and 2ml distributed simultaneously over the backs of each of 2 male Dutch white rabbits; after 28 days, 1ml of concentrated saliva (protein concentration, 5mg/ml) was injected intraperitoneally. Serum was collected 1 week after the last injection of saliva and that selected for use after appropriate testing was termed rabbit antiserum no.552 and no.564.

Determination of antigenicity of IgA in saliva-

This was carried out by the method of WORK and WORK (1969). The saliva used to develop rabbit antiserum O2-70 was diffused against this antiserum in conjunction with a 7S Hyland IgA standard (calibrated with the WHO standard) on standard immunodiffusion plates. The titre of anti-IgA in antiserum O2-70 was calculated as the last dilution of antiserum that produced a precipitin arc with the concentrated saliva; that arc giving a reaction of identity with the IgA standard.

Immuno-electrophoresis-

This was carried out as described in Chapter 3, using aliquots of the mixed saliva preparations previously employed for antiserum production.

Immunodiffusion-

0.3% Oxoid 'Ionagar' no.2 (0.1g/33ml distilled water) was layered onto alcohol-cleaned 2"x2" glass slides and allowed to solidify under cover to prevent dust settling. Plexiglass templates (with six 2mm or 1mm wells equi-distant from each other and a centre well by 4mm or 1mm respectively) were placed on the agar surface and the slides placed on a hotplate at 45°C for a few seconds to allow condensation to occur on the undersurface of the templates. Approximately 1ml of 1% 'Ionagar' (0.1g/10ml 0.85% (w/v) saline) incorporating a 1:10,000 dilution of merthiolate as a preservative, was flooded under the template, care being taken to avoid the production of air bubbles, and the slide removed from the hot

plate. The wells were filled with the reagents within 30 minutes of solidification of the agar and diffusion allowed to occur for 24-28 hours in a moist atmosphere at room temperature. The purified human plasma albumin used in this test was obtained from Hoescht Pharmaceuticals Ltd., Kew Bridge, Brentford, Middlesex.

RESULTS

Immuno-electrophoretic analyses-

A comparison of the immuno-electrophoretic pattern resulting from using mixed saliva and anti-plasma albumin serum with that using human normal serum (fig.13a) confirmed the presence of plasma albumin, or at least a protein bearing considerable immunological resemblance to plasma albumin in saliva, as previously reported (ELLISON et al. 1960; STOFFER et al. 1962; LEACH et al. 1963; SIMONS et al. 1964; BRANDTZAEG, 1965; MASSON et al. 1965 and STROBER et al. 1970). However, a sharper precipitin arc was obtained in the case of salivary albumin than plasma albumin, an observation also made elsewhere (BRANDTZAEG, 1965 and MASSON et al. 1965), but this might have resulted simply from differences in concentration of the albumin.

Immuno-electrophoresis of mixed saliva with anti-mixed saliva serum, resulted in the appearance of several arcs, including a single arc in the albumin region (fig.13b). On immuno-electrophoresis of normal human serum with anti-mixed saliva serum however, no arc in the albumin region was observed (fig.13b). 2, 10, 50 and 100 fold dilutions of normal human serum gave the same result. Accordingly, this

anomaly was investigated further. Fig.13c shows the immunoelectrophoretic pattern which resulted from reacting mixed saliva with anti-mixed saliva serum and anti-plasma albumin serum. The arcs produced in the albumin region clearly cross each other in a reaction of non-identity.

Mixed saliva was then adsorbed with anti-plasma albumin serum in order to remove the component which produced a precipitin arc on immunoelectrophoresis with anti-plasma albumin serum. Fig.13d shows the pattern produced with mixed saliva before and after adsorption with anti-plasma albumin serum, and shows that removal of the albumin was complete. However, on immunoelectrophoresis of the adsorbed mixed saliva with anti-mixed saliva serum, the pattern was unchanged (fig.13e), the arc in the albumin region still being present. These results are in agreement with those of SIMONS et al. (1964).

Examination of salivary albumin by double-diffusion-

Immunodiffusion was used as an additional method of investigating salivary albumin because cellulose acetate electrophoresis of salivary proteins also produced a component with the same mobility as plasma albumin, whilst the component corresponding to plasma albumin on isoelectric focussing in polyacrylamide gels appears as a sharp band rather than the heterogeneous group of bands characteristic of plasma albumin (BEELEY, 1972). This latter point warrants further analysis because of difficulties in this investigative technique, due to the differences in the molecular size of plasma albumin and anti-plasma albumin. However, results

Fig.13.

Immunoelectrophoresis slides. The photographs are orientated so that the anode is to the left and the cathode is to the right. (a) Mixed saliva, 35mg/ml(MS) and human normal serum (HNS) in wells; anti-serum to human plasma albumin (A-ALB) in trough. (b) MS and HNS in wells; anti-serum to mixed saliva (A-MS) from rabbit no.552 in trough. (c) MS in wells; A-MS from rabbit no.564 and A-ALB in troughs. (d) MS and MS adsorbed with A-ALB (MS') in wells; A-ALB in trough. (e) MS and MS' in wells; A-MS from rabbit no.02-70 in trough.

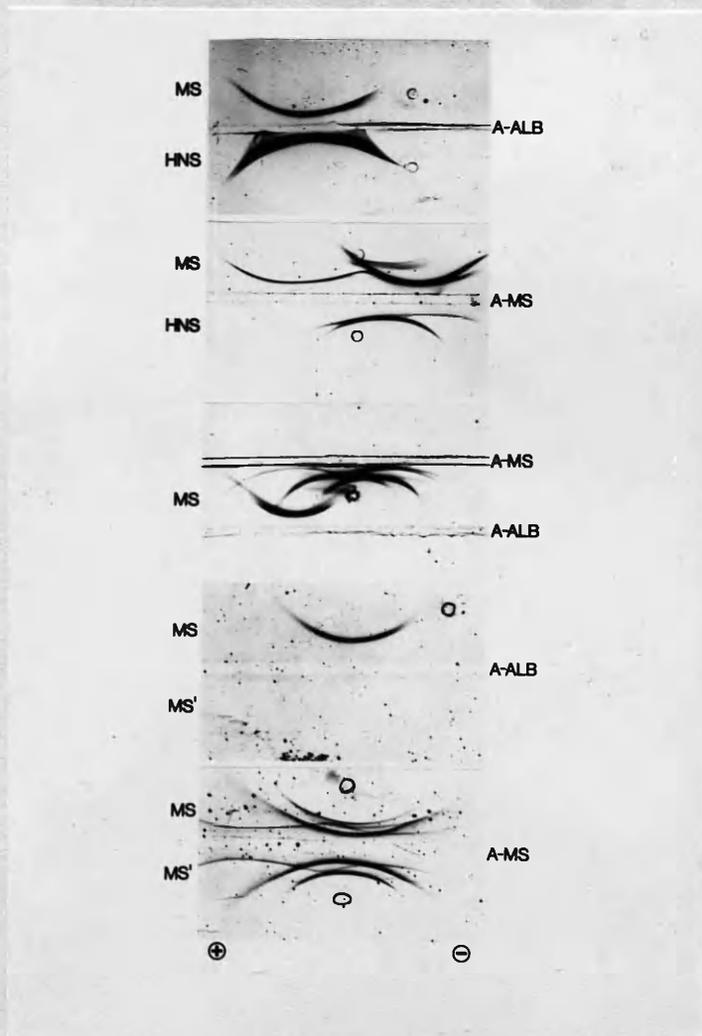
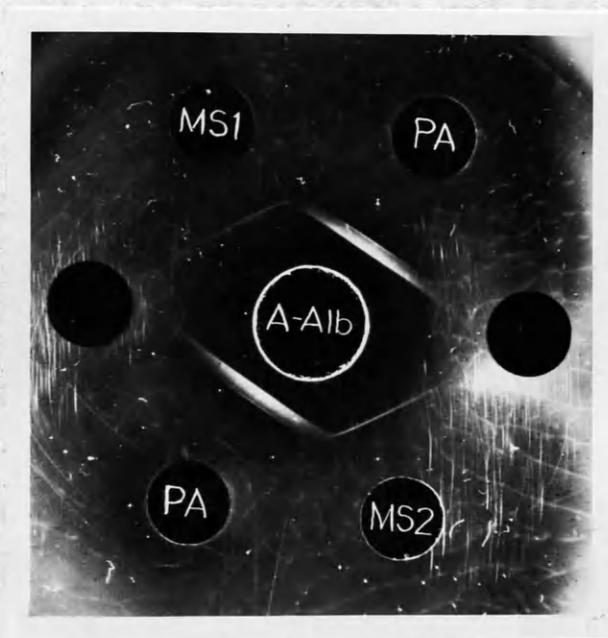


Fig. 14.

Double-diffusion. A-ALB, anti-serum to human plasma albumin; MS1, mixed saliva used for the preparation of antisera in rabbit no.552; MS2, mixed saliva used for the preparation of antisera in rabbit no.02-70; PA, human plasma albumin.



of a double-diffusion experiment showed a reaction of partial, if not total identity between plasma albumin and the albumin-like component of salivary albumin (fig.14), although a number of additional bands appeared between plasma albumin and anti-plasma albumin serum. This indicates that salivary albumin has one identical antigenic determinant with that of plasma albumin.

These results are reproducible and independent of the method of concentration of saliva or of antiserum production and all preparations correspond to those used in similar experiments by other workers (including those of BEELEY, 1972).

DISCUSSION

The findings reported by SIMONS et al. (1964) have been confirmed by the results of the experiments described in this study, by showing that anti-mixed saliva serum gives no precipitin arc corresponding to plasma albumin on immunoelectrophoresis with human normal serum. Furthermore, in contrast to the conclusion of ELLISON et al. (1960), the arc appearing in the albumin region on immunoelectrophoresis of mixed saliva with anti-mixed saliva serum shows a reaction of non-identity with the arc formed between mixed saliva and anti-plasma albumin serum.

From this observation it may be concluded that either the arc formed in the albumin region between mixed saliva and anti-mixed saliva serum does not in fact result from plasma albumin or that salivary and serum albumins are immunologically different. Adsorption of mixed saliva with anti-plasma albumin followed by immunoelectrophoresis with anti-mixed saliva serum

failed to eliminate the arc in the albumin region, therefore it may be concluded that the arc does not result from the presence of plasma albumin.

It thus appears that antiserum to mixed saliva does not contain antibody to whole plasma albumin. Although determinants of plasma albumin are clearly present in mixed saliva (fig.13a) they do not appear to be antigenic under the conditions used here. At present, the reason for this anomaly is uncertain, as it is unlikely that salivary albumin, although interacting with anti-serum to plasma albumin, is in itself non-antigenic, even if it were bound to a non-antigenic glycoprotein secretory piece. Although salivary albumin may be less heterogeneous than serum albumin, it does have the same electrophoretic mobility and double radial immunodiffusion has confirmed the presence of an identical component in salivary albumin and plasma albumin (fig.14). One possibility is that of denaturation or ageing with an accompanying increase in heterogeneity of the albumin in saliva and/or serum albumin (SOGAMI et al. 1969; NIKKEL and FOSTER, 1971) during antigen preparation, thus complicating antibody production and/or antigen-antibody interactions.

Another possible cause of these results is that the plasma albumin concentration of mixed saliva was too low to cause production of detectable quantities of antibody. Indeed, the saliva was obtained from volunteers with clinically healthy gingivae and thus should have contained little gingival exudate. In addition, the albumin concentration of "concentrated saliva" does not parallel the decrease in volume of the secretion, as was reported in the previous chapter with

salivary IgA and IgG; initial small amounts would therefore not be increased greatly in concentration relative to total protein concentration.

It cannot be concluded, however, that the proteins most readily detected by immunoelectrophoresis are the principal components of a mixture. The preparations used in these experiments had an albumin to IgA ratio ranging from about 0.9 to 1.8. IgA in the mixed saliva was found however, to be highly antigenic (titre 1:5,120) using an anti-serum to serum IgA (7S) although as no anti-11S IgA serum was available, the titre of secretory IgA in the mixed saliva could not be calculated. Furthermore, the albumin content of the saliva preparations was adequate to give a definite precipitin arc with anti-serum to human plasma albumin (fig.13a).

CONCLUSIONS

Further investigations will be required to clarify this problem, including an immunological and biochemical comparison of purified salivary albumin with plasma albumin, for although salivary and plasma albumin appear to have an antigenic determinant in common, plasma albumin has many more than the former and seems more antigenic. By making a rabbit tolerant to human plasma albumin and raising anti-serum to mixed saliva any antibody to 'albumin' should be to 'salivary albumin'; this arc, if produced on immunoelectrophoresis, could then be compared to the one at present found in the albumin region of the mixed saliva/anti-mixed saliva immunoelectrophoretic pattern, which has not yet been identified.

Some conclusions drawn from the results in Chapter 7, however, aid the clarification of this anomaly a little; see pp. 157-158.

CHAPTER 5.AN INVESTIGATION INTO THE SALIVARY AND
SERUM IgA LEVELS OF PATIENTS SUFFERING
FROM DIFFERENT TYPES OF PERIODONTAL DISEASE.INTRODUCTION

It appeared necessary to correlate salivary and serum immunoglobulin levels in patients presenting with acute and chronic inflammatory lesions of the periodontium and oral mucosa, in whom the dental caries state was also recorded simultaneously with their medical history. By doing so, changes in immunoglobulin levels could be attributed importance depending on the caries experience of the patient.

Patient immunoglobulin levels were compared to a control group matched as closely as possible and all results calculated on the basis of the WHO immunoglobulin standards (ROWE et al. 1970) to enable correlation of the results with those in other laboratories.

In addition, the survey was undertaken to define the problems and categories required for such an evaluation.

MATERIALS AND METHODSSubjects-

The series consisted of 69 individuals who presented with different forms of periodontal disease. They were aged from 9-65 years (mean, 34 years) and were compared to a control group of 23 individuals aged from 21-55 years (mean, 28 years). They were all assessed as shown in fig.15. The Modified Greene and Vermillion Oral Hygiene Index (O.H.I.) was used for scoring the mouths (GREENE and VERMILLION, 1964).

The scores ranged from 0.0-3.0 (mean, 0.950) in the patients compared to the values of 0.0-1.0 (mean, 0.246) in the control group. The average Russell Index (RUSSELL, 1956) for the mouth was also calculated to indicate the severity of the disease and the DMF values were recorded. The latter does not indicate the degree of active caries at the time of examination but indicates the overall carious experience of the patient. The broad groups into which the subjects were classified, the age ranges, oral hygiene indices, the average Russell values and the DMF values are shown in Table 5a and the subdivisions into the different types of periodontal disease are listed in Table 5b.

The serum and saliva samples from each patient were collected at the same time and in the case of those suffering from an acute condition, the 'convalescent' samples were taken 2 weeks after the first visit. The degree of dental caries activity was noted when analysing the immunoglobulin measurements; none of the controls had active caries although some had a high DMF score.

Collection of serum-

10ml of blood was withdrawn from the cubital vein into a sterile universal bottle, which was placed in a 37°C incubator for 30 minutes before transferring it to the refrigerator. When a clot had formed, the blood was centrifuges at 4,000 rpm (200g) for 10 minutes. The serum was removed and stored in 1ml aliquots at -20°C.

Collection, dialysis and concentration of saliva-

This was carried out as described in Chapter 3, using

Fig.15.

M.M. SURVEY

FORM.

SURVEY NUMBER: _____

M.M. SURVEY.

SURVEY CATEGORY: _____

NAME: _____ HOSPITAL NO.: _____ D.O.B.: _____

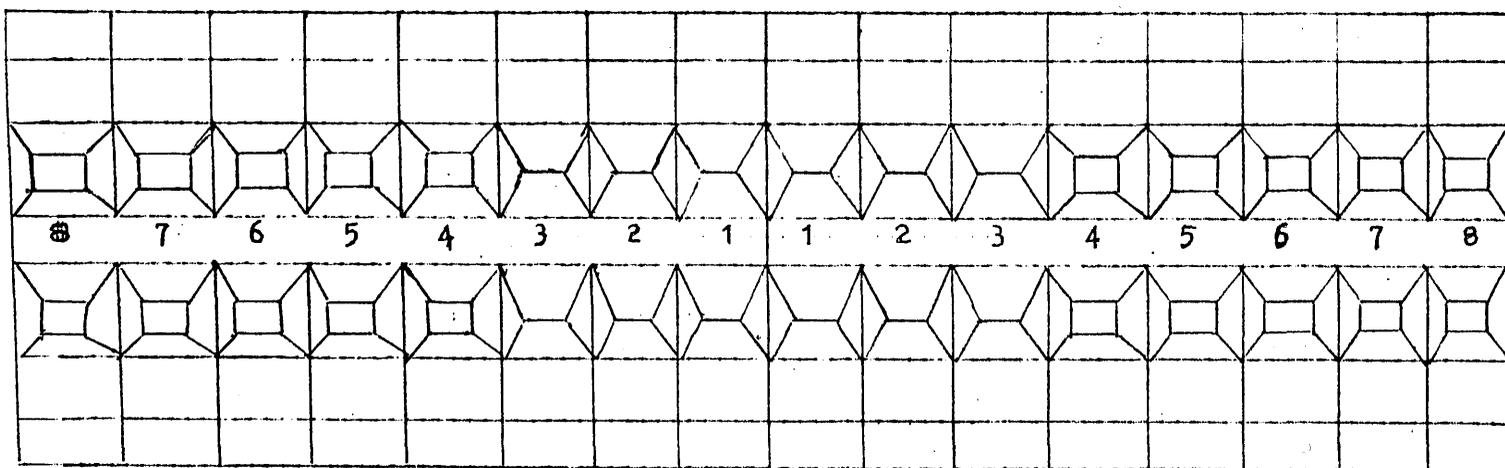
ADDRESS: _____ SEX: _____ RACE: _____

C.O.:

DENTAL HISTORY:

	Primary Condition.	Degree*.	Duration.	Secondary Condition.	Degree*.	Duration.
HERPES*						
A.U.G.*						
APHTHOUS*						
OTHER ULCERATION*						
CANDIDA*						
EROSIVE LESION*						
CHRONIC GINGIVITIS						
CHRONIC PERIODONTITIS						
PERICORONITIS						
DENTAL ABSCESS						
PERIODONTAL ABSCESS						
SORE TONGUE						
OTHER						

*refers to the severity of the condition; number of * indicates degree of severity.



	Upper jaw.	Lower jaw.		
NO. OF TEETH				
DECAYED			D.	
MISSING			M.	
FILLED			F.	
RUSSELL				
Total for jaw				
Ave. RUSSELL				
Ave. RUSSELL Mouth.				

O.H.I.			
--------	--	--	--

Ave. upper = / =
 Ave. lower = / =
 Ave. mouth = / =

RADIOGRAPHS	
CLINICAL PHOTOGRAPHS	

MEDICAL HISTORY:

	Condition.	Duration.
HEART		
CHEST		
SKIN		
ALLERGY		
RHEUMATISM		
DIABETES		
PERNICIOUS ANAEMIA		
G.I.		
G.U.		
OTHER		

DRUGS: _____

DURATION: _____

SAMPLES:

		Acute	Control	Convalescent	Chronic
SALIVA	SERUM				
	MIXED				
	PAROTID				
	STIMULATED				
	UNSTIMULATED				

Saliva stimulant used: _____

BACTERIOLOGY:

SWABS		Area.
SMEARS		
A69 FORM		

No.: _____

lyophilisation as the method of concentration. All saliva samples were decreased in volume 50 times in this way.

Quantitation of IgA in serum and saliva-

The technique used was that described in Chapter 3. The values of IgA in the 'concentrated' saliva could be extrapolated back to their original values in the unconcentrated secretion, if necessary, using the conversion factor obtained in the RESULTS of Chapter 3 (Table 4). A mean of at least 2 correlating results (\neq 4% difference) was taken for each sample measured.

Statistical analysis of results-

A Hewlett-Packard Calculator Model T91A (Hewlett-Packard Co., Hewlett-Packard Ltd., 224 Bath Road, Slough, Bucks.) was used for the following:

- 1) To calculate the best straight line through the values obtained from the known standards by minimising the sum of the squares of the deviations of the data points from the line (least squares fit) giving a straight line of the equation,

$$\text{Log}_e \text{ concentration} = 0.715 \times \text{diameter} - 0.413,$$
with a correlation coefficient of 0.973.
This was used as the standard curve for the known concentrations of IgA.
- 2) The student 't' test which calculates the t value for the distributions of 2 sets of samples.
- 3) The t values were then used to calculate the probability that the two distributions were the same.

Table 5a.

Age ranges, O.H.I. values, average Russell Indices and DMF scores of control and patient groups. The patient sample is broken down into its broad constituent groups.

Group	Sex	No	Age Range	Mean Age	O.H.I.	Mean O.H.I.	R.I.	Mean R.I.	DMF	Mean DMF
CONTROL	M	13	21-35	26	0.0-1.0	0.385	0.0-3.08	0.68	6-23	15
	F	10	21-55	30	0.0-0.67	0.167	0.0	0.0	7-32	17
PATIENT SAMPLE	M	35	17-54	32	0.0-3.0	0.965	0.30-6.0	2.70	2-32	16
	F	34	9-65	38	0.0-0.27	0.935	0.0-6.0	2.65	11-32	19
ACUTE	M	12	18-43	24	0.0-2.0	0.917	0.30-3.68	1.78	2-22	15
	F	10	9-65	24	0.0-2.0	1.000	0.0-5.95	1.99	17-32	22
ACUTE + CHRONIC	M	7	19-41	28	0.0-3.0	1.286	0.5-6.0	2.74	9-32	18
	F	3	20-38	30	0.17-2.17	0.888	1.48-3.67	2.79	11-16	14
CHRONIC	M	12	17-43	28	0.0-2.5	0.693	0.52-6.0	3.57	3-32	14
	F	16	17-53	31	0.0-2.67	0.958	0.50-6.0	3.17	14-28	22
RHEUMATOID	M	4	38-54	46	N o t K n o w n					
	F	5	48-66	61	N o t K n o w n					

KEY: M = Male

F = Female

No = Number

R.I. = average Russell Index score for the mouth

DMF = Decayed; Missing; Filled, value

O.H.I. = Oral hygiene index value

Table 5b.

Numbers and categories of test subjects
presenting within each group.

<u>Group.</u>	<u>Number.</u>		<u>Categories of disease in each group.</u>
ACUTE	22		
	<u>male</u> 12	<u>female</u> 10	AUG (including 1 male patient clinically and radiologically confirmed to be 'caries-free'); Herpes + AUG; Aphthous ulceration (including 1 female edentulous subject); Gingivitis; Other ulceration.
<u>ACUTE + CHRONIC</u>	10		
	<u>male</u> 7	<u>female</u> 3	AUG + Gingivitis; AUG + Periodontitis; Herpes + Gingivitis; Herpes + Periodontitis.
<u>CHRONIC</u>	28		
	<u>male</u> 17	<u>female</u> 11	Periodontitis (including 1 female patient with periodontitis + chronic herpetic stomatitis); Herpes; Gingivitis; Aphthous ulceration.

Key: AUG = Acute Ulcerative Gingivitis.

RESULTS

General Oral Health-

None of the patients in the control sample had active caries at the time of serum and saliva collection, although the mean DMF values were very similar to those of the test group; the average Russell Index scores for the mouth was much lower for the controls as was expected.

The patients in the 'Chronic' and 'Acute + Chronic' groups had high average Russell Index scores but the DMF values for these groups were not higher than those of the 'Acute' group. The average Russell Index scores showed the greatest variation in the 'Acute' group.

The O.H.I. scores of the control subjects was also much lower than those of the test subjects, whose values did not show much variation between the broadly classified groups.

The oral state of the Rheumatoid patients was not precisely evaluated as it was felt that it would cause too much distress to the patients, many of them being quite ill. However, none could be said to have healthy mouths and all had gingival inflammation to a certain extent.

The control and patient groups were quite evenly matched for age and sex.

Results of the statistical evaluation of the salivary and serum IgA levels-

The initial results of the immunoglobulin level estimations carried out by SRID (see fig.16) in the concentrated saliva and serum are shown in Tables 6a,b and c. The conversion factor to change from MRC units of the WHO standard

to mg/100ml was IgA, 1 unit = 1.2mg. The IgA values for saliva were based on the serum IgA standard.

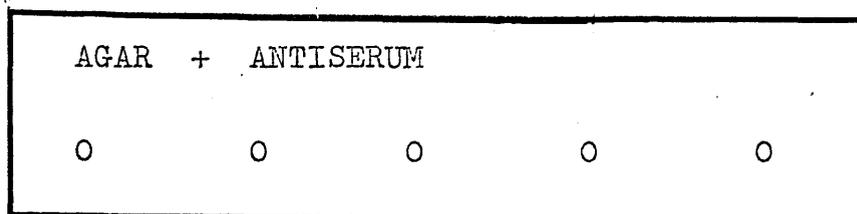
The serum IgA values are very high. This is due to the fact that the diameter of the precipitin rings in some of the samples was greater than that of the highest standard used. However, the same 'least squares fit' line was used for all estimations and therefore the probabilities will not be affected by the high values.

Because within each group, some of the samples were very high and some very low, the saliva and serum levels of individual diseases within each group were calculated giving the results in Tables 7a and b.

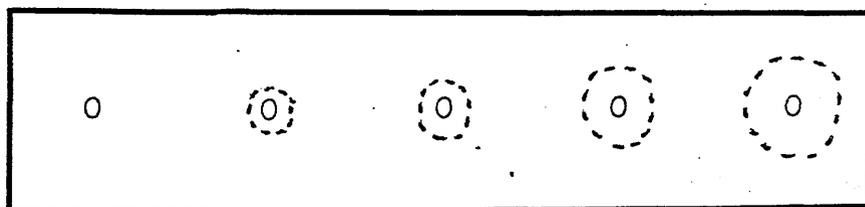
The male saliva values showed more deviation from the control values than did the female saliva; similar results were obtained with the serum values (Tables 6a and b). With male saliva, quite a high salivary IgA value was recorded in the first sample which showed a return to normal as the condition improved, as did the female in the Acute and Acute + Chronic conditions. With the serum samples, this result was paralleled in the male values, but the female values continued to fall.

As shown in Table 6c, those values that gave a significant difference with the male saliva were Acute (1) with a decrease in IgA, and Chronic, with elevated IgA levels. The Acute male 'caries-free' salivary IgA was very high in the first sample but it had returned to a value within normal limits on recovery. Female salivary IgA values showed no significant differences. Male serum showed a significant increase in IgA in Acute (1); female serum showed a very

S.R.I.D.



eg. 2 μ l saliva. 0 5 10 25 50 mg/100ml antigen.



Diameter of precipitin ring is related to antigen concentration.

A standard is included in every plate to standardise the test.

fig.16. Diagrammatic representation of Quantitative Immunodiffusion.

TABLE 6a.

IgA values in the 50x lyophilised saliva of the different subject groups.

SUBJECT GROUPS	MALE SALIVA				FEMALE SALIVA			
	No	Mean	Range	S.D.	No	Mean	Range	S.D.
Control	13	11.79	2.77-31.43	7.08	12	12.02	2.77-31.44	8.23
Acute(1)	11	22.59	6.29-38.96	11.63	7	15.73	4.56-37.59	14.20
Acute(2)	10	19.40	3.95-91.88	26.58	8	13.16	4.71-27.25	9.24
Acute+Chronic(1)	7	24.23	7.52-74.13	23.76	3	17.64	3.95-25.37	11.89
Acute+Chronic(2)	5	7.70	3.69-11.55	2.89	3	9.81	7.52-11.55	2.07
Chronic	12	18.58	11.15-34.99	7.26	16	10.23	4.09-27.25	7.13
Rheumatoid	4	17.91	4.89-31.44	11.08	5	14.73	4.09-44.95	17.18

KEY: No = Number of samples in each group

S.D. = Standard deviation of the sample values

(1) = the first lot of samples taken from that group of subjects

(2) = the second lot of samples taken from that group of subjects,

i.e. the 'convalescent' sample

All IgA values are in mg/100ml.

TABLE 6b.

IgA values in the serum of the different subject groups.

SUBJECT GROUPS	MALE SERUM				FEMALE SERUM			
	No	Mean	Range	S.D.	No	Mean	Range	S.D.
Control	13	549.73	113.86- 1122.15	297.78	12	690.15	288.44- 1044.71	232.30
Acute(1)	11	969.87	216.69- 1851.03	608.46	10	534.69	91.69- 2293.88	667.70
Acute(2)	12	539.67	232.75- 972.62	211.12	10	343.90	71.54- 680.27	201.88
Acute+Chronic(1)	7	593.96	232.75- 905.51	285.47	3	387.52	113.86- 589.63	245.82
Acute+Chronic(2)	4	442.58	151.56- 843.02	292.74	2	374.13	91.88 & 656.38	399.16
Chronic	12	595.67	91.88- 873.71	256.25	16	502.29	4.56- 1786.02	433.77
Rheumatoid	4	647.55	873.71- 730.67	69.98	5	1309.15	268.54- 2646.51	1049.97

KEY: No = Number of samples in each group

S.D. = Standard deviation of the sample values

(1) = the first lot of samples taken from that group of subjects

(2) = the second lot of samples taken from that group of subjects,

i.e. the 'convalescent' sample

All IgA values are in mg/100ml.

TABLE 6c.

Probability values of the means of the different subject groups compared to the mean of controls. The values are given for both 50x lyophilised saliva and for serum IgA values.

SUBJECT GROUPS	Male saliva	Female saliva	Male serum	Female serum
	P cf C	P cf C	P cf C	P cf C
Acute(1)	0.0104	0.4753	0.0384	0.4579
Acute(2)	0.3334	0.7759	0.9244	0.0114
Acute+Chronic(1)	0.0920	0.3454	0.7519	0.0668
Acute+Chronic(2)	0.2635	0.6615	0.5375	0.1243
Chronic	0.0262	0.5459	0.6849	0.1861
Rheumatoid	0.2034	0.6606	0.5531	0.0625

KEY: (1) = the first set of samples taken from that group of subjects
 (2) = the second set of samples taken from that group of subjects,
 i.e. the 'convalescent' sample

P cf C = the probability that the values of the subject groups listed are the same as the values of the control group subjects.

A P value of less than 0.0500 is significant.

TABLE 7a.

Combined Male and Female salivary IgA values from 50x Lyophilised saliva, compared to those of the control group, specifying the different types of disease within the groups. *The values of IgA are in mg/100ml.

SUBJECT GROUPS	No	Mean*	Range*	S.D.	P.
Control	25	11.89	2.76-31.43	7.49	-
ACUTE (1)					
AUG	8	19.71	5.26-37.59	10.81	0.028
Herpes	5	12.03	4.55-31.43	11.43	0.975
Herpes+AUG	2	33.59	28.24&38.95	7.57	0.0006
Apthous ulcers	2	34.51	31.43&37.59	4.35	0.0003
Calculus formation	1	4.55	-	-	0.346
ACUTE (2)					
AUG	9	20.59	4.89-91.88	28.06	0.157
Herpes	5	13.41	5.26-25.36	7.35	0.6827
Herpes+AUG	2	16.44	5.65&27.24	15.26	0.443
Apthous ulcers	2	7.10	4.89&9.32	3.13	0.387
ACUTE+CHRONIC (1)					
AUG+gingivitis	4	16.61	7.52-27.24	10.28	0.274
AUG+periodontitis	4	19.44	10.75-29.26	9.24	0.080
Herpes+periodontitis	1	3.95	-	-	0.309
Herpes+gingivitis	1	74.14	-	-	23x10 ⁻⁹
ACUTE+CHRONIC (2)					
AUG+gingivitis	4	8.94	3.68-11.55	3.71	0.451
AUG+periodontitis	2	7.13	6.75&7.52	0.54	0.386
Herpes+periodontitis	1	10.37	-	-	0.905
Herpes+gingivitis	1	7.52	-	-	0.572
CHRONIC					
Periodontitis	17	15.04	4.09-34.99	9.20	0.230
Herpes	2	13.43	4.89&21.98	12.08	0.788
Gingivitis	7	9.88	4.27-17.12	4.20	0.504
Apthous ulcers	2	17.16	11.55-22.78	7.94	0.349
RHEUMATOID	9	16.14	4.56-31.43	14.01	0.261

KEY: No = Number of subject samples

S.D. = Standard deviation

AUG = Acute ulcerative gingivitis

P. = probability that the means of the patient samples in the different groups are the same as the mean of the control samples; P less than 0.0500 is significant.

TABLE 7b.

Combined Male and Female serum IgA values from the different subject groups compared to those of the control group, specifying the different types of disease within the group. *The values of IgA are in mg/100ml.

SUBJECT GROUPS	No	Mean*	Range*	S.D.	P.
Control	25	617.23	113.85-1122.14	272.42	-
ACUTE (1)					
AUG	10	772.89	91.88-1851.03	608.09	0.328
Herpes	5	948.19	216.69-2293.88	947.03	0.134
Herpes+AUG	2	575.35	106.00-1044.71	663.76	0.849
Apthous ulcers	3	720.80	459.09-972.62	256.90	0.537
Calculus formation	1	232.75	-	-	0.179
ACUTE (2)					
AUG	11	386.47	71.54-633.33	189.88	0.015
Herpes	5	552.51	383.94-843.02	200.47	0.619
Herpes+AUG	2	547.46	122.30-972.62	601.26	0.748
Apthous ulcers	3	524.56	475.80-548.94	42.22	0.567
Calculus formation	1	232.75	-	-	0.179
ACUTE+CHRONIC (1)					
AUG+gingivitis	4	642.72	232.75-905.51	305.54	0.865
AUG+periodontitis	4	425.17	113.85-843.02	325.50	0.211
Herpes+periodontitis	1	459.09	-	-	0.574
Herpes+gingivitis	1	589.63	-	-	0.921
ACUTE+CHRONIC (2)					
AUG+gingivitis	2	497.29	151.56-843.02	488.93	0.571
AUG+periodontitis	2	267.42	91.88-442.96	248.25	0.091
Herpes+periodontitis	1	656.38	-	-	0.889
Herpes+gingivitis	1	332.78	-	-	0.316
CHRONIC					
Periodontitis	17	626.25	4.56-1786.02	440.19	0.934
Herpes	2	403.16	216.69-589.63	263.70	0.294
Gingivitis	7	392.94	174.86-548.94	130.60	0.044
Apthous ulcers	2	490.66	412.40-568.92	110.67	0.526
RHEUMATOID	9	105.13	268.54-2646.51	821.36	0.038

KEY: No = Number of subject samples
 S.D. = Standard deviation
 AUG = Acute ulcerative gingivitis
 P. = probability that the means of the patient samples in the different groups are the same as the mean of the control samples; P less than 0.0500 is significant.

significant difference in Acute (2) where the IgA value fell markedly. The Acute + Chronic male serum values showed a decreasing trend (Table 6b) and the Acute + Chronic IgA values were lowered and the Rheumatoid IgA values were raised in both male and female serum.

On examining differences within the individual groups, compared to the controls, more significant differences were obtained. Males and females were grouped together in this case. With salivary values (Table 7a), Acute AUG (1), Acute Herpes + AUG (1), Acute Aphthous ulceration (1) and Acute Herpes + Chronic gingivitis (1) showed very significant differences in their IgA values. The Herpes salivary value was raised (not significantly) from the normal, but when combined with gingivitis, gave a greatly increased salivary IgA value. Herpes + periodontitis showed lowered IgA values (not significantly).

With the serum IgA values, there were only three significant differences recorded. They were in lowered IgA in Acute AUG (2), a lowered IgA in Chronic gingivitis and a greatly increased IgA in the Rheumatoid patients.

This division of the groups into their constituent diseases, gave a better indication of the changes in salivary and serum IgA that occurred as the disease progressed or regressed.

DISCUSSION

It was decided to measure IgA levels in serum and saliva of patients presenting with a range of periodontal complaints compared to control subjects free from such disease on

clinical examination. IgA was estimated because it is the predominant immunoglobulin in saliva and overall evidence collected by HEREMANS (1968) shows that IgA plays a major role in contributing protection to the sero-mucous surfaces of the body.

The discovery on the first statistical analysis of the quantitation of serum and salivary IgA, in the control and test subjects, was interesting, in that there were differences in the results between the sexes. If the two groups of male and female were assessed together, significant differences apart from those of the Acute (1) group, were lost. The DMF values and average Russell Index values were similar for both sexes in each group and the numbers with each disease in both groups were quite evenly matched; there were also no outstanding differences in the available medical histories of the two groups.

Some differences in immunoglobulin levels may have been due to the patients' susceptibility to caries as raised IgA values have been attributed to greater caries-resistance than caries-susceptibility (LEHNER et al. 1967; ZENGO et al. 1970, 1971). The total DMF values do not indicate the present degree of decay; however, the control group, although having no active caries, had varied DMF values and the IgA values in relation to susceptibility to caries should have been matched with the test subject groups.

The salivary values for the groups as a whole, vary depending on the type of disease, as already noted by LEHNER (1969a,b). An increase in salivary IgA on increasing severity of the disease might have been expected; on the

other hand, more of the immunoglobulin might be taken up with the attempted control of the disease process and so be removed from the saliva, giving lowered values. The type(s) of antigen(s) in each disease must therefore be considered, especially in a virus-caused disease such as Herpes, where it is probably the Secretory IgA levels that are important (GENCO and TAUBMAN, 1971), a rise in titre in the convalescent stage indicating a Herpes infection. It is interferon production that is initially concerned with the action to overcome virus infections, antibody being produced in larger quantities in the recovery phase of the illness.

Of the salivary values, both groups show a return to normal IgA levels in the convalescent state of Acute and Acute + Chronic infections. The Chronic values are raised in the males and reduced in the females. However none of the values was significant in the saliva apart from the male Acute (1) and male Chronic; it may be concluded therefore that the values in the other groups remain more or less normal in these diseases, when taken as a whole.

The values of IgA in the individual diseases show little variation from the normal as LEHNER (1969,b) reported. Herpes + gingivitis has a greatly increased IgA level in the acute phase, which returns to normal in the convalescent stage, but as this was only in one subject, this change cannot be taken as always being the case. The results of increased IgA in major Aphthous Ulceration in both serum and saliva, corroborated those of LEHNER (1969,b) as did the increased serum values in Herpes, although not significantly. LEHNER (1969,b) did not take into account the caries state

or the amount of gingival or periodontal disease when examining Herpes and Aphthous Ulceration.

The presence of an acute plus a chronic condition makes analysis of the results more difficult as either raised or lowered levels may result from one or the other condition or both.

The amount of endotoxin produced by the plaque can have an inhibitory effect on antibody production (FRANZL and McMASTER, 1968; McMASTER and FRANZL, 1968) depending on the route, dosage and time of injection compared to other antigens. Poor oral hygiene results in the accumulation of plaque which contains more Gram negative organisms and subsequently increased amounts of endotoxin, as the plaque ages (GIBBONS, 1963; RITZ, 1964; GENCO et al. 1969; LYNCH et al. 1969 and GOLDBERG, 1970). Those Gram negative organisms may thus prevent immunoglobulin synthesis, resulting in lowered IgA levels in both saliva and serum.

This may in part account for the fact that serum IgA levels fall in the Acute (2) measurements, where some of the patients may not have been convalescent but in a more acute phase, with their excessively lowered immunoglobulin values affecting the results as a whole; those immunoglobulins normally produced, being used in the immune reaction and then being catabolised as normal with no subsequent stimulus for increased production.

An additional fact is that IIS IgA plus Complement (which is present in crevicular fluid) plus Lysosyme (which is a constituent of saliva) lyse Gram negative bacteria (ADINOLFI et al. 1966), releasing additional bacterial

substances which may be harmful to the host. Increased values of IgA in serum and saliva, on the other hand, especially in the acute phase of eg. AUG plus periodontitis or Aphthous Ulceration, may be the natural result of the defence mechanisms of the body to the antigen in these cases.

Increased levels in Rheumatoid Arthritis are commonly found and apart from the usual secretory mechanisms into the saliva IgA is especially synthesised by the labial salivary glands in Sjögren's syndrome (TALAL et al. 1970).

50x lyophilised saliva was used throughout the complete patient survey. Because there were so many samples to be concentrated within a short space of time, it was felt that both time-wise and financially, lyophilisation would be best. At this level of 'reduction in volume' there is little difference in the final degree of concentration (see Tables 3 and 4). As all samples were processed in an identical manner before immunoglobulin estimation, the results should be comparable.

CONCLUSIONS

An accurate measurement of salivary IgA is difficult because of the presence of both 7S and 11S molecules, and measuring total IgA assumes that both types are involved in specific antibody production in oral disease. HOBBS (1970) recommends multiplication by 1.4 as 11S diffuses 70% of 7S but this assumes that salivary IgA is all the 11S type and this is not so (BRANDTZAEG et al. 1970). Presumably, the most efficient method would be to measure it against both a 7S and an 11S standard so as to gauge the full IgA concentration

in the saliva. Other immunoglobulins may be important in conjunction with IgA (LEHNER, 1969a,b), depending on the disease.

Serum levels may be confusing as they mirror the general health of the individual and are influenced by minor as well as major ailments. Antibodies may be present in serum to organisms related to (and therefore exhibiting some antigenic characteristics in common with) the organisms implicated in oral diseases. Thus their measurement may not give the correct serum antibody level to the oral disease-causing organisms.

The use of different standards and methods in different laboratories leads to confusion in the comparison of results, therefore experimental limits and data must be well-defined and detailed. To enable as much correlation as possible, a) choice of groups into which test subjects are divided, b) standard methods of analysis, and c) comparison of immunoglobulin levels with universal immunoglobulin measurements and standards, would be an advancement in the elucidation of the connection of salivary immunoglobulin levels with defined oral diseases.

The fact that other oral disease eg. dental caries, and systemic disease, are bound to play a role in determining the overall immunoglobulin levels in serum and saliva must be taken into account. LEHNER and SHILLITOE (1972) reported that they chose not to examine caries but only chose those subjects who had no other oral or systemic diseases, when examining immunoglobulins in crevicular fluid; there are few reports where it is even mentioned whether these other

factors are taken into account or not. A rise in serum immunoglobulin in systemic disease may cause a concomitant rise in salivary levels by leakage into the oral cavity if there is tissue destruction due to periodontal disease. These factors confuse the issue, and the results indicate that salivary immunoglobulin levels may be only really relevant and informative where a single disease state may be examined, although trends towards a change in immunoglobulin levels could be observed in the categories listed above.

However, in conclusion, it must be noted that measurement of immunoglobulin levels in both the serum and saliva by SRID is NOT a measure of the antibody levels to the antigen(s) causing the different forms of oral disease. Variation in immunoglobulin levels may be due to an increased or decreased rate of catabolism or production of the immunoglobulins and is not necessarily an indication of the amount of antibody produced in the disease. It may solely indicate impaired immune functions in these individuals with immunoglobulin levels that vary from the normal, although a trend to changes in immunoglobulin levels in groups of patients with different oral diseases is evident: particularly when these diseases are analysed singly, rather than grouped under the same condition (eg. acute; chronic) with a sub-division into male and female.

CHAPTER 6.THE EFFECT OF IMPLANTATION OF CARIOGENIC
STREPTOCOCCI IN THE RABBIT MOUTH.INTRODUCTION

Rodents were used for this investigation because of ease of (a) implantation of microorganisms in the oral cavity and (b) mixed saliva collection. The response of the oral environment to this change in flora, both by salivary and gingival tissue immune mechanisms was estimated and compared to the human response to microorganisms present in the mouth, which are implicated in the pathogenesis of oral disease.

MATERIALS AND METHODSCultivation of bacteria-

The cariogenic streptococci Strep. mutans OMZ 176 E were obtained from Dr. B. Guggenheim (Zurich, Switzerland). They were grown anaerobically on horse blood agar plates on which they produced a narrow zone of ~~haemolysis~~ haemolysis, or in Brewers Medium (CRUICKSHANK, 1965). These organisms are Erythromycin resistant and therefore incorporation of 1mg Erythromycin (Eli Lilly & Co. Ltd.; supplied by The Lilly Research Centre Ltd., Erlwood Manor, Windlesham, Surrey) /ml medium prevented growth of other streptococci.

Laboratory animals-

In each experiment, three male Californian rabbits weighing 2-3 Kg were used. They were 3-4 months old on initial implantation.

Cariogenic diet for implantation of microorganisms-

The following cariogenic diet was devised by KEYES and JORDAN (1964).

<u>Diet 2,000</u>	Confectioners sugar	56%	Powdered alfalfa	3%
	Skim milk powder	28%	Liver powder	1%
	Whole wheat flour	6%	Sodium chloride	2%
	Brewers yeast	4%		

This was fed with a weekly supply of equal parts of apple, carrot and kale, blended into a puree; about 10g/animal/week.

10g of the dry ingredients of the diet was dissolved in the drinking water of rabbits 7 and 8 (the test animals) each day, while rabbit 1 (the control animal) received the normal drinking water. This diet was fed throughout the duration of the experiment.

Implantation of microorganisms-

A sterile swab was rubbed over a heavy growth of organisms on a blood agar plate. This swab was then rubbed over the upper and lower incisors and surrounding gingivae of rabbits 7 and 8, every third day for the duration of the experiment. Rabbit 1 received no organisms.

Determination of the success of implantation-

Before commencing the experiment, swabs were taken from the incisor area of the rabbit mouths and were plated out on blood agar plates. The swabs were also placed in Brewers medium containing 1mg Erythromycin/ml medium. No streptococci grew in the latter although some α haemolytic streptococci

were present in the normal rabbit oral flora and were detected on the blood plates. No OMZ 176 E were present in the rabbit mouths. Each day, before swabbing with the organisms, swabs were taken to test whether implantation had occurred. These swabs were plated on blood agar to test for β haemolytic colonies and were placed in Brewers medium plus Erythromycin (1mg/ml); growth in this medium indicated the implantation of OMZ 176 E in the rabbit mouth.

Collection of rabbit serum-

The ear was shaved and the vein enlarged by swabbing with xylene. A 1" 20G B-D Vacutainer needle (Becton-Dickinson, Division of Becton, Dickinson & Co., Rutherford, New Jersey) was inserted and the blood allowed to flow through the needle into a sterile bottle. After cessation of bleeding, the ear was thoroughly washed with water to prevent necrosis of the tissue by the xylene. The blood was centrifuged at 4,000 rpm (200g) for 10 minutes; the serum was removed and stored in 1ml aliquots at -20°C .

Collection of rabbit saliva-

Mixed saliva was stimulated by an intra-peritoneal injection of 5mg Pilocarpine Hydrochloride (BDH Chemicals Ltd., Poole, Dorset, BH12 4 NN, England) /Kg Body weight of the rabbit (after BROWN and QUINTON, 1957). Increased salivary flow was apparent after about 10 minutes and the fluid was collected in a sterile universal bottle as it dripped from the rabbit mouth. The saliva was stored at 4°C until centrifugation at 15,000 rpm (20,000g) for 40 minutes. The

supernatant fraction was removed and stored at -20°C until required.

Concentration of rabbit saliva-

The saliva supernatant was dialysed against distilled water for 24 hours at 4°C , in a similar manner to human saliva (see Chapter 3). The volume of dialysed saliva was decreased 10x or 20x by Lyophilisation, as described in Chapter 3. Protein concentration was measured by the method of Lowry (LOWRY et al. 1951). Ultrafiltration, as described in Chapter 3, was also carried out.

Immuno-electrophoresis and Immunodiffusion-

The methods used were those described in Chapters 3 and 4 respectively. The rabbit serum and concentrated rabbit saliva were tested for immunoglobulin content using Goat anti-rabbit gamma globulin (Hyland) and goat anti-rabbit IgA, IgG and IgM (Miles-Yeda Ltd., distributed by Miles Research Products Division, Miles Laboratories, Inc., P.O.Box 272, Illinois 60901, USA). The antisera were tested by immuno-electrophoresis to check that only specific anti-heavy chain activity was present.

Pathology of implantation-

After six months, the three rabbits were killed by intravenous injection of Nembutal (Abbot Laboratories Ltd., Queenborough, Kent), after the final swabs had been taken and serum and saliva collected. The upper and lower incisors including the surrounding gingivae, of all the rabbits were

removed immediately after death, into neutral buffered formal saline. The teeth were decalcified and the teeth (with the attached tissue) were embedded in wax. The sections cut from the upper incisors plus gingivae of rabbit 1 and rabbits 7 and 8 were stained H and E and Gram.

Examination of rabbit serum and saliva by
Immuno-fluorescence methods-

I. To estimate if serum and/or salivary antibodies were produced in rabbits 7 and 8 against the implanted organisms, the following immuno-fluorescence techniques were employed.

1. Smears of OMZ 176 E were made on cleaned glass slides and allowed to air dry before fixing in neutral buffered formal saline for 10 minutes at 4°C.
2. The slides were washed in isotonic Phosphate Buffered Saline (PBS), pH 7.1 for 2-3 minutes.
3. The experimental rabbit serum or 10x Lyophilised rabbit saliva was layered onto the slides which were left in a dark, moist atmosphere for 30 minutes.
4. The slides were washed in PBS for 10 minutes.
5. Goat anti-rabbit gamma globulin (fluorescent) (Hyland or Fluorocan, Winthrop Biologicals Ltd., Winthrop House, Surbiton-on-Thames, Surrey) was layered onto each slide and left in a dark, moist atmosphere for 30 minutes.
6. The slides were washed in PBS for 10 minutes.
7. The slides were mounted in neutral buffered glycerol, pH 7.1, using ultra-thin coverslips before examination under the UV microscope.

Negative controls were set up in the following ways:

- a. Smears were heated in boiling water for 10 minutes before processing as in 2.-7 above.
- b. The technique was carried out as in 1.-7 above, substituting 0.85% (w/v) saline in 3. Human serum was not used to block the reaction because of the reported cross-reactivity between rabbit and human serum proteins (JOHNSON and YODER, 1970). (Illustrated in fig. 12b, 4 vs 7).

II. To estimate the class of immunoglobulin (if any) producing immunoglobulin antibody, the technique was as follows:

1. As in 1.-4 above.
2. Goat anti-rabbit IgG, IgA and IgM (Miles-Yeda Ltd.) were layered onto each slide and left in a dark, moist atmosphere for 30 minutes.
3. The slides were washed in PBS for 10 minutes.
4. Rabbit anti-goat gamma globulin (fluorescent) (Difco, Difco Laboratories, Detroit 1, Michigan, USA) was layered onto each slide which were left in a dark, moist atmosphere for 30 minutes.
5. As in 6. and 7. above.

Negative controls were set up as above, with the additional control of substituting physiological saline at part 2. of II, above.

Immuno-fluorescence studies on human oral streptococci, human serum and saliva as a comparison with the implanted animal model-

A strain of Strep. sanguis was obtained from the Bacteriology Department, Glasgow Dental Hospital and School,

which had been isolated on blood agar, from a patient oral swab. Serum and saliva (50x Lyophilised) were collected in the usual way from both patients and controls; some patients and controls were selected from the Patient Survey samples (Chapter 5).

The immuno-fluorescence studies were carried out as described in I. of the previous section, substituting human serum and saliva in 3., and Hyland fluorescent anti-human IgG, IgM and IgA (goat) in 5. Negative controls were set up as described before.

RESULTS

Macroscopic and microscopic examination of the oral state on implantation-

A fortnight after commencing implantation, the organisms OMZ 176 E were recovered from the swabs of the incisal area of rabbits 7 and 8. Gram stains were made of the implanted organisms and those recovered from the Brewers medium containing Erythromycin (fig.17). From then until the rabbits were killed, these organisms formed part of their oral flora.

Whilst being fed the cariogenic diet, the rabbits developed a redness in the gingivae (which may have been caused by the trauma of swabbing) and a film over the incisors which was not evident in control rabbit 1.

The teeth and gingival tissues were examined microscopically following sacrifice.

(i) Gram stain: The control rabbit had a few bacteria on the tooth surface which stained Gram positive and had

the appearance of cocci. The experimental rabbits had large numbers of bacteria on the teeth. These were mostly Gram positive Cocci; the high carbohydrate diet had caused a thin pellicle to be laid down on the tooth surface to which the bacteria could adhere more easily than when fed normally. This mimics the early stages of human plaque formation. Fig.18 illustrates this observation.

- (ii) H and E stain: The gingivae of rabbit 1 appeared normal (figs.19 a and b). That of rabbits 7 and 8 showed an inflammatory cell infiltrate (figs.20 a and b) which could have resulted from the bacterial build-up at the gingival margin.

No dental caries was evident on the incisors of either the control or experimental animals.

Concentration of rabbit saliva-

Rabbit saliva total protein concentration when measured by Lowry, after reduction in volume, gave good corresponding concentration values compared to that of human saliva. Pilocarpine stimulation probably resulted in a more dilute secretion that was more easily concentrated as reduced viscosity results in less loss of protein. The results are recorded in Table 8. The estimates were carried out a minimum of twice on each sample.

As with human saliva, protein appeared to adhere to the UM-20 membrane on Ultrafiltration, causing loss of protein. Concentration by Lyophilisation resulted in good concentration and this method was subsequently employed.

Fig.17. Strep. mutans OMZ 176 E (x1520).



Fig.18. Strep. mutans OMZ 176 E on the surface of the upper incisor of rabbit 7. (x1516.8).

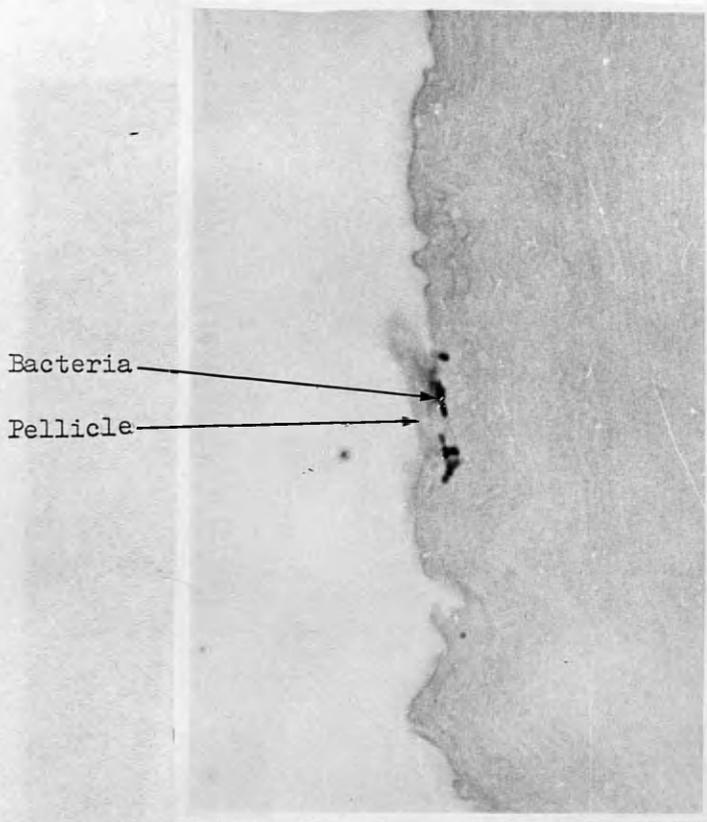


Fig.19a. Gingivae of rabbit 1 (control) adjacent to upper incisors. (x41.44).

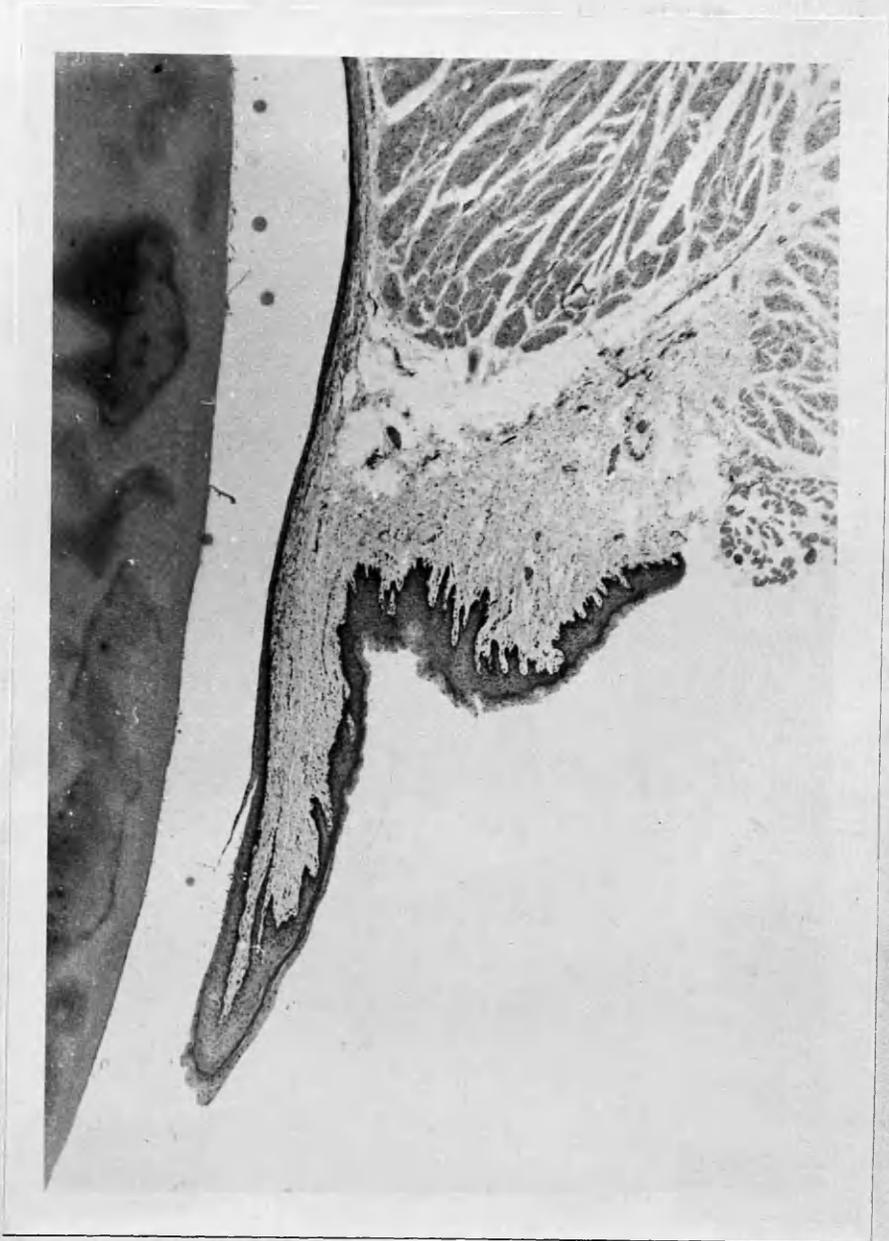


Fig. 19b. High power of gingivae of rabbit 1. (x360).

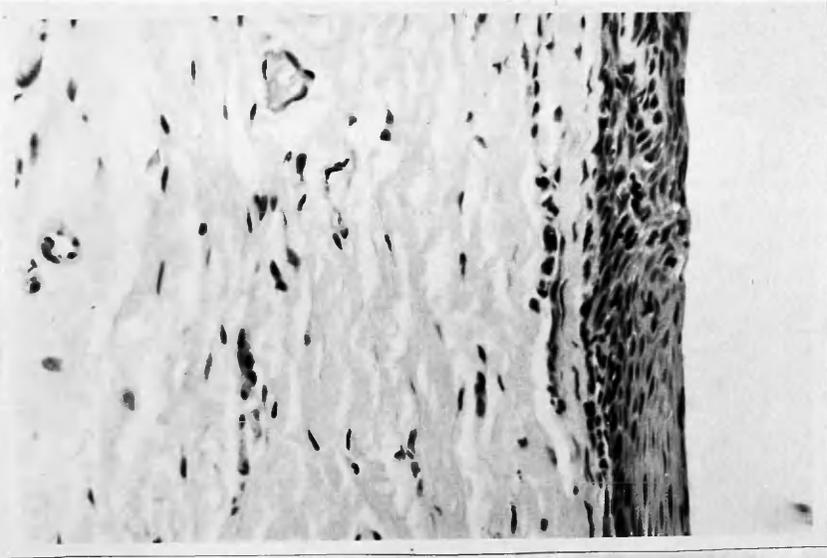


Fig. 20a. Gingivae of rabbit 7 (implanted) adjacent to upper incisors. (x83.2).

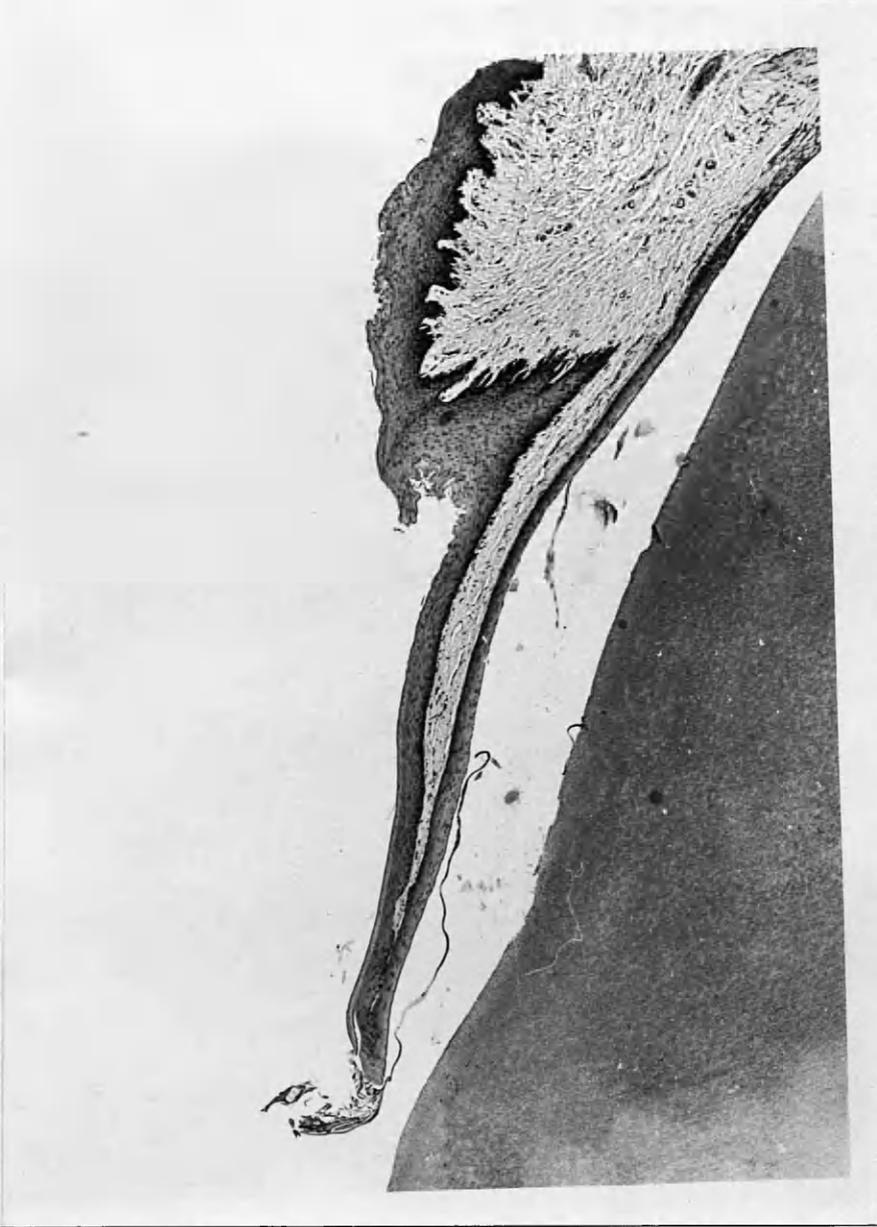
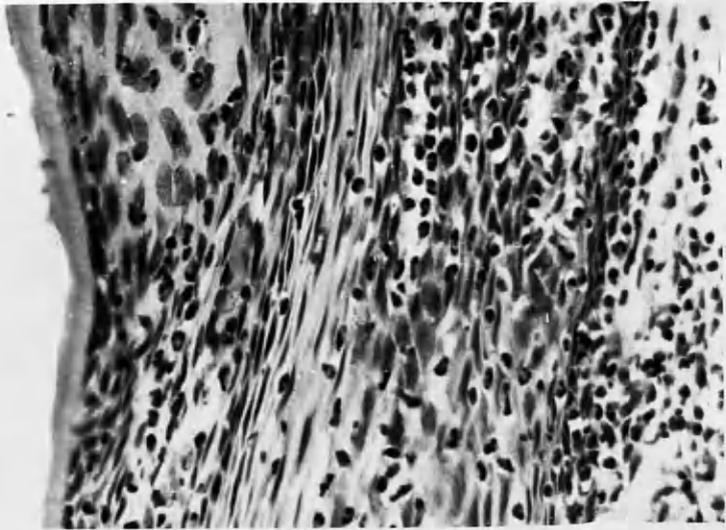


Fig. 20b. High power of gingivae of rabbit 7. (x360).



Examination of rabbit serum and saliva by immunoelectrophoresis and immunodiffusion-

On analysis of rabbit serum by immunodiffusion (fig.21) when it was diffused against anti-rabbit gamma globulin, a number of precipitin lines developed. Not as many were produced as on immunodiffusion of normal human serum against anti-human normal serum.

Immunoelectrophoretic analysis using antiserum specific for the individual rabbit serum gamma globulin fractions produced precipitin arcs indicating the presence of IgG, IgA and IgM in the rabbit serum (fig.22). On immunoelectrophoresis of unconcentrated saliva, no immunoglobulins were detected. When the secretion was concentrated 10x by Lyophilisation, an IgG arc appeared and on 20x reduction in volume by Lyophilisation, all three immunoglobulins were seen to be present, the IgM arc appearing as a very faint band (fig.23). The appearance of IgG as the immunoglobulin in greatest concentration in rabbit saliva corroborates the report of TAUBMAN and GENCO (1969) that this is the predominant immunoglobulin in rabbit saliva.

Estimation of antibody production to the implanted streptococci by immune fluorescence.

After applying the serum and concentrated saliva of the control rabbit 1 and test rabbits 7 and 8, plus the specific fluorescent anti-serum, the slides were examined for the presence of fluorescing organisms. The controls were negative as were the experimental slides; no fluorescence was apparent from either the serum or saliva of any of the rabbits.

Table 3. Concentration of rabbit, mixed saliva supernatant by Lyophilisation and Ultrafiltration; measurement of total protein by Lowry.

Saliva and methods of concentration	Protein conceh. mg/100ml.	Concentration factor.
Rabbit saliva	114.0	-
Rabbit saliva after centrifugation and dialysis	95.0	-
R. saliva after volume decreased 20x by Ultrafiltration	840.0	8x
R. saliva after volume decreased 10x by Lyophilisation	891.0	9x
R. saliva after volume decreased 20x by Lyophilisation	1140.0	13x

Results of analyses on pooled saliva samples from six healthy rabbits. This was used to correspond as closely as possible to the human situation.

Fig.21. Normal immunodiffusion pattern of rabbit serum. (x4).

1. 1:10 Flow rabbit serum; 2. 1:10 rabbit 1 serum
3. 1:10 rabbit 7 serum 4. 1:10 rabbit 8 serum
5. 1:10 rabbit anti-human serum
6. 1:10 normal human serum
7. 1:3 goat anti-rabbit serum (Hyland)

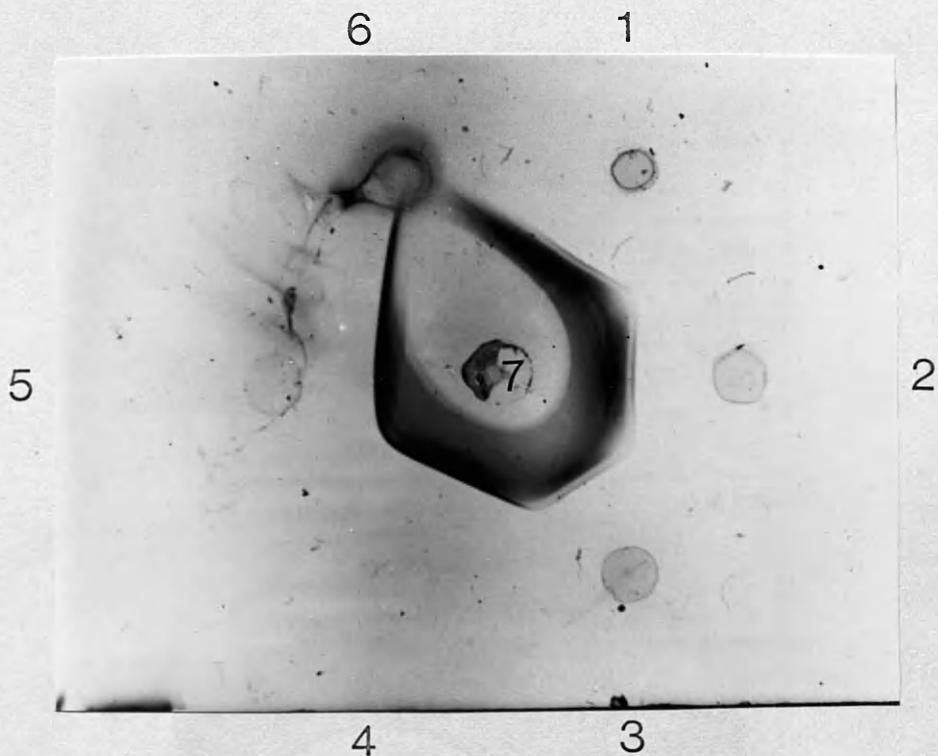


Fig.22. Normal immunoelectrophoretic pattern of rabbit serum. (x2).

The anode is to the right and the cathode to the left. All the wells contain normal rabbit serum.

1. Hyland goat anti-rabbit gamma globulin
2. Miles-Yeda goat anti-rabbit IgA
3. Miles-Yeda goat anti-rabbit IgM
4. Miles-Yeda goat anti-rabbit IgG

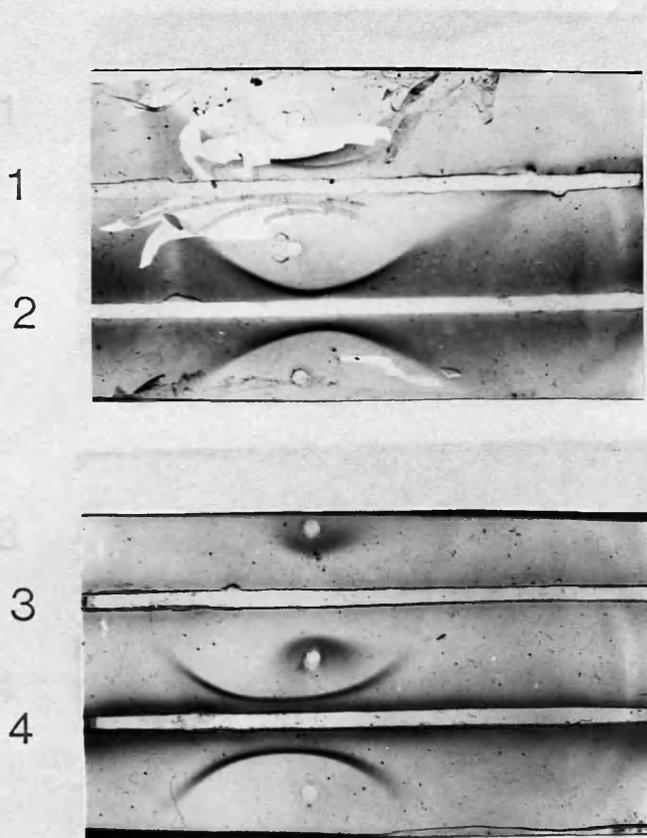
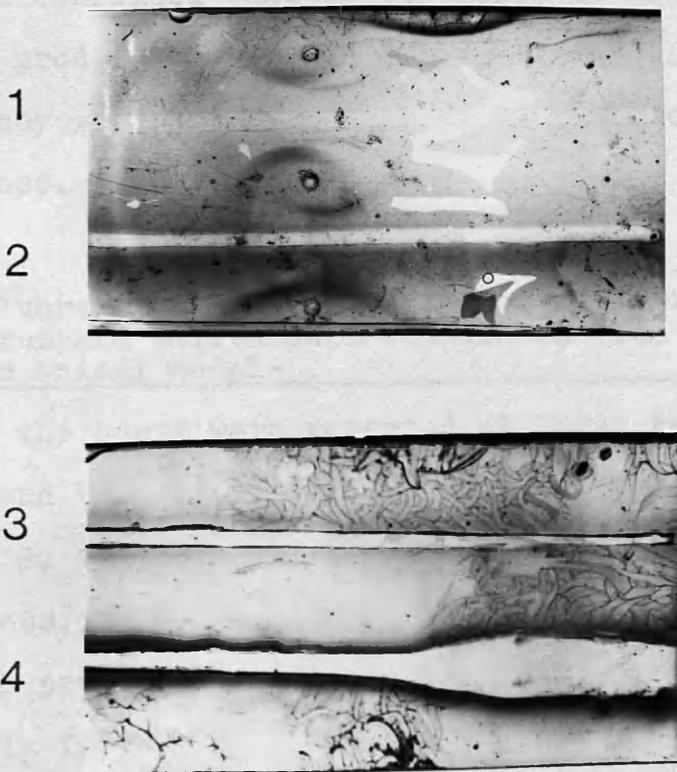


Fig.23. Normal immunoelectrophoretic pattern of 20x
Lyophilised rabbit saliva (x2)

The anode is to the right and the cathode to the left.
All the wells contain rabbit saliva.

1. Hyland goat anti-rabbit gamma globulin
2. Miles-Yeda goat anti-rabbit IgA
3. Miles-Yeda goat anti-rabbit IgG
4. Miles-Yeda goat anti-rabbit IgM



To increase the sensitivity of the reaction, antiserum specific for the individual rabbit immunoglobulins was applied. Again, all the smears were negative.

These tests were repeated a number of times and on all occasions they proved negative. Positive controls demonstrated that the system was working, as fluorescent-labelled antiserum applied to a smear that had already had specific streptococcal grouping antiserum applied in an identical manner to the above, gave positive fluorescence. It was therefore concluded that if any serum or salivary antibodies had been produced to the implanted OMZ 176 E in rabbits 7 and 8, they were in insufficient quantity to be detected by this method.

Immuno-fluorescence studies on human oral streptococci, human serum and saliva as a comparison with the implanted animal model-

All the tests were repeated at least twice on the three patient and three control samples. The results, recorded in Table 9, followed the same pattern that had been observed in previous, trial immuno-fluorescent studies.

Both patient and control groups had antibodies, especially IgG class in their serum, to the isolated organism. The control subjects, who were free from periodontal disease and had no active caries, although one had a history of a previously high caries rate, had salivary IgA antibodies. The presence of antibodies in the saliva of the test subjects, one of whom had no active caries, was doubtful, as fluorescence was very low. The fluorescence produced by the IgA antibodies in saliva was positive when compared to the positive

Table 9. Results of fluorescence studies on human serum and saliva (concentrated 50x by Lyophilisation) from individuals estimated to have clinically healthy gingivae and those with some form of periodontal disease. The diseased subjects were taken from the patient sample (reported on in Chapter 5) as were the controls taken from that control sample.

SUBJECT	SERUM ANTIBODIES		SALIVARY ANTIBODIES	
	IgG	IgA	IgG	IgA
Patient with A.U.G.	++	+	-	±
Patient with Herpes and Pericoronitis.	++	+	-	±
Patient with aphthous ulcers.	++	+	-	±
Control 1.	++	+	-	+
Control 2.	++	+	-	+
Control 3.	++	+	-	+

Number of + indicates the degree of fluorescence.

- indicates negative fluorescence.

± indicates doubtful fluorescence.

The organism used was Strep. mutans OMZ 176 E.

and negative controls, but did not fluoresce as brightly as the antibody in the positive sera.

The fluorescent antibody technique is not a precise quantitative test, but it appeared from the amount of fluorescence that:

- 1) Patients and controls had high serum IgG and good serum IgA antibody titres to the streptococci.
- 2) Neither patients nor controls had salivary IgG antibody to the streptococci.
- 3) Controls showed the presence of more IgA antibody to the streptococci than test subjects whose saliva resulted in a low level of fluorescence.

DISCUSSION

The aim of this series of experiments was to discover what effect an implanted organism, that was foreign to the normal flora of the rabbit mouth, would have on the appearance of the teeth and gingivae, both macroscopically and microscopically and also to assess its effect on serum and salivary antibody production.

It was proved relatively easy to implant the cariogenic streptococci, swabbing the rabbit mouths in the same way as Guggenheim et al. (1970) whilst they were being fed the cariogenic diet. The organisms could be identified in the plates (fig.24a) and in the medium containing Erythromycin (fig.24b), thus preventing growth of other streptococci. Both types of media were used for isolating the organisms from the swabs used for testing for implantation.

It was obvious on macroscopic appearance, that a film was

Fig.24a. Blood agar plates showing growth of Strep.
mutans OMZ 176 E.

1. shows the original culture with β haemolysis
2. shows the culture of implanted and recovered organisms with fainter β haemolysis.

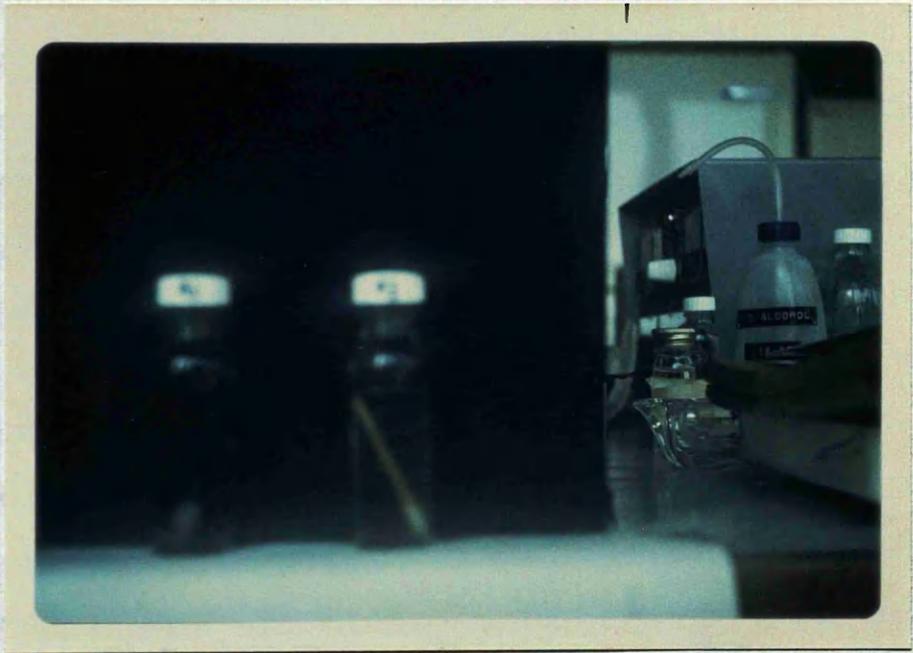


1

2

Fig.24b. Brewers medium containing Erythromycin (1mg/ml).

1. shows the clear medium indicating no growth from the swab of control rabbit 1.
2. shows the cloudiness indicating growth from the swab of test rabbit 7.



1

2

developing over the incisors of the implanted rabbits that did not appear in the non-implanted, therefore Diet 2,000 of KEYES and JORDAN (1964) was instrumental in allowing implantation. The diet was stopped for a fortnight with rabbit 8 and OMZ 176 E disappeared but was reimplanted on readministration of the diet. Therefore it may be concluded that a high sucrose diet was responsible for implantation in this case.

As reported in the results, those rabbits with implanted organisms 'foreign' to their normal flora, developed a surface layer on their incisors and at the dento-gingival margin, resulting in the appearance of inflamed gingivae. The bacteria that adhered in this area had not penetrated the intact epithelium to invade the tissue at all. This fact that bacteria do not invade the intact tissue has been observed at Electron Microscope level by FREEDMAN et al. (1968) and SUSSMAN et al. (1969), but it does not exclude the possible penetration of bacterial metabolites and/or toxins into this area.

In agreement with this, it was observed that the normal appearance of the rabbit gingiva at the labial mucosal-gingival site exhibited few inflammatory cells, whereas in the implanted animals, there was a large inflammatory cell infiltrate (figs.20a and b). Either bacterial products were acting as antigens and causing an immunological response in the gingiva, and/or the accumulation of debris (including bacteria and the diet deposit) on the dento-gingival margin, was causing trauma of the tissue, resulting in inflammation and gingivitis. This immunological response demonstrates the

ability of the tissue at the site of irritation to initiate a protective response to overcome the irritation. It appears that in this case, as well as in the human situation, the protective immune mechanism results in the release of substances including eg. lysosomal substances, pharmacological reagents and antibody with the capacity to bind to tissue, that are detrimental to the host during the process of combating local infection.

Examination of the rabbit serum and saliva by immunoelectrophoresis and immunodiffusion revealed the presence of immunoglobulins, as has already been described by CEBRA and ROBBINS (1966); CEBRA and SMALL (1967); CEBRA et al. (1968); GENCO and TAUBMAN (1969) and TAUBMAN and GENCO (1971). The fact that these IgG, IgA and IgM immunoglobulins are present in both serum and saliva, though in different proportions from the human situation, indicates that the animals are capable of producing antibodies to foreign proteins, as has already been reported by BERGLUND et al. (1969), GENCO and TAUBMAN (1969) and TAUBMAN and GENCO (1971).

The immuno-fluorescence studies were carried out to determine if either serum or salivary antibodies had been produced, but none were detected. This means that either none were produced or that any that were produced in a primary response were immediately combined with the antigen before it could effectively provide enough antigenic stimulation to elicit a high titre of antibody in a secondary response. Alternatively, it could mean there was a lymphocyte-mediated immune response.

In comparison, in the human situation, antibodies to a

streptococcus that is thought to play a causative role in the initiation of periodontal disease were present in the serum of both periodontally diseased patients and control subjects. Salivary antibodies appeared to a greater extent in the healthy controls than in the diseased. Serum antibody levels may not be important in this case. Salivary antibodies could have some significance in that they might possibly be adsorbed to the greater numbers of accumulated bacteria in the diseased mouth and therefore be unable to carry out a protective role at the surface of the oral tissue although they are protecting the mouth from further bacterial attack. It seems that possibly tissue mechanisms might play a more important role in combating oral disease than salivary mechanisms, although those people with periodontal involvement may have impaired secretory immunoglobulin mechanisms.

DICK and TROTT (1969,b and 1971) have demonstrated that antigen penetration of the gingiva cannot always be demonstrated due to efficient ingestion and inactivation by the host defence cells, which thus prevent the activation of antibody-forming cells. Any antibody formed as a result of minor leakage would enhance the phagocytic activity of the polymorphonuclear leukocytes (PMNs) (HAMP and FOLKE, 1968) in the irritated gingival tissue. However, the bacteria themselves did not appear to penetrate the gingivae in this experiment, therefore it was concluded that the bacterial products caused the production of an inflammatory cell infiltrate in this case, to overcome the noxious agents. Any salivary antibody produced due to their accumulation on the surface of the teeth on adsorption to the surface of the

bacteria (BRANDTZAEG et al. 1968,b) would render nil, the possibility of its detection by the method used.

CONCLUSIONS

It appears that the continued presence of an organism in persons with a more competent immune system enables health to be maintained; implantation of a new organism does not result in sufficient quantities of salivary antibody to overcome its effect.

It seems probable from these results that it is necessary to implant the antigen into the pocket as did RIZZO and MITCHELL (1966) and RANNEY (1970) or to cause irritation with an inert substance such as cervical ligature (ROVIN et al. 1966) in order to allow sufficient antigen penetration to stimulate the production of detectable amounts of antibody in the serum or saliva and in the tissue locally. In the human situation, the PMN infiltration is followed by antibody-producing plasma cells, on continued irritation of the gingiva (ZACCHRISSEN, 1968; PORTEOUS, 1969; PLATT et al. 1970 and BERGLUND, 1971).

In animals, previously sensitized with the antigen, the tissue antibody response would be much greater, and the antibody would combine with the antigen and the resulting complexes would attract PMNs. Their action, although removing the antigen, would lead to tissue destruction.

This might be the case in the human mouth, with continual sensitization of the gingiva by bacteria and their metabolites, some of which might have adjuvant activity, and

micro-ulceration of the gingival epithelium, leading eventually to tissue destruction by the body's endeavour to overcome this infection locally.

CHAPTER 7.STUDIES ON THE LOCAL IMMUNIZATION
OF RABBITS.INTRODUCTION

Immunization against periodontal disease and/or dental caries is attractive. Results of immunization studies to date (WAGNER, 1966; 1967 and BOWEN, 1969,a) are encouraging even if the mechanisms cannot yet be fully explained.

Rodents were chosen for this investigation because of ease of examination of the oral cavity; the possibility of producing antibody in rabbit saliva against a single antigen was studied.

Dental plaque is so complex and the oral microflora so varied, that no single antigen has so far been identified as the causative agent of the different oral diseases. Any plaque antigen chosen would be removed from its usual environment and could well have a different effect from that exerted normally in the human mouth and might not necessarily be antigenic in the rabbit mouth (cf. CEBRA and ROBBINS (1966) who failed to elicit antibody in colostrum to Ferritin injected in the rabbitmammary gland). Active immunization was therefore attempted with a single solution of human gamma globulin to investigate the best route of inoculation that would give maximum local antibody production. Immunization passively, with human plasma albumin, human serum and horse anti-human IgG was also attempted.

MATERIALS AND METHODSLaboratory animals-

Six male Californian rabbits weighing 2-3 Kg. were used

in each series of immunization experiments. They were fed their normal diet.

Collection of rabbit serum and saliva-

Serum and saliva samples were collected as described in Chapter 6.

Concentration of rabbit saliva-

The rabbit saliva was concentrated as outlined in Chapter 6.

Immuno-electrophoresis and Immunodiffusion-

The same techniques were employed as described in Chapter 6.

Active immunization schedules-

A control rabbit that was not immunized or was ^{sham-}immunized with physiological saline was included in each series of immunization experiments. The injection schedules are outlined in Tables 10a,b,c. Kabi gamma globulin was the antigen (Ag) used. Immuno-electrophoretic examination of the serum and saliva from the rabbits before injection, revealed that they had no natural antibodies to this antigen; haemagglutination was also used to confirm this fact (Vide infra).

'Passive' immunization schedules-

A minimum of two rabbits was used in each test.

1. 1.0ml of normal human serum was injected intra-venously.
2. 0.65ml of Horse anti-human IgG (Nordic Pharmaceuticals

and Diagnostics, 91 Amhurst Park, London N16) was injected intra-venously.

3. 0.2g of Human plasma albumin (Behringwerke AG, Hoechst Pharmaceuticals, Hoechst House, Salisbury Road, Hounslow, Middlesex, England) in 0.6ml sterile, distilled water, was injected intra-venously.
4. 0.2g of Human plasma albumin (Behringwerke AG) in 0.6ml sterile, distilled water, was injected intra-peritoneally.

2 uninjected rabbits were used as controls; the saliva of the animals used for injection was tested beforehand for the presence of antibodies to the antigens used, by immunoelectrophoresis and haemagglutination. If antibodies were already present, they would combine with the substance being injected and prevent any transport into the saliva.

Haemagglutination-

The method in 'Handbook of Experimental Immunology' (edited by WEIR, 1967) was followed; see fig.25.

(i) This test was used to determine the serum and salivary antibody titres produced in response to active immunization with gamma globulin. 20mg of gamma globulin was coated onto the red blood cells. All tests were repeated to check the results obtained.

(ii) This test was also used to check for the presence in saliva of the proteins it was proposed to administer parenterally. 20mg of each of the following proteins was coated onto the tanned red cells to determine whether any of the substances to be injected as outlined in the previous

Table 10a. Active Immunization schedule 1.

<u>Rabbit number</u>	<u>1st injection</u>	<u>-28days-</u>	<u>2nd injection</u>	<u>-10days-</u>	<u>3rd injection</u>
1.	8mg Ag+FCA i-m		5mg Ag i-v		2mg Ag i-left cheek
2.	As in 1.		1.0mg Ag i-lymph node		As in 1.
3.	As in 1.		10mg Ag/day in drinking water		As in 1.
4.	5mg Ag/Kg body weight i-gingiva		1.0mg Ag i-right cheek		rabbit died
5.	As in 4.		10mg Ag/day in drinking water		1.0mg Ag i-left cheek
6.	As in 4.		1.0mg Ag i-lymph node		2mg Ag i-left cheek

The antigen (Ag) used was Kabi gamma globulin; serum and saliva were collected 10 days after injection in each case.

Key: FCA = Freund's Complete Adjuvant

i = intra

i-v = intra-venously

i-m = intra-muscularly

Table 10b. Active Immunization schedule 2.

<u>Rabbit number</u>	<u>1st injection</u>	<u>-28days-</u>	<u>2nd injection</u>	<u>-10days-</u>	<u>3rd injection</u>	<u>-10days-</u>	<u>4th injection</u>
1.	5mg Ag/Kg body weight i-left S-M gland		2mg Ag i-left cheek		2mg Ag i-left cheek		2mg Ag i-left cheek
2.	As in 1.		10mg Ag/day in drinking water		As in 1.		As in 1.
3.	As in 1.		2mg Ag i-left cheek		As in 1.		As in 1.
4.	As in 1.		10mg Ag/day in drinking water		As in 1.		As in 1.
5.	4 i-m injections of 10mg Ag + FCA		5 mg Ag i-left S-M gland		As in 1.		As in 1.
6.	As in 5.		5mg Ag i-v		No injection		0.2ml NaCl (physiological) i-left cheek

The antigen (Ag) used was Kabi gamma globulin; serum and saliva were collected 10 days after injection in each case.

Key: FCA = Freund's Complete Adjuvant

i = intra

i-v = intra-venously

i-m = intra-muscular

S-M = sub-mandibular

Table 10c. Active immunization schedule 3.

<u>Rabbit number</u>	<u>1st injection</u>	<u>-28days-</u>	<u>2nd injection</u>	<u>-14days-</u>	<u>3rd injection</u>	<u>-14days-</u>	<u>4th injection</u>
1.	16mg Ag + FCA i-m in 2 sites		5mg Ag i-v		0.2ml NaCl (physiological) i-left parotid gland		No injection
2.	As in 1.		5mg Ag i-left parotid gland		2mg Ag i-left parotid gland		2mg Ag i-left parotid gland
3.	As in 1.		As in 2. + 1.0mg Ag i-left cheek		As in 2.		As in 2.
4.	As in 1.		As in 3.		As in 2.		As in 2.
5.	As in 1.		5mg Ag i-left parotid gland + 1.0mg Ag i-m		As in 2.		As in 2.
6.	12mg Ag + FCA i-m in 2 sites		As in 5.		As in 2.		As in 2.

The antigen (Ag) used was Kabi gamma globulin; serum and saliva were collected 10 days after injection in each case.

Key: FCA = Freund's Complete Adjuvant

i = intra

i-v = intra-venously

i-m = intra-muscularly

section was already present in the rabbit saliva.

1. Hyland goat anti-human normal serum.
2. Kabi gamma globulin.
3. and 4. Hyland goat anti-human plasma albumin.

All tests were repeated to check the results obtained.

In (i) and (ii) the dilute or concentrated saliva, and serum, collected from the rabbits was termed the 'antiserum' in these experiments and was absorbed with tanned red cells to remove non-specific organisms. Positive and negative controls were always set up concurrently with the tests. The negative control consisted of tanned red cells plus the antiserum used in the test; the positive control consisted of red cells coated with a substance known to react with the test antiserum. Other controls were set up as follows:

- a. Washed red blood cells (RBCs) plus antigen.
- b. Washed RBCs plus rabbit serum* (absorbed and inactivated with sheep red blood cells).
- c. Washed RBCs plus saline (PBS).
- d. Washed RBCs plus 1% rabbit serum*/PBS.
- e. Tanned RBCs plus PBS.
- f. Tanned RBCs plus rabbit serum* (not absorbed and inactivated).
- g. Tanned RBCs plus rabbit serum* (absorbed).
- h. Tanned RBCs plus 1% rabbit serum*/PBS.
- i. Tanned RBCs plus antigen (absorbed).
- j. Coated RBCs plus PBS.
- k. Coated RBCs plus rabbit serum* (not absorbed and inactivated).
- l. Coated RBCs plus 1% rabbit serum*/PBS.

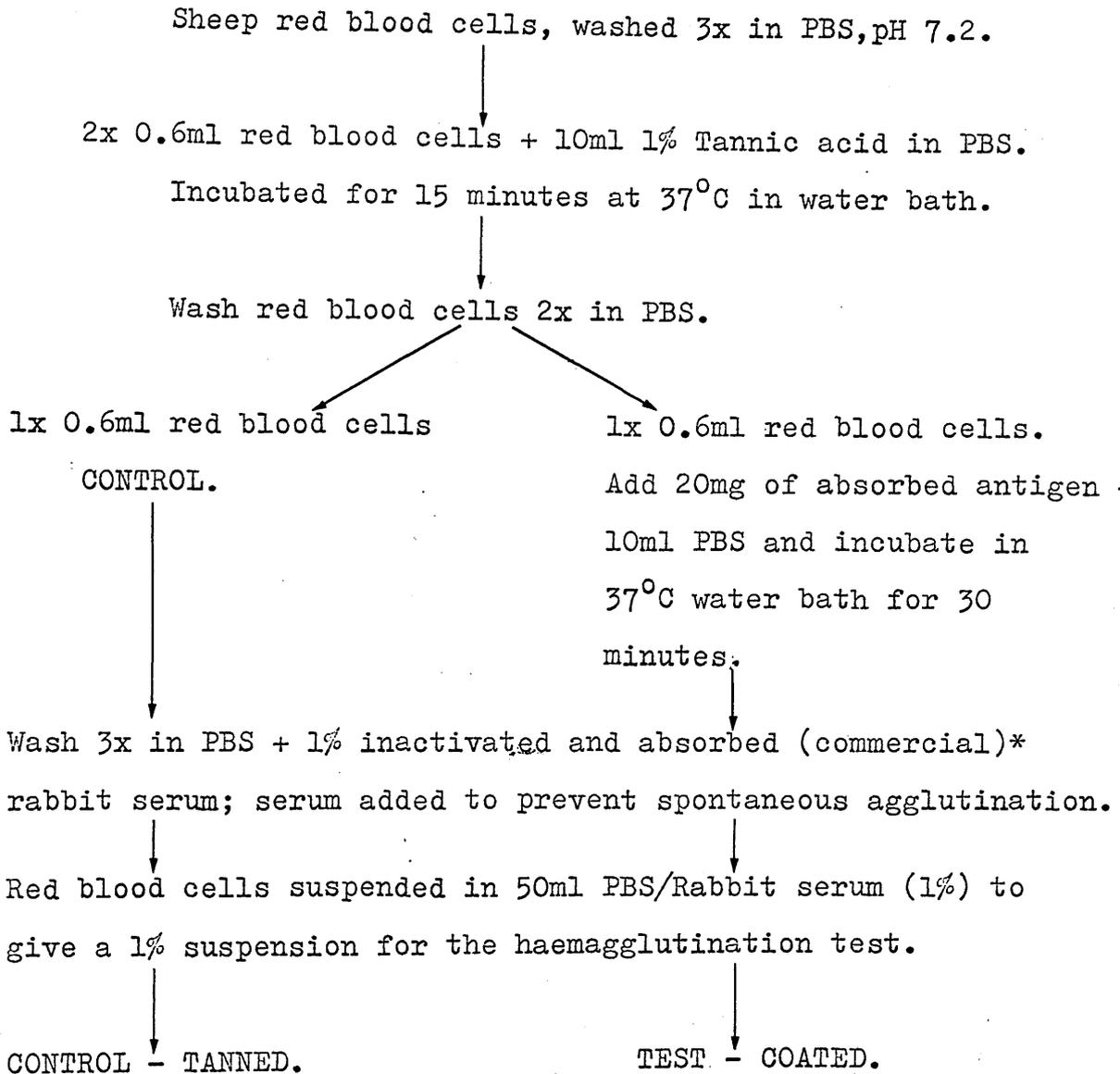
HAEMAGGLUTINATION.

Fig.25 Flow diagram to outline the method used in the haemagglutination reaction, to prepare the red blood cells.

Key: *The rabbit serum was obtained from Flow Laboratories Ltd., Victoria Park, Heatherhouse Road, Irvine, Ayrshire, Scotland.

PBS = Phosphate Buffered Saline, pH 7.1.

The haemagglutination reactions were carried out in plastic agglutination plates, with wells 1.5cm in diameter (Flow Laboratories Ltd.). 0.1ml of a 1% suspension of sensitized (i.e. coated) cells was added to 0.1ml of a doubling dilution of the absorbed 'antiserum'; the doubling dilutions were made in physiological saline. The results were read after overnight incubation at room temperature, in a moist atmosphere to prevent drying out. The end point (i.e. titre) was estimated as that dilution of 'antiserum' causing the formation of a pellet of red blood cells with a surrounding ring.

The haemagglutination test was used because rabbit saliva is rich in IgG (unlike human saliva) (TAUBMAN and GENCO, 1969) and should therefore be a sensitive test for any antibody produced, being more specific for IgG antibodies than IgA.

Immuno-fluorescence studies on rabbit sub-mandibular salivary gland-

The left sub-mandibular gland was removed from a rabbit with a salivary anti-human gamma globulin titre of 1:10 after local administration of antigen in the oral cavity. Frozen sections were cut at -20°C in the cryostat, fixed in neutral buffered formal saline for 15 minutes at 4°C and then washed in PBS, pH 7.1 for 10 minutes.

The presence of anti-human gamma globulin producing cells was tested using the method outlined in Chapter 6, plll, section I, using the gamma globulin in the concentration in which it was injected, on the section, as in stage 3; Hyland goat anti-human IgG (fluorescent) was added at stage 5.

The class of rabbit immunoglobulin that might produce antibody in the salivary gland was determined by the method outlined in Chapter 6, pl11, section I, using goat anti-rabbit IgG and IgA (Miles-Yeda Ltd.) at stage 3, and rabbit anti-goat gamma globulin (fluorescent) (Difco), at stage 5.

Negative controls were set up as before to eliminate the possibility of non-specific fluorescence.

RESULTS

A. ACTIVE IMMUNIZATION.

Examination of serum and saliva by immunodiffusion-

On analysis by immunodiffusion, it was observed that there were no serum or salivary titres to the Kabi gamma globulin, used as antigen, before injection, as no precipitin lines appeared in the agar.

Examination of serum and saliva by haemagglutination-

The serum and salivary titres obtained in the three active immunization schedules are shown in Table 11. Fairly high serum titres were obtained in agreement with BERGLUND et al. (1969) who induced serum antibody to the lipopoly-saccharide somatic antigen of E. coli on intra-mucosal injection; they did not however, report salivary titres. None of the injection schedules used was successful in producing high salivary antibody titres although low titres were achieved.

The best salivary antibody titre was obtained with an intra-muscular injection to prime the animal followed by local immunization (schedule 1); purely local immunization

Table 11. Serum and salivary antibody titres on measurement by haemagglutination after active immunization.

<u>Rabbit number</u>	<u>Serum titre</u>	<u>Salivary titre</u>	<u>Serum titre</u>	<u>Salivary titre</u>
SCHEDULE 1.		After 2nd injection	After 3rd injection	
1.	1/1280	-	> 1/5120	1/20
2.	1/5120	-	> 1/5120	1/20
3.	> 1/5120	-	> 1/5120	1/10
4.	1/1280	-	Rabbit died	
5.	1/20	-	1/160	-
6.	1/1280	-	> 1/5120	1/10
SCHEDULE 2.		After 3rd injection	After 4th injection	
1.	1/10	-	1/640	-
2.	1/40	-	1/640	-
3.	1/160	-	1/640	-
4.	-	-	-	-
5.	1/160	1/10	1/640	1/10
6.	1/640	-	1/640	-
SCHEDULE 3.		After 3rd injection	After 4th injection	
1.	1/20	-	1/40	-
2.	1/10	-	1/40	1/2
3.	1/160	-	1/320	1/4
4.	1/40	1/4	1/40	1/2
5.	1/160	-	1/160	-
6.	1/160	-	1/160	1/2

Key: > = greater than

did not result in the presence of any measurable titre of salivary antibody with the techniques presently employed (schedule 2). Schedule 3 was difficult to carry out as the parotid gland is in a very inaccessible site in the rabbit mouth.

Examination of serum and saliva by immunoelectrophoresis-

The serum and saliva (10x Lyophilised) of rabbits 1-6 on injection schedule 3 after the 3rd injection were examined immunoelectrophoretically for the presence of antibodies to the gamma globulin, used in the quantities in which it was injected. For example, rabbit 3 had a serum titre of 1:160 and rabbit 4 had one of 1:40; precipitin arcs appeared in the gamma globulin region, indicative of antibody production (fig.26a). On examination of the concentrated saliva, it was noted that although rabbit 4 had a 1:4 titre by haemagglutination, no precipitin arcs appeared in the gamma globulin region. None appeared, as expected with rabbit 3 as it had a zero haemagglutination titre (fig.26b). Therefore it was confirmed that haemagglutination was a more sensitive method than immunoelectrophoresis for the detection of antibody production in the serum and saliva of rabbits.

Immuno-fluorescence studies on rabbit sub-mandibular salivary gland after active immunization-

Immuno-fluorescent antibody pictures obtained on examination of the injected sub-mandibular salivary gland indicated that antibody to the injected gamma globulin was produced by the inner cells of the intra-lobular salivary ducts in which some brightly fluorescing cells were observed.

Cells producing both rabbit IgA and IgG were present in this site.

B. 'PASSIVE' IMMUNIZATION.

Examination of rabbit saliva by haemagglutination-

This test was used, before passive immunization was commenced, as the most sensitive test to determine if antibodies were already present to the substances to be injected parenterally. All tests proved negative using 10x Lyophilised saliva.

Examination of rabbit saliva by immunoelectrophoresis and immunodiffusion-

It appears that after injection of 1. Human serum, 2. anti-human IgG and 3. plasma albumin, intra-venously, and examination of the 20x Lyophilised saliva collected after injection, that some of the substances when administered parenterally do appear in the saliva, which is collected for 1 - 1½ hours after immunization. This confirms the report of SCHEIN and TUNG (1962) who detected parenterally injected albumin in pilocarpine-stimulated rabbit saliva, by immunodiffusion analysis.

Examination of the patterns obtained on immunodiffusion and immunoelectrophoresis of the saliva against the antiserum to the injected substances, led to the following observations.

- a) Some of the human serum protein appeared in the saliva, as a precipitin arc appeared between the rabbit saliva and the anti-human serum, although not all the human serum protein components did (fig.27).
- b) There was a faint precipitin arc between the saliva of

Fig.26a. Immunelectrophoretic examination of rabbit serum containing antibodies to gamma globulin after active immunization (x2).

Wells contain Kabi gamma globulin

1. Rabbit 3 serum; 2. Rabbit 4 serum.

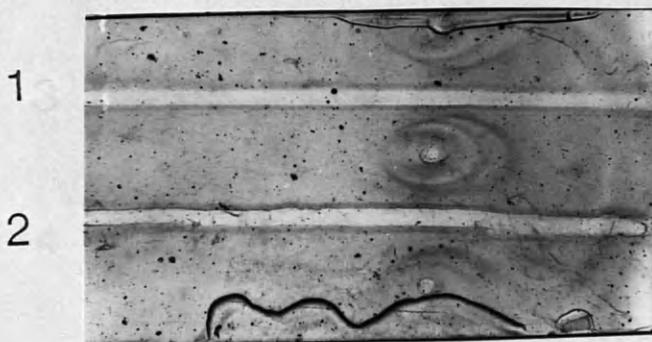
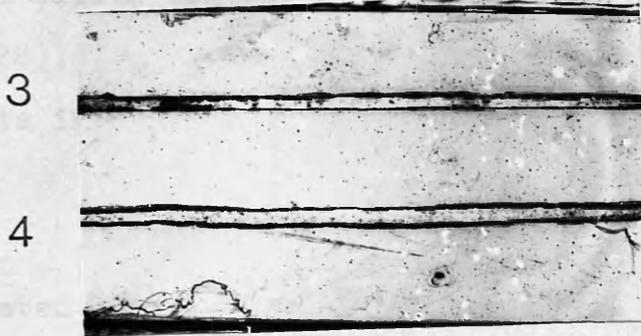


Fig.26b. Immunelectrophoretic examination of rabbit saliva with and without antibodies to gamma globulin after active immunization (x2).

Wells contain Kabi gamma globulin

3. Rabbit 3 saliva. 4. Rabbit 4 saliva.

In both cases, the anode is to the left and the cathode is to the right.



the rabbit injected parenterally with anti-IgG, and the IgG itself on examination of the rabbit saliva by immunodiffusion and immunoelectrophoresis. This result was not convincing.

- c) Human plasma albumin appeared in the saliva of the rabbits intra-venously injected; it was readily detected by immunoelectrophoresis and immunodiffusion of the rabbit saliva against anti-human plasma albumin and a line of identity was produced between the albumin in the rabbit saliva and the albumin injected into the rabbit (fig.28). No albumin was detected in the saliva of the rabbits injected intra-peritoneally.

DISCUSSION

As stated before, the ultimate aim of this work was to endeavour to produce a satisfactory form of injection schedule as a means of inducing immunity to, and thus preventing oral disease.

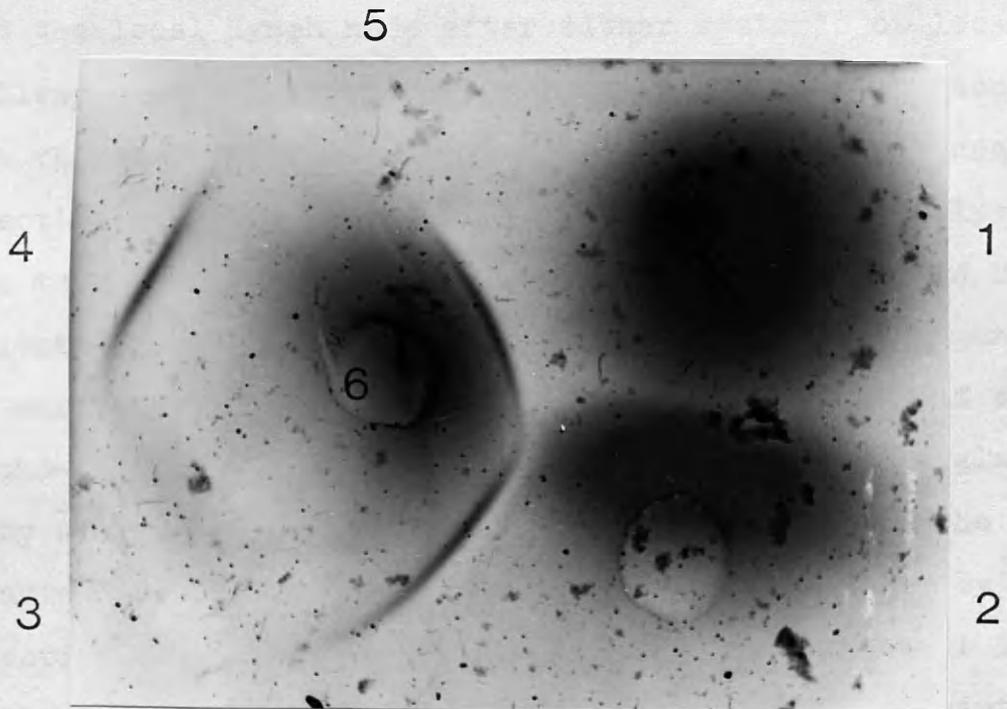
A. ACTIVE IMMUNIZATION.

As can be seen from the results obtained, none of the injection schedules employed, using gamma globulin as antigen, was highly successful in producing salivary antibody, as even titres of 1:10 and 1:20 do not seem sufficiently high to overcome a challenging agent, except as an ancillary mechanism to other protective immune processes. Quite high serum titres were obtained in some cases however. High titres were not recorded either by RANNEY and ZANDER (1970) on measurement of serum antibody after sensitization of squirrel monkeys.

Fig.28. Immunodiffusion of rabbit saliva, after parenteral (i-v) immunization with human plasma albumin, against anti-albumin (x4).

1. Human plasma albumin; 2. rabbit serum from immunized animal.
3. unconcentrated rabbit saliva
4. 20x Lyophilised saliva after i-v immunization.
5. 20x Lyophilised saliva before i-v immunization
6. Hyland goat anti-human plasma albumin

Key: i-v = intra-venous.



Haemagglutination was used to measure the antibody titres because, unlike human saliva, IgG is the predominant immunoglobulin in rabbit saliva (GENCO and TAUBMAN, 1969) whilst IgG is the predominant immunoglobulin in both human and rabbit serum. It was therefore felt that this should be a sensitive test. As mentioned in the results, neither immunoelectrophoresis nor immunodiffusion was considered to be assensitive a method for the detection of antibody, even in 'concentrated' saliva. This is not surprising as it is not a quantitative test and is not sensitive enough to detect minute quantities of an antigen.

The greatest salivary titre was obtained by injecting into the local lymph node after either systemic or local challenge by the antigen. This would seem to be in accordance with the results of GOUDIE et al. (1966) who recommended injection of very small amounts of antigen into the lymph node to produce a high antibody response. The second best salivary titre was obtained by injection of the antigen into the sub-mandibular gland (supported by the results of the immuno-fluorescent studies on rabbit sub-mandibular gland) or by administering it in sufficient quantities in the drinking water after primary systemic challenge, or by injecting the antigen into the local lymph node after local challenge. However, it may not be feasible to administer antigen in this way in the human situation because of the possible atrophy or allergic response of the glands or lymph node when directly injected.

EMMINGS and GENCO (1972) have reported that they obtained a response after injecting dinitrophenylated bovine

serum albumin directly into surgically exposed sub-mandibular glands of rabbits with and without adjuvant and also with and without intra-peritoneal pre-immunization. They detected the antibody formed as a result, by immunofluorescence techniques. Serum titres to this antigen were measured but salivary titres were not recorded. The fluorescence results obtained as mentioned on page 147 also showed that antibody was produced to the injected gamma globulin, indicating that a mechanism for antibody production may exist in the gland on direct stimulation after initial systemic challenge. The antibody that was produced was not predominately of a specific class, as compared to the results of the above-mentioned authors who found that injection of the antigen in saline resulted in a 30-90% IgA response; pre-immunization, or antigen injected in adjuvant, resulted in a predominance of IgG. All results show that route of injection determines the response. It is not yet known if the parotid gland reacts in the same way after direct injection.

CONCLUSIONS (A.)

It is concluded at this stage that although serum titres can be obtained to a simple antigen, it is difficult to produce a parallel salivary titre using the above methods of immunization and detection. There could be a number of reasons for this failure:

- 1) The wrong antigen might be being used, as CEBRA and ROBBINS (1966) discovered when they failed to elicit an antibody response to Ferritin in rabbit colostrum even though

^ their secretory

mechanism may be identical to that of the human salivary glands which are unable to produce high concentrations of salivary antibody.

5) Antibodies do not readily enter saliva because they may be too large to pass through the salivary gland, gingival fluid perhaps being the source of these substances in the mouth.'

serum titres were obtained, after local injections of the antigen into the mammary gland.

- 2) Non-optimal routes of injection might have been employed.
- 3) Non-optimal detection methods might be employed, even though the haemagglutination test measures 0.003 μ g of antibody Nitrogen.
- 4) The rabbit may not be able to produce large amounts of salivary antibody; λ

If it is the secretory IgA that is important in protecting the rabbit mucous membranes as it seems to be in humans, then even though it is produced in increased amounts on local immunization, it would possibly not be detected by haemagglutination as this is not a very specific test for this immunoglobulin, especially as it is not the predominant immunoglobulin in either rabbit saliva or serum.

It must be remembered that an antigen must be non-toxic and a disadvantage of active immunization is that if any active principle was found that contained streptococcal components, there would be a danger in producing sub-acute bacterial endocarditis. Plaque is so complex and although it has been shown to contain at least two toxins (LEVINE, 1972), it will be difficult to produce a non-toxic, active antigenic principle from it.

B. 'PASSIVE' IMMUNIZATION.

The 'passive' immunization schedules were more successful. On examination of the rabbit saliva after intra-venous injection it was discovered that some component of human serum, and definitely human plasma albumin (when injected

singly) appeared in the rabbit saliva, collected shortly after injection. Horse anti-human IgG was not so evident in the saliva after it was injected intra-venously and human plasma albumin, after intra-peritoneal injection did not appear at all in the saliva.

There therefore appears to be a diffusion from the serum into the saliva of certain substances, depending on their molecular weight. That of albumin is 69,000; other human serum components have molecular weights of under 100,000 (Spl. Table 2)* but the antibody molecules such as Horse anti-human IgG have a molecular weight of at least 160,000. Therefore the smaller molecular weight substances can appear in the saliva, and also fragments of larger molecules which may still be detected immunochemically in this case, if the antigenic part of the antibody molecule i.e. the F_c piece (Spl fig. 1)* diffuses through. This would explain the presence of albumin, some of the serum components and the variable presence of anti-IgG in the saliva. In addition, in disease and the resulting tissue breakdown, more serum components will pass into saliva, than when the glands and tissue are intact.

The immunodiffusion results of the passively injected human plasma albumin proved interesting in connection with the immunological investigation of human salivary albumin (Chapter 4).

In these experiments, salivary albumin against anti-human plasma albumin (fig. 14) gave an arc whereas plasma albumin against anti-human plasma albumin gave a number of arcs. A similar result was obtained on immunodiffusion of

*Spl = Supplement

the albumin which was injected intra-venously in the rabbit, which appeared in the rabbit saliva. This albumin gave one band with anti-plasma albumin and a line of identity with human plasma albumin, indicating that it was the injected albumin that appeared in the saliva. However, a number of other bands (faintly-staining because of the low concentration of albumin in the well) also appeared in this diffusion of control albumin against anti-human plasma albumin (fig.28) giving the same picture as in fig.14, and emphasising the greater heterogeneity of plasma albumin.

CONCLUSIONS (B).

It may be concluded from these results that although there is salivary albumin in saliva, giving an arc in the albumin region on immunoelectrophoresis of mixed saliva against anti-mixed saliva (fig.13c) there is also a diffusion of one antigenic component, or a modified antigenic component, of plasma albumin into human saliva. This is probably more evident in the saliva of patients with periodontal disease. As the anti-mixed saliva used in Chapter 4 was prepared from saliva from volunteers with clinically healthy mouths, little plasma albumin would be present in it and so result in the pictures obtained there.

Finally, it appears that passive immunization might be successful if the antibody to the toxic substances in plaque that cause the most harm could be digested with pepsin, to allow the F_{ab} fragment (the antigen-binding part of the molecule) to diffuse into saliva after intra-venous injection. The main disadvantage is that continual passive immunization

can result in serum sickness (see Supplement, p. 15).

Final analyses-

From the results of this study, it would appear that continued experimental work in both active and passive immunization would be valuable, especially if the 'active' toxin(s) of plaque (once characterized) could be utilized, eg. in the form of a toxoid. The toxoid could be used for the production of an antiserum or as the antigen in active immunization.

Active immunization studies in animals on the route of injection to stimulate maximal local antibody production is important. As only smaller molecules appear to infiltrate into saliva from serum, this is more important than producing high systemic antibody titres.

Passive immunization studies with the 'Active' principle of an antiserum for short-term immediate protection must also be pursued. Therefore studies on the production of an antiserum to toxic plaque antigens, with subsequent fractionation of the antiserum to produce the small molecular weight, antibody-binding fraction (F_{ab} part: see Spl. fig. 1) for injection, are important.

CHAPTER 8.GENERAL DISCUSSION.

Ever since the days of Louis Pasteur and rabies vaccination, medical research has been concerned with experimentation on immunization as part of the whole research in the science of immunology. The immune system is concerned with defence against foreign substances, with discriminating between 'self' and 'non-self', with inflammation and with repair. Some of these reactions, although all have the ultimate aim of benefiting the person concerned, may result in processes detrimental to the host due to their intrinsic properties. They may also malfunction and thus induce autoimmune disease.

Immunization studies are employed to investigate antibody responses and their role in overcoming disease. However, in an area as complex as the oral cavity, it cannot be assumed that an antibody response is the sole existing immune mechanism. However, it was felt that a study of immunization in connection with oral disease, to investigate if local antibodies could be induced and so enhance one of the existing oral immune mechanisms, would be of value.

As stated in the introduction, saliva is a complex substance with varied and fascinating properties; the complexity of these and the problems arising from their investigation is amply illustrated in Chapters 3 to 7.

Saliva contains non-specific immune mechanisms. Its viscosity protects the mucous membranes but this very property can give rise to experimental difficulty in that it provides a base for the deposition of oral debris and growth of

bacteria. It also contains enzymes such as lysosyme, which help to combat infection. Bacteria are always present in the mouth and when there is a rise in salivary pH, due to their action or by loss of CO_2 , there is precipitation of salivary constituents resulting in their deposition on the teeth. Bacteria adhere to this deposit, and if it is not removed, their numbers increase and bacterial plaque is formed (see Chapter 2). Also LEACH and MELVILLE (1970) have described the mechanism whereby the terminal sialic acid is removed by enzyme cleavage from the carbohydrate side chains of the glycoprotein; accordingly a bacterial deposit is formed.

Bacterial plaque accumulates in the absence of effective oral hygiene measures with the potential to cause periodontal disease and dental caries. Secretory IgA antibodies to Strep. mutans prevent, in the absence of complement, the colonising of the teeth (TAUBMAN and GENCO, 1971). However, irritation of the gingiva allows exudation of complement-containing crevicular fluid, and colonisation of the teeth can be initiated. The subsequent build-up of organisms and debris causes continual irritation and stimulation of the immune system, in order to cope with these 'non-self' substances. This may result in paralysis of the immune system. Continual low-level stimulation results in low levels of antibody which combines with the antigen thus removing the stimulation of the competent cells. New antigens can then leak in from plaque and the process is repeated to induce tolerance.

Alternatively, large amounts of antigen overload the

system, which cannot cope with the continual onslaught of concentrated antigen, and this results in immune-paralysis. From work outlined by COHEN (1970), on low zone and high zone paralysis of immune reactions, it appears that there are low affinity and high affinity competent antibody-producing cells, the former having higher site numbers for the attachment of antigen than the latter. Cells with high affinity have more chance of capturing antigen and being stimulated to proliferate and produce antibody. With high doses of antigen, such as from large amounts of plaque, even low affinity cells combine and give antibodies of low average affinity. COHEN (1970) believes that the antigen is processed to make it immunogenic, all competent cells having receptors enabling this immune reaction to take place.

Interaction of a single molecule of antigen with 1 receptor leads to paralysis; interaction with more than one antigen molecule induces antibody production. Higher doses of antigen saturate the cells with low site numbers first and then leave them in their tolerant state. As antigen concentration increases, there is a fall below the activation threshold of low affinity cells first (higher site numbers) and then the activity of the high affinity cells is picked up and so the average affinity of the antibodies is increased i.e. when the animal or human escapes from high zone tolerance or is partially tolerant, the antibody will be of low affinity. This could explain the lack of significant change in the salivary and serum IgA levels in the different periodontal disease states, especially in chronic conditions where the immune system is continually overloaded with

^ Erratum: ppl63 and 176.

TAUB et al (1970,b) should read

DRESSER, D.W., TAUB, R.N. and KRANTZ, A.R.

Immunol. 18 (1970,b) 663-670.

antigens.

Immune paralysis seems a likely explanation for the continued presence of periodontal disease as the antigen(s) are always present. The paralysis may explain why there is no response that overcomes the plaque antigen(s) as there is in eg. a streptococcal infection of the throat.

This theory may also account for the low levels of antibody produced in the immunization schedules, as the dose of antigen required to give maximum response is crucial.

TAUB et al (1970 a and b) experimented with the injection of substances directly into the lymph node (in mice). Injection of substances with extrinsic adjuvanticity caused paracortical expansion and hyperplasia of the nodes. This was partly due to augmented cellular traffic with a net flux of recirculating lymphocytes into these areas. Substances with intrinsic adjuvanticity led to a marked influx of lymphocytes. In both cases, therefore, there was an increase in the number of cells in contact with antigen. This may be the reason for the higher degree of success in producing local antibody on injection into the lymphnode and suggests that further studies on immunization in this area would be justified. Injection of substances with extrinsic adjuvanticity would be less likely to cause irritation of the node. It is apparent therefore, that the route of injection, as well as antigen concentration and composition, is of the utmost importance.

Induction of high serum titres by active immunization might not necessarily be of great value, if the antibody molecules are too large to diffuse into the mouth, which

might be the case as concluded from the results of Chapter 4 and Chapter 7. As stated in the latter, passive immunization with the active binding part of the antibody molecule would be of more value, for immediate treatment of an acute condition, as in eg. tetanus or german measles infection, although this could be harmful. Serum-sickness can be induced if passive immunization is used for long-term therapy.

Apart from the variation in serum and salivary antibodies, another notable feature of periodontal disease is the change in the pathology of the periodontium. The initial rise in polymorphonuclear leukocytes, PMNs, illustrated in rabbits in Chapter 6, may indicate an Arthus-type reaction, because of the repeated exposure of the gingivae to bacterial antigens and plaque deposits in concentrations within those levels that may induce immune-tolerance. Arthus activity is decreased on depletion of PMNs in rats (WARD and COCHRANE, 1965) even though complement and immune reactants are present. Rats and guinea pigs vary in their response with complement present, depending on their complement-fixing capacities 'in vitro' but their results outline the importance of continued PMN presence for the Arthus-type reaction.

An alternative mechanism may be due to the fact that antigen in sensitized tissues rapidly combines with any antibody produced to it. These complexes attract PMNs and lead to phagocytosis and therefore destruction of antigenic properties (BOYDEN, 1962). However, tissue destroyed in this process would allow further inward leakage of plaque antigens. With the resulting rapid introduction of the antigens in

large numbers, insufficient antibody could be produced in sufficient time to complex all the antigen and some of the latter would remain free, to promote further immune reactions.

An increase in the number of plasma cells after PMN infiltration may result in a further inflammatory response of an anaphylactic type. The breakdown of the tissue after initial sensitization could result in the second shocking dose of plaque antigen(s) being administered. Complement is present in the crevicular fluid and serum and a local anaphylaxis could occur. Salivary IgA on infiltration into the destroyed tissues may aid the anaphylactic reactions. GREEN et al (1970) outlined an interaction between monovalent antigen and antibody at the mast cell surface, which in the absence of complement can provide an anaphylactic reaction. Also, if antibody is adsorbed first to the cell surface, complement is not necessary. IgA does not fix complement and could therefore aid the response in this additional type of anaphylactic process. The release of pharmacologically active substances would further destroy the periodontal tissues.

LEHNER, in his 1972 'Review of Cell-mediated immune responses in connection with oral disease', provides substantial evidence for the involvement of these mechanisms, to varying extents, in connection with the different types of disease. The local tissue is capable of eliciting a cellular response to the bacterial antigens present in the plaque, with a subsequent breakdown of tissue.

From the results obtained in this work reported in this thesis, only the surface of the problem has been investigated,

but the results obtained can be seen to illustrate several of the reactions outlined above. It appears that no single mechanism of all those present in the oral cavity, is involved. The diversity of the responses the local environment is capable of making is another factor that causes the tissue destruction and inflammation that is representative of periodontal disease in its different manifestations. Salivary antibodies have a role in these processes but their action is perhaps more confined to the dental caries process i.e. salivary IgA is removed from the saliva by its adherence to oral bacteria (BRANDTZAEG et al. 1968) i.e. as plaque accumulates, the antibodies are less able to cope. It also seems to be generally accepted that people less prone to caries have more IgA in their saliva than those who are caries-susceptible (see Chapter 2). However, they may play an important role in the pathogenesis of periodontal disease, as is mentioned above.

Thus, from the theories already outlined, the most fitting conclusion appears to be that proposed by RANNEY and ZANDER (1970): "Thus it may be hypothesised that periodontal disease may in some instances be a by-product of a successful endeavour by the immune system to protect the body as a whole from foreign materials, with the periodontium undergoing progressive chronic destruction with eventual loss of support from the teeth as a result of the local injurious nature of the immune reactions". Control of these immune mechanisms must be sought to lessen their destructive properties and to aid recovery from oral diseases.

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BASIC CONCEPTS IN IMMUNOLOGY

SUPPLEMENT

to

A STUDY OF IMMUNE MECHANISMS IN THE
MOUTH IN RELATION TO ORAL DISEASE

by

FRANCES JANE McKEAN, B.Sc. (Hons)

DEPARTMENT OF ORAL MEDICINE AND PATHOLOGY
UNIVERSITY OF GLASGOW DENTAL SCHOOL

THESIS PRESENTED TO THE UNIVERSITY OF GLASGOW
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BASIC CONCEPTS IN IMMUNOLOGY.

It was felt that a resume of some basic immunological concepts, and the immunological mechanisms pertinent to the subject would be of use for reference when dealing with the interpretation and discussion of the results and with the general conclusions.

IMMUNITY

Vertebrates, excluding the most primitive, when they come in contact with a 'foreign' agent or material, can to a varying extent, exhibit an immune reaction which can be 'non-specific' or 'specific'. Non-specific immune mechanisms include innate, which is rationally or genetically determined; racial differences in susceptibility to disease; age, as the old and the very young are more susceptible to disease; and hormones, which if inefficient in their job render one more susceptible. Skin and mucosae act as both mechanical and biochemical barriers to infection. If infective agents enter the body, anti-bacterial substances in the blood and lymph attack them and polymorphs attempt phagocytosis. The specific immune reaction may be mediated through the production of soluble proteins (immunoglobulin antibodies) or by cells (lymphocytes). It may be aided by 'Cytophilic' antibody which attaches to macrophages and is analagous to the 'cytotropic' antibody that binds to the surface of mast cells and mediates anaphylactic responses.

PROPERTIES OF ANTIBODIES

Antibody is produced in response to the presence of an

antigen (or immunogen) which may be a substance of molecular weight greater than 20,000; alternatively it may have a smaller molecular weight and act as a hapten by being covalently linked to a heavier protein. An antibody molecule is a glycoprotein, which does not bind to the whole of the foreign substance but with the antigenic determinant. These determinants may all be different as with proteins, or may be common to certain substances, as in bacteria of the same type.

The basic property of antibody molecules is the ability to bind individual molecules or particles of the antigen, into large aggregates, which are then more rapidly phagocytosed, and destroyed, 'in vivo'. Individual classes of antibodies are adapted to perform extra functions apart from binding to immunogens. eg. IgG, in man, has a structural feature enabling it to cross the placenta and confer protection on the newborn; also, the IgA in man produced in sero-mucous secretions, has an additional 'Secretory' or 'Transport' piece enabling it to be transferred to the surface of the mucous membrane where it is probably important in counter-acting local infection. These different properties can be utilised in identifying the class of immunoglobulin the body is producing eg. complement fixation identifies IgG. Immunoglobulins may be characterised also, by their ability to move through a supporting medium with or without an electric current; a property useful for their identification. Some of their biological activities determined to date, are listed in Table 1.

These different antibody activities were all named according to function eg. one having the ability to effect the

lysis of bacteria was called a bacteriolysin. It is now known that antibodies with the same basic structure but varying specificity, induce these different effects.

ANTIBODY CONCENTRATION

Antibody concentration in serum is constant with time i.e. is in a steady state. Antibodies of a given specificity behave like the total mass of non-specific immunoglobulins and break down exponentially. 'In vivo', IgM and IgA antibodies are broken down more quickly than IgG and the degradation rate is characteristic for a given class of immunoglobulin in a particular species.

Antibodies and their functions have been extensively studied in serum and their levels measured in a number of diseases (HOBBS, 1970). See also Table 2.

ANTIBODY STRUCTURE

The antibodies identified to date have been divided into five classes, namely IgG, IgM, IgA, IgD and IgE. All classes have unique heavy chains but all have either κ or λ light chains. The basic molecule has a molecular weight of 150,000, the two heavy chains being 50,000 each and the two light chains, 25,000 each. The biological properties other than antigen-binding, vary according to class and are controlled by structures in the F_c part of the molecule (fig.1). IgG exists as a monomer, IgM as a pentamer and IgA as a dimer or trimer (in most cases).

PROPHYLAXIS

The main function of an antibody is to protect a host

Table 1. PROPERTIES OF HUMAN IMMUNOGLOBULINS.

(after JEFFERIS, 1972).

PROPERTIES	IGG	IGA	IGM	IGD	IGS
Biological					
Serum conc. (mg.%) (1) ^a	800-1600	140-420	50-190	0.3-40(2)	0.1 -0.14
Synthesis rate (mg./kg./d.)	20-40 (3)	2.7-55 (4)	3.2-16.9(5,6)	0.03-1.49(6)	
Catabolic rate (% I.V. pool/d)	4-7 (3)	14-34(4)	14.25(5,6)	18-60(6)	
Distribution (% in I.V. pool)	48-62(3)	40(4)	65-100(5,6)	63-86(6)	
Antibody activity	+	+	+	+	+
Complement fixation	+	0	+	0	0
Placental passage (7)	+(14)	0	0	0(2)	
Presence in cerebrospinal fluid(8)	+	+	0		
Selective seromucous secretion ^b	0	+	0		
Skin sensitization					
Heterologous species	+(9)	0(10)	0(9)	0	+
Homologous species	0	?	0		+
Immunological					
Light-chain types	κ, λ	κ, λ	κ, λ	κ, λ(2) ^c	κ, λ
Heavy-chain classes	γ	α	μ	δ	ε
types ^b	4	2	2		
Allotypes, Gm	+	0	0	0(11)	
Inv	+	+	+		
Physicochemical					
S _{20,W}	6.5-7.0	7,10,13,15,17	18-20,30	6.2-6.8(11)	8.2
Ammonium sulphate precipitation (molar conc)	1-49-1.64(13)		1.64-2.05(13)		
Total carbohydrate (%) (12)	2.9	7.5	11.8		10.7
Hexose (%)	1.10	3.2	5.4		
Acetylhexosamine (%)	1.30	2.5	4.4		
Sialic acid (%)	0.30	1.8	1.3		
Mucos (%)	0.20	16.2	0.7		

against infection (fig.2). On first exposure, primary response, there are no cells capable of making antibody and the body takes time to start manufacture. Antibodies can be detected 3-4 days after injection of erythrocytes, 5-7 days after soluble proteins and 10-14 days after bacterial cells. The role of lymphocytes and the derivation of effector cells in this response is illustrated in fig.3.

There have been a number of selection theories as to the production of antibody. The first theory was proposed by PAULING* (1940) and his predecessors who believed that non-specific polypeptide chains were moulded after synthesis to assume a specific immune pattern by physical contact with the antigen. In 1955, the Natural Selection Theory was put forward by JERNE*; 'natural antibody' already present in the host was thought to unite with antigen and this complex then ingested by a phagocytic cell and more antibody then produced to the pattern of that ingested. LEDERBERG* (1959) proposed the Subcellular Selection Theory by which each cell possesses the preexistent capacity to produce large numbers of antibody patterns. The entry of antigen would cause the selection of the appropriate pattern for the expression of antibody. The most favoured theory to date is that of BURNETT (1959) which is termed the Clonal Selection Theory in which, stem cells by a process of randomized somatic genetic change develop different immune patterns, of which one only is expressed for each cell and clone. Antibody formation or any other specific response is provoked by contact with the corresponding antigen.

It is known that many cells rapidly divide in the lymph

*Reference: BURNETT

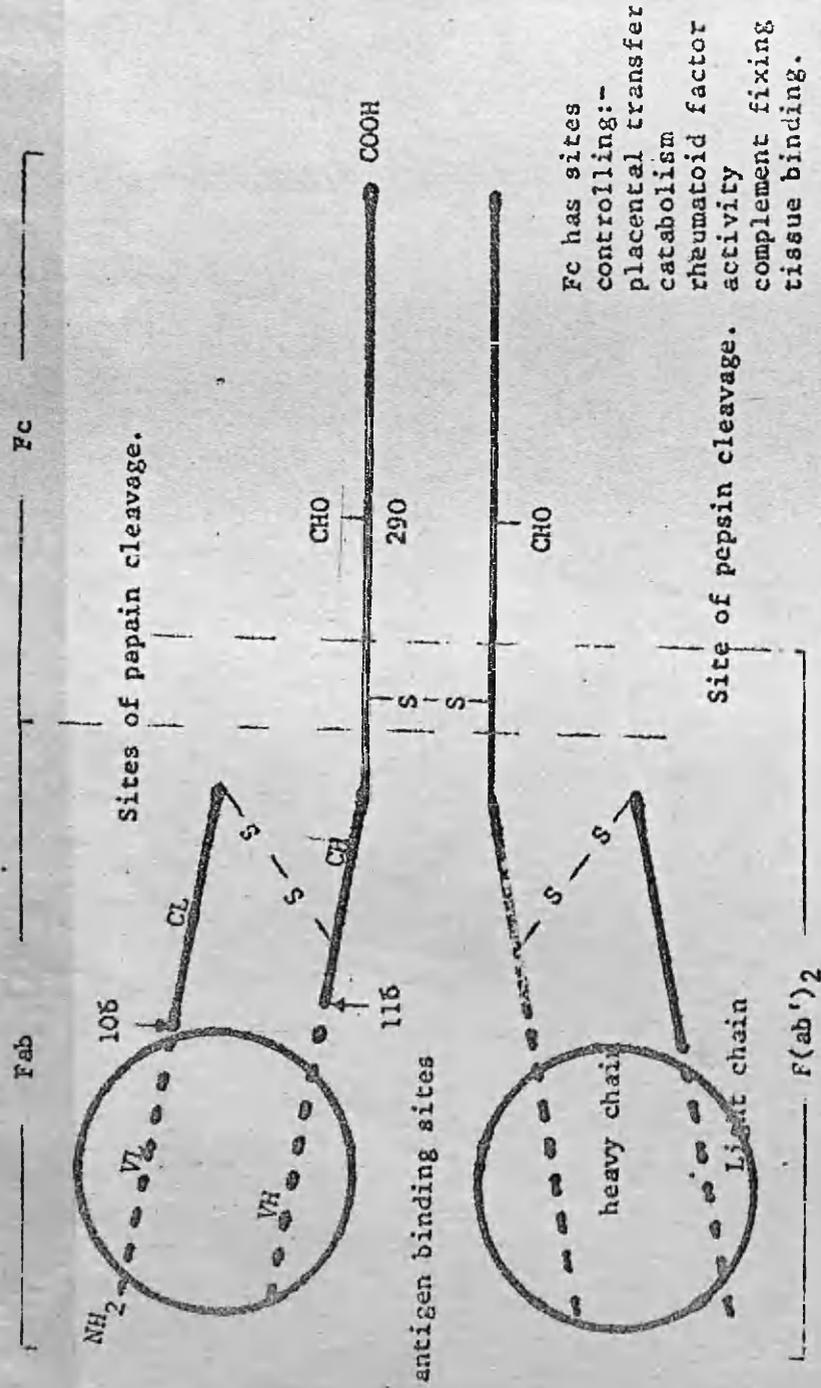
Table 2. PROPERTIES OF SERUM COMPONENTS INCLUDING ANTIBODIES.

(Behringwerke AG. 'Table of Proteins of Human Blood Plasma'.)

Proteins (synonyms)	Molecular weight	Peptide content %	mg/100 ml serum (plasma)		Biological function (Inherited variants)	Abnormalities (Hereditary deficiency)
			mean value	range		
Prealbumin	61 000	99	25	10-40	Thyroxin binding	Reduced in severe liver diseases
Albumin	69 000	100	4 400	3 500-5 500	Osmotic function; protein reserve, transport of ions, pigments, etc. (Bisalbuminaemia)	Reduced in cirrhosis, nephrosis, etc. (Analbuminaemia)
α_1 -acid glycoprotein (orosomucoid, α_1 -seromucoid)	44 100	62	90	55-140	(Electrophoretic polymorphism)	Increased in chronic inflammatory conditions, rheumatoid arthritis, malignant neoplasms
α_1 -lipoprotein (high-density lipoprotein)	200 000	45	360	290-770	Transport of lipoids, hormones, etc.	Reduced in liver diseases (Tangier disease)
α_1 -antitrypsin (α_1 -3,5-glycoprotein)	45 000	86	290	200-400	Proteinase inhibitor (trypsin, chymotrypsin) (Electrophoretic polymorphism)	Increased in inflammatory conditions (Hypo- α_1 -antitrypsinaemia in emphysema)
Gc-globulin (group specific component)	50 800	96	40	30-55	(Electrophoretic polymorphism)	Reduced in severe liver disease
Ceruloplasmin	160 000	89	30	20-45	Copper binding, oxidase	(Reduced in Wilson's disease)
α_2 HS-glycoprotein (Ba- α_2 -glycoprotein)	49 000	87	60	40-85		
Haptoglobin	(100 000)	81	160	50-220	Haemoglobin binding; peroxidase (Polymorphism: Hp 1-1, 2-1, 2-2)	Reduced in liver diseases and haemolytic anaemias; increased in inflammatory conditions
α_2 -macroglobulin	820 000	92	♂ 240 ♀ 290	150-350 175-420	Proteinase inhibitor (plasmin), hormone binding (Xm-allotopy)	Increased in liver disease, nephrosis, diabetes
Plasminogen (profibrinolysin)	143 000	~91	30	20-40	Proenzyme of plasmin (fibrinolysin), proactivator	Reduced during fibrinolytic treatment
β -lipoprotein (low-density lipoprotein)	3 200 000	~19	530	290-950	Transport of lipoids, cholesterol, hormones, etc.) (Polymorphism: Ag, Lp, Ld)	Increased in nephrosis (Abetalipoproteinaemia)
Haemopexin (β_1 B-globulin)	80 000	77	100	70-130	Haemin binding	Reduced in haemolytic anaemias
β_1 C-globulin (C3-component)	S _{20,w} ^v = 9.5 S	97	110	80-140	Complement factor (in serum converted into β_1 A- + α_2 D-globulin)	Reduced in auto-immune diseases (glomerulonephritis, lupus erythematosus, etc.)
Transferrin (siderophilin)	90 000	95	295	200-400	Iron binding and transport (Electrophoretic polymorphism)	Reduced in nephrosis and malignant neoplasms
Fibrinogen (coagulation factor I)	341 000	97	(300)	(200-450)	Coagulable protein	Reduced in parenchymal liver damage, hyperfibrinolysis (Afibrinogenaemia)
C-reactive protein				< 1		Demonstrable only in acute inflammatory conditions
β_2 -glycoprotein I	40 000	81	20	15-30		(Familial deficiency of β_2 -glycoprotein I)
γ G-globulin (IgG; γ_2 , 7S- γ -globulin)	160 000	97	1250	800-1 800	Antibodies (Structural polymorphism: Gm, InV)	Increased in liver disease, chronic infections, myeloma. Reduced in antibody deficiency syndrome.
γ A-globulin (IgA; γ_1 A-, β_2 A-globulin)	160 000 and polymers	92	210	90-450	Antibodies (especially in secretions) (Structural polymorphism: InV)	Increased in cirrhosis, chronic infections, myeloma. Reduced in antibody deficiency syndrome, ataxia telangiectasia.
γ M-globulin (IgM; β_2 M-, 19S- γ -globulin)	1 000 000 and polymers	88	♂ 125 ♀ 160	60-250 70-280	Antibodies (isoagglutinins, etc.) (Structural polymorphism: InV)	Increased in chronic infections (trypanosomiasis, etc.), Waldenström's macroglobulinaemia, liver diseases. Reduced in antibody deficiency syndrome, myeloma.
γ D-globulin	150 000		3	0.3-40	Antibodies?	

Fig.1. Basic Immunoglobulin Structure.

(after STANWORTH, 1972).



Enzyme digestion:-

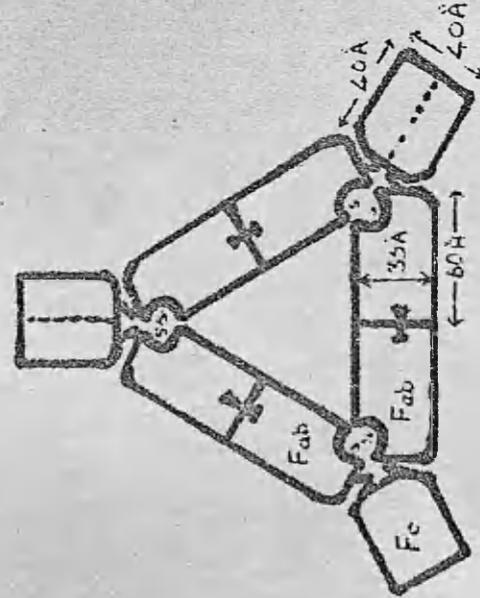
- Papain IgG 2 Fab + Fc
- Pepsin IgG F(ab')₂ + small peptides
- F(ab')₂ = Fab + small portion of Fc.

VL = region of variable amino acid sequence in the light chain.

CL = region of constant amino acid sequence in the light chain.

VH & CH refer to similar regions in the heavy chain.

CHO carbohydrate.



nodes near the injection or initial site of infection. Large basophilic cells, full of RNA and therefore producing vast amounts of protein divide to give intensely basophilic progeny - plasma cells; the antibody producers. Generally the first immunoglobulin produced is IgM and IgG is detected shortly afterwards. Injection of an adjuvant eg. Freund's complete adjuvant, which is a water in oil emulsion of extracts of the cell walls of tubercle bacilli, along with the antigen gives a better and more prolonged (though possibly less specific) response. The antigen molecules are slowly released from the emulsified antigen/adjuvant mixture, resulting in continual exposure of antigen molecules and more prolonged antibody production.

Exposure to the antigen for a second time induces a very rapid response (secondary response) which is characterized by, (a) lowering of the threshold dose, (b) decrease in the lag phase, (c) greater rate of synthesis of antibody, (d) prolonged persistence of the antibody produced and (e) greater affinity of the antibody for the immunogen compared to that synthesised at a similar time after the initial stimulus.

The amount of antibody produced is also dependent on the host's innate ability in this system. Several syndromes i.e. the combination of defects that influence humoral and/or cell-mediated antibody production, may be exhibited where there are defects in the mechanism of production of gamma globulins. These people have little or no resistance to infection and are subject to continual bacterial infection unless gamma globulin is injected regularly in humoral

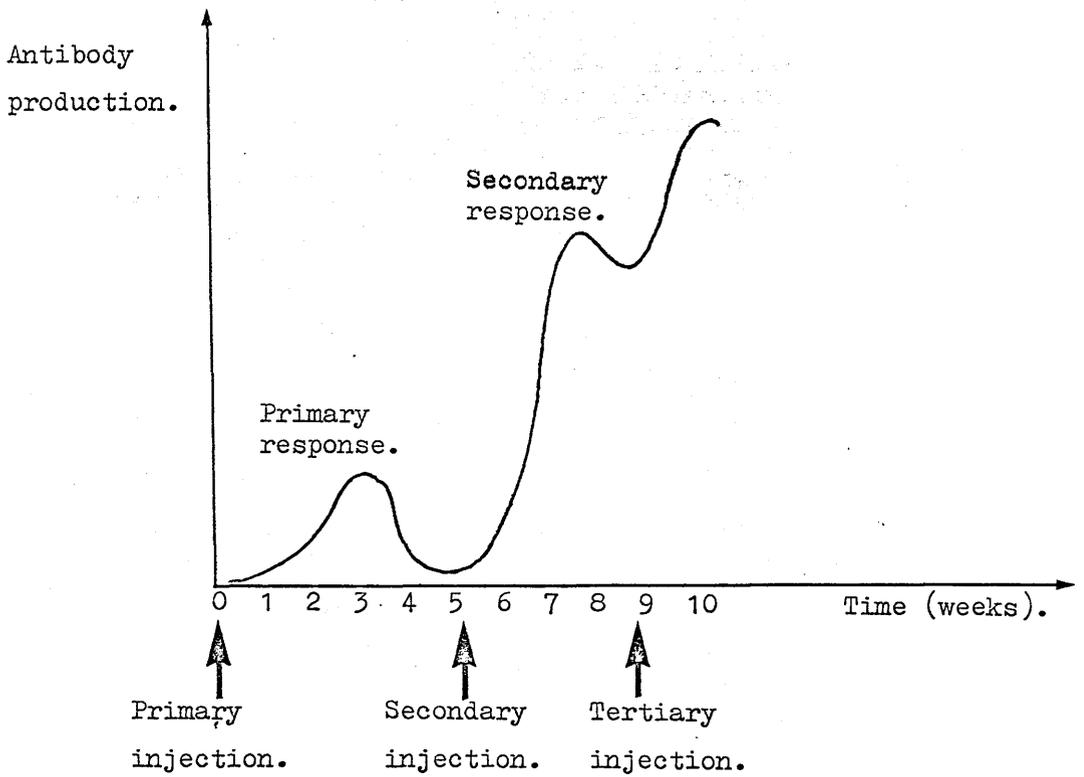


Fig.2. Serum antibody response after primary, secondary and tertiary infection or injection.

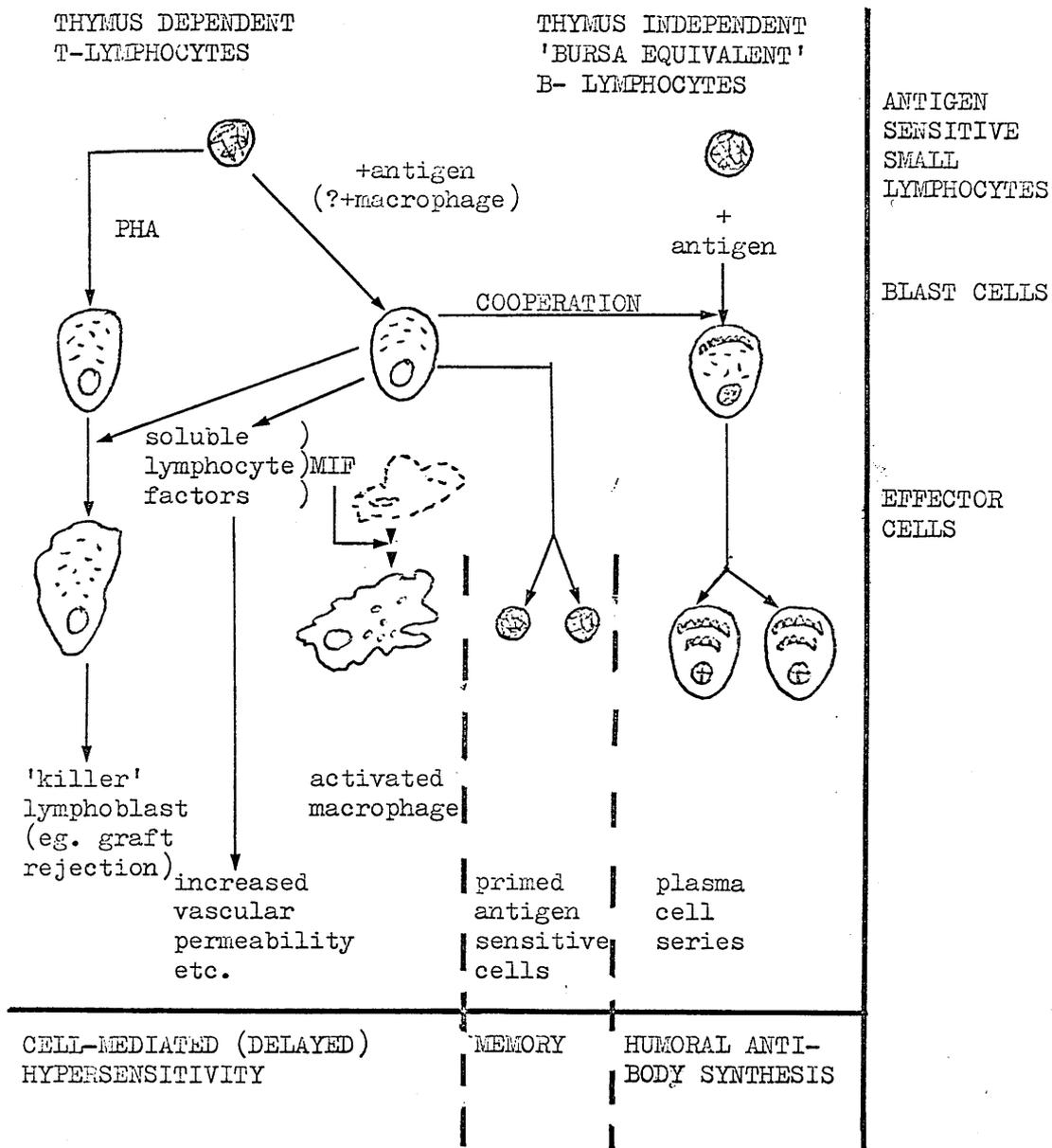


Fig.3. Role of T-lymphocytes and B-lymphocytes in immunological responses, from ROITT et al (1969).

Many stages involve active cell proliferation but this is only indicated at two stages, for simplicity.

deficiencies. When cell-mediated responses are also affected, treatment is much more difficult and infection increases in severity.

Basically, therefore, antibody production occurs after (a) natural exposure to a foreign agent or antigen including ingestion and inhalation and (b) deliberate injection of the antigenic substance i.e. immunization.

IMMUNIZATION

It has been proved beyond doubt, that prophylactic immunization is of benefit both to the individual and the community as a whole. It can give some manifestations of the disease itself but confers partial or total immunity.

Immunity may be classified as follows:

- 1) Natural acquired immunity - through infection or transmission by mother to foetus.
- 2) Artificial acquired immunity - by injection. Artificial can be further subdivided into ACTIVE and PASSIVE.

Active is long-lived as the body itself, manufactures antibodies after injection. Passive is short-lived as the antibodies (prepared in another host) are themselves injected to neutralise the infection.

After prophylactic immunization, the number of people in a community susceptible to a disease falls as there are only a few left in whom the organism can proliferate, therefore it can no longer act as an aggressor. As a result, if prophylaxis is continued in ensuing years, the disease may die out due to the HERD effect, even though there is not 100% immunization.

If the critical number of 3:1 are immunized, the disease can be overcome.

ACTIVE IMMUNIZATION

Some bacteria are better immunogens than others.

Bacterial antigens can be subdivided as follows:

- a) organism dependent where the killed or live, attenuated organism is injected. The antigenic determinant is the same as that of the virulent organism eg. enteric organisms and pertussis. The disease process is more subtle, therefore less certain and less long-lasting immunity is obtained. Dead vaccines need reinjection at smaller intervals than live.
- b) toxin dependent in which the toxin produced by the organism is treated to form a toxoid without change in antigenicity. There is a high success rate as the antigen is a soluble, diffusible protein, of which larger doses can be administered, eg. tetanus, diphtheria and scarlet fever.

In giving multiple injections, it must be taken into account that one antigen may affect the other, therefore a balance between them must be obtained to induce maximum immunity against the different antigens.

FACTORS AFFECTING RESPONSE IN THE RECIPIENT

There are also a number of factors in the recipient that affect the response to the immunogen, eg.

- a) the child may be immunologically immature, agammaglobulinaemic and/or thymus deficient. Immunologically deficient people may get massive infections with the organisms used as antigen that give very mild infections to immunologically mature recipients.
- b) the recipient may have already been exposed to the antigen or had a mild infection. A child may have a low titre from his mother. The subsequent secondary response, on immunization, will be good.
- c) if there already is a high titre of circulating antibody, the antigen is mopped up before it reaches the antibody-manufacturing cells and therefore continual production of antibody is not obtained.
- d) continual exposure to low doses of the antigen from an early age induces a state of tolerance to that antigen.
- e) the general state of health of the recipient is important.

PASSIVE IMMUNIZATION

In this case, the antibody to the disease agent is administered directly. An alcohol or ether fractionation of immune serum is carried out and is injected i-m in eg. german measles, small pox and tetanus, to give immediate protection as the antibodies to the antigen would be produced with too long a delay to be effective as a cure. Another example is that of Rhesus negative mothers who are injected with a small dose of antibody to the Rhesus antigen at the time of birth to prevent subsequent manufacture of large

doses of antibody to the Rhesus factor.

Thus, the recipient's immunological mechanism is not actively engaged in manufacturing protective antibody and the effect is short-lived but very necessary when administered.

CELL-MEDIATED IMMUNITY (delayed hypersensitivity)

Immunological reactions are not always advantageous or protective. In specific cell-mediated responses, where plasma cells are little concerned, lymphocyte infiltration with the ensuing reaction may be dangerous (and unpleasant) to the host.

Sensitization of the T lymphocytes takes place as illustrated in fig.3. On returning via the blood stream to the site of stimulation they are responsible for such reactions as contact sensitivity, the homograft rejection reaction, tumour immunity and runt disease.

ADDITIONAL DETRIMENTAL IMMUNE REACTIONS

These other reactions take place as a result of antigen/antibody interaction.

Anaphylaxis is the antithesis of prophylaxis in that the antibodies produced are not protective. If a shocking dose of antigen is administered after a time-lapse has occurred since the sensitizing dose, a severe reaction occurs. The sensitizing and shocking antigens need only have cross-reactivity; the initial dose is administered systemically whilst the subsequent dose is given intra-venously or intra-peritoneally. The F_c piece causes attachment of the

circulating antibody peripherally and subsequent antigen attaches to the antibody, and the mast cell is lysed in the presence of complement. Pharmacological agents such as heparin, serotonin (SRS-A) and histamine are released from the mast cells.

Serum shock is an analogue of anaphylaxis although different pharmacological agents (or their proportions) are employed. This can occur, eg. with penicillin, where the patient goes into coma, has respiratory difficulty and dies in a few seconds. The penicillin or its derivatives combine with body protein to give macromolecular antigens.

Serum sickness develops when large amounts of foreign serum are injected, eg. Horse anti-toxin for diphtheria. The injection of the foreign serum protein (often i-v) causes, in a few days, a rash, temperature increase, enlargement of the lymphnodes where injected, damage to the kidneys resulting in the excretion of protein and albumin and some swelling of the joints to give local arthritis, i.e. it leads to the formation of toxic antigen/antibody complexes which cause a severe and generalized inflammatory response.

The Arthus phenomenon occurs when antigen is repeatedly injected at a local site, corresponding to local serum sickness, giving a severe inflammatory response. The antibody differs from that of anaphylaxis as it is the heavy chain of the antibody that precipitates with the antigen and it therefore does NOT depend on the F_c piece in this case.

Hay fever and similar allergic reactions occur with elevated IgE levels. A severe allergic reaction occurs after secondary contact with the immunogen or allergen in persons who, after initial contact have produced the IgE tissue-binding class of antibody (reagin), the F_c piece having endowed it with a high avidity for mast cells, resulting in bronchial spasm and acute hypotension. This is a manifestation of anaphylaxis.

The autoimmune response occurs when an individual forms demonstrable antibodies to a self-antigen or exhibits an allergic reaction when tested with it, i.e. there is a loss of self-tolerance or an altered distribution of self-antigen.

There are many other deleterious immune reactions, eg. transfusion reactions due to antibody reacting with red cells, white cells or platelets, haemolytic disease of the newborn, haemolytic anaemia. These are initiated by the antibody reacting with either an antigenic component of a tissue cell or an antigen or hapten which has been intimately associated with tissue cells. Complement is usually, but not always, necessary. These may be termed cytolytic or cytotoxic reactions.

CONCLUSION

This brief outline of immunological mechanisms is intended to give a general outline of their characteristics and functions at serum and tissue levels, where they apply to general human disease and the body's attempt to combat infection.

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