A COMPARATIVE STUDY OF THE SALIVA AND OF THE BLOOD OF THE CARIES SUSCEPTIBLE AND CARIES RESISTANT STRAINS OF HUNT-HOPPERT RATS

By

MELVIN A. BENARDE

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INTRODUCTION

Approximately ten years ago Hunt and Hoppert (31) published the results of many years of experimentation. They succeeded in evolving two distinct strains of albino rats; one was caries resistant, the other caries susceptible. Both groups were maintained on the Hoppert caries producing diet. Their work proved that, for these animals, heredity is an important factor in the development of dental caries.

The fundamental nature of their work serves as a basis from which many problems may be drawn. In fact, the research problem to be described presently is a direct consequence of their findings. Because of the complex nature of the overall problem, discretion necessitates partition into smaller but specific areas of investigation. These investigations are, therefore, only several facets of this larger problem.

Since the carious condition is, in part, one result of heredity, it is permissible to speculate that certain physiological and chemical qualities have been altered in one of the groups to a degree sufficient to initiate caries. The immediate problem can be succinctly stated. What are the differences between these two groups of rats disposing one of them to dental caries?

It is not often that two such ideal groups of animals are available for observation, each serving as a control for

the other. Specimens taken for study from both groups at the same time, under the exact conditions, receiving equivalent treatment, could be expected to yield significant results whether differences or similarities were found between the two strains. It is understood, however, that only differences between the two strains might possibly explain the disposition of the susceptible group to caries.

Added direction for this study was obtained from the early recognition of wide bacteriological differences in the oral microflora of these animals. Rosen et al (59) were able to recover lactobacilli more frequently and in greater numbers from susceptible animals than in the resistant group.

Utilizing the Hunt-Hoppert animals and Rosen's findings as a point of departure, it was deemed logical at this time to undertake a study of the saliva and blood of these animals. These two materials would be subjected to a variety of tests in an attempt to discover any significant qualities that might explain the difference between the two strains of rats.

The caries time (the number of days elapsing from the date on which the animal was placed on the cariogenic diet to the date on which the first carious cavity could definitely be established in a lower molar) is approximately 70 days for the susceptible animals and 300-500 days for th e resistant animals. The procedure adopted was to collect

the necessary specimens from four developmental levels: the pre-caries stage, the period of caries development, the period of advanced caries, and the period when caries appear in the resistant strain. In this way a complete picture depicting the conditions prevailing at strategic stages of growth would be obtained.

EXPERIMENTAL METHODS AND MATERIALS

For this study to proceed according to adopted protocol, adequate quantities of saliva for the various procedures had to be obtained. The immediate problem was to devise a method for the collection of saliva.

After several unsuccessful attempts in which various types of suction devices were used, chemical inducement of salivation was employed with fair success. It was desirable not only to obtain a sufficient quantity of saliva but also to assure the survival of the animals since they would be required for more than one test.

A necessary piece of equipment in which to hold the animals while anesthetized and salivating had to be designed. Figure 1 shows the multiple unit animal holder with and without animals.

The holder has the following measurements.

Overall length	-	$33\frac{1}{4}$ inches
overall width	-	$14\frac{1}{4}$ "
height with cover	-	$8\frac{1}{2}$ "
number of sections	-	8
width of each section	-	4 inches
width of separation	-	3/16 inch
head opening	-	$1\frac{1}{2}$ inch diameter
width of bottle rack	-	2-5/16 inches
individual bottle holder	-	$1\frac{3}{4}$ inch outside dia.
height of bottle	-	3 inches
angle of incline of unit	-	8 degrees

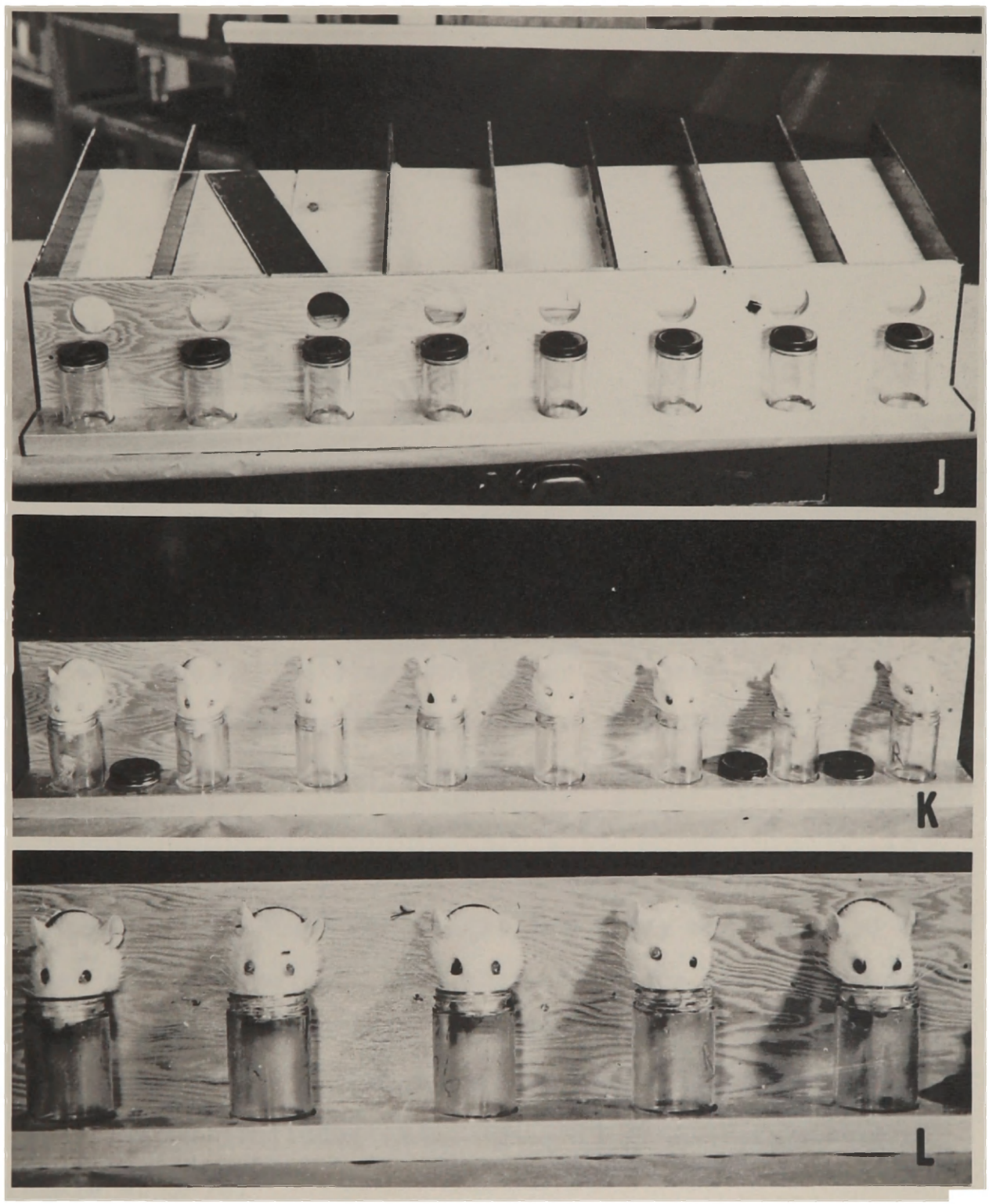
The sides and dividing partitions were made of Masonite.

Figure 1. Photographs of multi-unit animal container

J. Multiple unit animal container (empty)

K. Container full and cover in place

L. Close-up view of animals salivating


J
K
L

Before the anesthetized animal is placed in the unit, a piece of brown wrapping paper can be slipped into the compartment. This reduces the necessity of cleaning to a minimum.

The Masonite cover prevents the animals leaving their units. The size and lightness of weight of the holder enables anyone to carry it about easily and to set up operations in small quarters.

Animals Employed

The animals used in this investigation were obtained from the colony maintained by Hunt and Hoppert. These albino rats have been selectively bred; one strain for caries susceptibility and the other for caries resistance.

The data to be presented were obtained from 107 animals. This does not include the animals used in preliminary trials required to perfect techniques. Of the 107 animals used, 78 were males and 29 were females. Of these, 90 animals were used for the saliva study and included 65 males and 25 females. Only three of these animals were reused for saliva studies. Forty-five animals were used for the serum studies of which 17 were not used in the saliva experiments. Included among these 45 were 35 male and 10 female animals.

For the bulk of the studies reported, pools of saliva and serum from four animals were used. In the case of very young animals, individual samples or pools from two animals

saliva were subjected to the various tests because of the scarcity of animals in that category during the time of this investigation.

Drug Schedule

When mechanical devices failed to yield sufficient saliva, a chemical stimulant was sought. Holck (23), writing in Griffith and Farris, lists many drugs used in rats. From this list pilocarpine nitrate was chosen as a salivary stimulant. Nembutal (a barbiturate) was selected to produce light anesthesia as it was discovered early in this work that a struggling animal would not yield saliva.

Holck's list is a compilation culled from the literature and can only be used as a point of departure. His list suggests that 40-160 mg per kilo of body weight would yield the typical action of pilocarpine. One hundred milligrams was administered along with nembutal at 40 mg per kilo of body weight. Nembutal was given first by intraperitoneal injection to bring on the anesthesia. This was followed by subcutaneous injection of the pilocarpine only after the anesthetic had taken effect. This dosage resulted in many deaths. The pilocarpine was reduced to 50 mg per kilo and the nembutal to 20 mg per kilo. This schedule also caused enough deaths to warrant further study. It was observed that after injection of the pilocarpine the animal becomes more flaccid than from nembutal alone. Pilocarpine appears to enhance the effect of nembutal. This observation

led to a reduction of pilocarpine to 5 mg per kilo. This was sufficient to induce adequate salivation. In fact, it was equal to that obtained with 100 mg per kilo. Pilocarpine was equally effective at the 5 mg per kilo level for all ages and weights and for both sexes.

An initial injection of 20 mg per kilo of body weight was administered. In cases where this dose did not achieve the desired anesthesia additional 5 mg per kilo increments were given until the anesthesia was obtained. A large variation in response was found among the animals. Some were anesthetized by the initial 20 mg per kilo, others required as much as 50 mg per kilo. This variation did not appear to be determined by sex, weight or age. The response of animals to nembutal was unpredictable. This variation may be charged to "constitutional differences" among individual animals.

When the dose was standardized the animals responded well with approximately one death in eight each time a group was processed.

After the drug was injected the animals were placed in the holder and allowed to salivate until sufficient quantities were obtained. This procedure was employed for all saliva used in this investigation. Collections were made in the animal house and shortly thereafter the samples were brought to the laboratory for analysis.

The saliva and serum were subjected to various tests. These are listed in outline form. Each procedure constitutes a unit and is described separately.

OUTLINE OF PROCEDURES

Bacteriological

I. (a) Total counts of oral microflora

(b) Lactobacilli and streptococci counts

II. Antibacterial properties of saliva

Biochemical

I. Amylase determinations of saliva

II. Moving boundary electrophoresis of sera

Serology and Immunology

I. Agglutination tests

Saliva and sera

II. Determination of leucocytes in saliva

Physical Measurements

I. Refractive index - saliva

II. Surface tension - saliva and sera

III. Viscosity - saliva

IV. Specific Gravity - saliva

I. BACTERIOLOGICAL STUDIES OF HUNT-HOPPERT RAT SALIVA

The microflora of the oral cavity has long been associated with dental caries. It was not until 1882 that substantial experimentation removed these observations from the realm of pure speculation.

W. D. Miller (44, 45) promulgated a theory of caries production that is popularly held today. He noted that the source of acids which decalcify teeth is carbohydrates degraded by oral bacteria.

Bunting (10) was so convinced that Lactobacillus acidophilus was the prime agent of dental caries that he wrote, "The presence or absence of Bacillus acidophilus in the mouth constitutes a definite criterion of the activity of dental caries that is more accurate than any clinical estimation can be . . . . And there was a spontaneous cessation of caries coincident with the disappearance of B. acidophilus from the mouth."

Arnold and McClure (3) identified lactobacilli in 90 per cent of cases of dental caries. They presented a positive correlation between the number of organisms in saliva and caries activity.

Becks (5) noted that a high caries incidence was related directly to a high aciduric micro-organism count.

Rosen and his group (59) showed that lactic acid bacteria are a permanent microflora of caries susceptible rats whereas the same organisms do not persist in caries

resistant rats.

Rosebury (57), on the other hand, stated that of 40 healthy albino rats all had L. acidophilus as a part of the normal oral flora. He believed that these aciduric bacteria may be present in the mouth without giving rise to dental caries.

Streptococci have also been incriminated as etiologic agents in caries. Belding and Belding (6) made the observation that Streptococcus odontolyticus is the principal agent in dental caries.

Canby and Bernier (12) suggested that, since streptococcus is rarely isolated from deep carious dentin, great emphasis must not be given them as an agent of caries.

Bibby, Volker and Von Kesteren (8) using three different media estimated the organisms present in saliva. They found that in carious mouths lactobacilli made up about 1/2000 of the total numbers and less than 1/1300 of the acidogenic aerobic organisms. Streptococci were present in 20 times greater numbers than the lactobacilli but appeared to be numerically unimportant. They believed that the acid production in the mouth is due to acidogenic types (mainly streptococci) which were not identified in their study. They found that the streptococci produced acid more rapidly than the lactobacilli.

From these few reports, which are typical of the bulk of the literature, it is plain that although acid production in the mouth is generally held to be a direct causal factor in caries production, the organism or organisms involved are uncertain.

The present study was undertaken with the intention of describing the condition of the saliva with respect to both the total numbers of micro-organisms and the numbers of lactobacilli and streptococci present at each stage of caries activity.

Procedure

Saliva was obtained in a manner previously described. Ten-fold serial dilutions of the saliva were made in sterile distilled water. Platings of 0.1 ml portions were then made on four different media.

For an approximation of the types and numbers of aerobic bacteria to be found in saliva, tryptone glucose extract agar (Difco), a general purpose medium was selected. To enumerate the lactobacilli present the specific lactobacillus medium of Rogosa (BBL) and tomato juice agar special (Difco) with 0.2 per cent sodium azide added to inhibit the growth of gram-negative forms were employed. Azide dextrose broth (Difco) with 1.5 per cent agar added was employed for the selective cultivation of streptococci.

One-tenth milliliter portions of the various dilutions of saliva were dropped onto the agar surfaces and spread by means of a sterile glass rod bent at approximately a 45° angle.

Incubation was carried out at 37° C. for from 24 to 72 hours. The colonies on tryptone glucose extract agar (TGE) were counted after 24 hours; the colonies on lactobacillus selection medium (LBS), tomato juice agar special (TJAS) and azide dextrose agar (ADA) were counted after 72 hours.

Results

Table 1 shows that the resistant animals at each level of caries activity exhibit a lower total count than do the susceptible strain. Lactobacilli and streptococci are present in both strains with the streptococci far outnumbering the lactobacilli. This observation is in accord with the general observations noted in the literature.

The total numbers increase successively from group to group in both the resistant and susceptible animals. At the pre-caries stage the susceptible strain shows a three-fold higher incidence of oral bacteria. In the developmental period this same strain exhibits a 2.5 times greater incidence in total numbers. A six-fold greater occurrence of oral bacteria in the advanced caries stage and a nine-fold increase in the oldest group marks the susceptibles as consistently higher with respect to total numbers.

Lactobacilli were present in greater numbers in the susceptible animals especially during the period of caries development. There was a ten-fold increase when lactobacillus selection medium was used and a 17-fold increase when tomato juice agar was employed. The zero counts obtained in both strains of animals on lactobacillus selection medium during the pre-caries stage is probably not a true reflection of the prevailing condition.

The azide dextrose agar discloses fairly large numbers of streptococci present in all stages. The susceptible strain during the developmental period exhibits a nine-fold increase over the resistants.

It may be significant that between the pre-caries and developmental stages of caries activity, "the sensitive stage," large increases in acidogenic bacteria become apparent. The possibility of sustained acid formation during this "sensitive stage" may initiate the carious process.

It was also noted that the increase in total numbers of bacteria from the pre-caries level to the period when caries appeared in the resistant animals was 60-fold. Should total numbers be more significant than any one organism this observation may be of considerable importance.

Table 2 shows the per cent relative incidence of certain bacteria found in the saliva. The predominance of streptococci over lactobacilli is evident.

Table 1. Bacterial counts per ml of Hunt-Hoppert rat saliva at different stages of caries development.

Medium	Pre-caries level		Developmental period		Advanced caries		Caries appear in resistants	
	R*	S*	R	S	R	S	R	S
T G E (total numbers)	5,800	15,400	70,000	165,500	100,000	628,000	111,333	915,000
LB S (lactobacilli)	0	0	1,175	11,800	550	500	415	720
T J A S (lactobacilli)	550	1,000	1,380	20,000	1,070	14,000	740	12,330
A D A (streptococci)	15,000	11,050	48,750	400,000	73,500	170,000	93,500	28,000

*R = Resistant
S = Susceptible

T G E = Tryptone glucose extract agar
L B S = Lactobacillus selection medium
T J A S = Tomato juice agar special
A D A = Azide dextrose agar

Table 2. Per cent occurrence of oral lactobacilli and streptococci isolated on selective media at different stages of caries development.*

Medium	Pre-caries stage		Developmental period		Advanced caries		Caries appear in resistants	
	R	S	R	S	R	S	R	S
L B S	0	0	1.6	7.1	0.55	0.07	0.37	0.07
T J A S	9.5	6.1	1.9	12.0	1.07	2.20	0.66	0.13
A D A	258.0	71.0	69.6	242.0	73.50	26.10	85.70	3.05

*Comparison is based on total counts obtained on tryptone glucose extract agar (Table 1).

II. ANTIBIOTIC ACTIVITY OF SALIVA

At the outset of the investigation, when the problem area was outlined, it became quite obvious that a study of the antibacterial properties of saliva should be undertaken. When the general defensive mechanism of the body is outlined, as regards the response of the host to the parasite, saliva assumes an important role. The belief that saliva contains some healing qualities dates back to antiquity. Hippocrates considered its value and, of course, there is biblical reference to the dogs that licked the wounds of the beggar. Throughout the history of early empirical medicine the use of saliva was common practice in the treatment of surface wounds.

Forty-five years ago Black (9) stated, "The logical inference is that the cause of the differences in the liabilities of individuals to caries of the teeth is something in the constitution operating through the oral fluids and acting upon the active cause of caries, hindering or intensifying its effect."

The bulk of the literature as it pertains to the problem at hand can be divided into three main groups: (a) those reports indicating that saliva possesses activity against the oral flora; (b) reports indicating that saliva has antibacterial properties, but only against bacteria other than those present in the mouth; and (c) reports

claiming that saliva has no antibacterial effect.

Several in each category are presented. Claims for and against the antibacterial nature of saliva have been forthcoming since the earliest reported observations of Robertson (55) who, speaking of saliva, said, "by its decidedly antiseptic properties, it prevents the process of putrefaction."

Sanarelli (61) concluded that saliva possesses a certain bactericidal power which is able to deal with small quantities, or isolated organisms, but that this power is quantitative and is insufficient to deal with large numbers.

Florain (20) attributed a direct though weak antiseptic action to saliva and Hugenschmidt (30) observed that the bactericidal action of saliva was "problematical."

A case report by Rigolet (54) of a 44-year old man with complete absence of salivary secretion for three years and showing extensive caries of all the teeth, is attributed to the lack of saliva with its antiseptic nature.

Miller (46), after extensive experimentation, concluded that saliva, filtered or unfiltered, did not retard fermentation or putrefaction. In a later report (47) he concluded that saliva possessed no specific antiseptic action against the organisms commonly found in the mouth. But he demonstrated the presence of bacterial antagonism of salivary fluids to other bacteria.

Clough (14) tested forty-one different salivas against L. acidophilus on Kulp's tomato juice agar. All but one inhibited growth. The zones of inhibition around the deep wells varied from 0.5-8.0 mm. Filtered saliva showed no inhibitory effect. In a later work his group reported zones of inhibition against L. acidophilus from 250 of 260 salivas tested.

Dold et al (19), in Germany, reported on substances found in saliva which they termed "inhibines." They were found to be active particularly against the diphtheria bacillus. Activity was destroyed by heating at 100° C. for 1 minute or 56° C. for 3 to 5 minutes. If left standing for ten days at room temperature, the activity is lost and is not regained by adding small amounts of fresh saliva. These inhibines do not pass through Seitz filters and there does not appear to be a relation between inhibitory activity and the bacterial count of saliva.

Clauberg (13) observed that saliva contained a substance that would inhibit the diphtheria and the influenza bacilli.

The following year Pesch and Damm (52) reported that saliva kills or makes the pneumococcus avirulent, but that it does not change the type. Heating to 56° C. or passing through Seitz filters inactivated the saliva.

Von Kesteren et al (68) noted that saliva contained at least two antibacterial agents. One of these, resembling

lysozyme, was effective against one group of organisms, the other, distinct from lysozyme, was effective against another group of organisms.

Kesel and his group (35) indicated that the inhibiting agent of saliva is a volatile substance which he found to be ammonia. He correlated directly the amount of ammonia nitrogen with the degree of inhibition. This group later reported (36) that persons with no caries experience possessed saliva with greater inhibitory properties as compared with saliva from carious mouths. The saliva from non-carious persons increased in activity on heating to 37° C. and maintained its activity after Seitz filtration.

Thompson and Shibuya (65) claimed that the inhibitory action obtained against diphtheria bacilli is due not to the saliva but to the streptococci contained in the saliva.

Thompson (66), in an earlier report, showed that the lysozyme in saliva could not be a factor in inhibition of bacterial growth.

Granados et al (22) suggested the presence of a factor in saliva of caries free people capable of preventing caries. One group of hamsters was given water containing saliva obtained from a caries negative individual. A second group received water containing saliva from a caries positive individual. A third group received water along. The first group receiving water with saliva from a caries negative individual showed less caries than the others.

Bonicke and co-workers (11) obtained saliva from subjects as they awoke. This was termed "night saliva." It showed a higher antibacterial activity than that obtained during the day. The agent in the night saliva is chemically and physically equal to Dold's inhibine. The factors from both day and night saliva were independent of the bacterial cell count. They attributed the greater activity of night saliva to a higher concentration of the antibacterial factors because of the slow flow during sleep.

The seventeen representative papers mentioned clearly indicate a turbid, if not confused, role for saliva as a defense against oral microflora.

The claims and counter claims for the part saliva plays in dental caries made a study of this type mandatory, as was previously stated.

Procedure

When the literature had been searched and procedures for this specific study adapted, one criterion was imposed. The technique employed should approximate conditions as they appear in the animal mouth.

Saliva was obtained as previously described. One-tenth to 0.2 ml of pooled saliva was pipetted into deep wells cut into agar plates. Two wells were cut into each of the media used in order to observe the action or effect of susceptible and resistent salivas under equivalent conditions. These plates were seeded with organisms isolated

directly from the teeth of caries susceptible animals.

The three lower right molars of 5 to 7 caries susceptible rats were vigorously swabbed with standard cotton-tipped applicator sticks moistened in one per cent peptone water. Each of these applicators was used to streak the surfaces of the following three media:

1. LBS medium (BBL) for the isolation of lactobacilli.
2. Tomato juice agar special (Difco) with 0.2 per cent sodium azide for the isolation of lactobacilli.
3. Azide dextrose broth (Difco) with 1.5 per cent agar for the isolation of streptococci.

The plates were incubated at 37° C. and examined daily for four days for signs of inhibition.

Two other techniques were used to determine if the rat saliva possessed any inhibitory nature. As Kesel suggested that the antibacterial factor was a volatile substance, a different approach was taken. Porous cups were warmed in a bunsen flame and placed on the agar of all three media. Two-tenths ml of saliva was placed in the cups. A sterile cover glass was placed on top of the cup and quickly sealed with natural color nail polish. The Petri cover was replaced and the plates incubated as usual.

Filter paper discs impregnated with 0.1 ml of saliva were also employed. Two filter paper discs 12.7 mm in

diameter were placed on each of the three media. One disc contained the resistant and the other the susceptible saliva.

Results

Zones of clearing around the deep wells, porous cups or filter paper discs were not observed. The seeded organisms grew up to the lip of the wells and to the edge of the cups and discs. Apparently the saliva of both strains of rats exerts no inhibitory effect upon the lactobacilli or streptococci tested.

It may be of interest to note that Thompson (66) demonstrated growth inhibition by human saliva when the saliva was diluted. Rosen (57) recently observed this same phenomenon with saliva obtained from Hunt-Hoppert rats against Bacillus subtilis. No explanation for this can be offered at this time. Since this condition of diluted saliva does not prevail in the mouth, it appears to be of only passing interest here.

III. AMYLASE

The determination of amylase was not in the original protocol. The results of viscosity measurements suggested inclusion of this enzymatic study.

As early as 1912 Pickerall (53) suggested that ptyalin (amylase) may protect against caries by its rapid digestion of starch accumulated on the teeth so that the soluble maltose would be rapidly swept away. He believed that no amylase at all would be better than a scanty amount because the intermediate sticky dextrins would not be formed.

Hubbell (29) found the diastatic activity of saliva to be greater in the presence than in the absence of caries in children.

Day (17) made a similar observation but did not believe that the diastatic power of saliva was important in caries production.

Florestano (21) and his group believed that "the diastatic activity of saliva may serve as an index of caries susceptibility."

Turner and Crane (67) reported the time of salivary hydrolysis of starch to be inversely related to the number of cavities in fifty-one cases studied.

McClure (42) found no significant difference in reducing sugars from salivas of children with or without caries.

Bàràny (4) estimated the amylase content of saliva. No significant differences were obtained between persons with "much caries" and little caries."

Hess and Smith (27) also measured the amylase activity of human saliva. They similarly found no significant difference between the salivas of carious and non-carious persons.

Should the prevailing theory of caries production prove to be correct then the presence of an amylase may be a factor in the ultimate formation of acid from starch.

A general outline of the chemistry of the ultimate acid production can be represented as follows:

$$(C_6H_{10}O_6)_x + \xrightarrow[\text{amylase}\ \text{bacterial or salivary}]{H_2O} C_{12}H_{22}O_{11}\ (\text{maltose})$$

$$C_{12}H_{22}O_{11} + \xrightarrow[\text{maltase}\ (\text{bacterial})]{H_2O} 2C_6H_{12}O_6\ (\text{glucose})$$

$$C_6H_{12}O_6 \xrightarrow[\text{Fermentative enzymes}\ (\text{bacterial})]{} 2C_3H_6O_3\ (\text{lactic acid})$$

Should food particles be retained by teeth, starch would be present as a potential source of acid. The amount of amylase present in the mouth might be an important factor in determining the amount of acid produced.

Procedure

A preliminary study was made to determine the presence or absence of amylase in the saliva of the animals.

Saliva was harvested and used in the achromic end point test as described in Hawk, Oser and Summerson (25).

It was found that the end point (disappearance of blue color) was reached approximately three times faster with the saliva from susceptible animals than with that of the resistant animals, indicating that the susceptible animals produced more amylase.

However, due to the limitations of the accuracy in determining end points, the method of Stark et al (62) was adopted for use. This is a simple yet strikingly demonstrative test.

Filter paper discs placed on starch agar gels are impregnated with a calculated amount of test saliva. This is allowed to incubate for ten hours after which the plate is flooded with a dilute iodine solution and the excess poured off. Diameters of the colorless circular areas (against a deep blue background) are easily measured with a millimeter rule.

Two discs were used on each starch agar plate; one containing the saliva of resistants, the other of the susceptible strain. This was done in order to obtain measurements under the same conditions. Tests were carried out in duplicate and an average of the zones obtained.

Results

The measurements of zones of hydrolyses, measured in millimeters, appear in Table 3. It is evident that group for group the resistant strain exhibited smaller zones of hydrolysis, which indicates that less amylase is present in the saliva of this strain.

If these data portray the condition prevailing in the mouths of most of the animals, it may bear directly on the caries problem. If the amylase present can split sufficient starch molecules to maltose rapidly enough for the oral microflora to degrade the maltose and produce a sustained acid action, one of the conditions for caries production would be satisfied.

As pointed out in another section, the saliva of susceptible animals contained far greater numbers of bacteria than did that of resistant animals. The combination of amylase and large numbers of acidogenic bacteria being present at the same time in the susceptible animals is doubtless a condition favorable to caries activity.

If the results of this study are borne out in further studies on a larger number of animals, this evaluation would take on greater significance. For the present such a concept is only suggested.

Table 3. Amylase determination of pooled caries resistant and caries susceptible Hunt-Hoppert rat saliva at different stages of caries development.

	Diameter of zones in mm	
	Resistant	Susceptible
Pre-caries	29.5	31.5
Developmental period	27.7	33.2
Advanced caries	24.5	27.0
Caries appear in resistant animals	24.5	28.1

Figure 2. Photographs showing zones of hydrolysis produced by amylase present in the saliva of resistant and susceptible rats.

M. Pre-caries level

Upper clear zone represents resistant saliva; lower zone, susceptible saliva.

N. Period of caries development

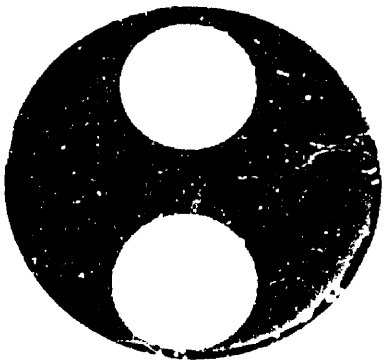
Upper zone represents resistant saliva; lower zone, susceptible saliva.

O. Caries appear in resistant level

Upper zone represents resistant saliva; lower zone, susceptible saliva.

F. Advanced caries level

Upper zone represents susceptible saliva; lower zone, resistant saliva.



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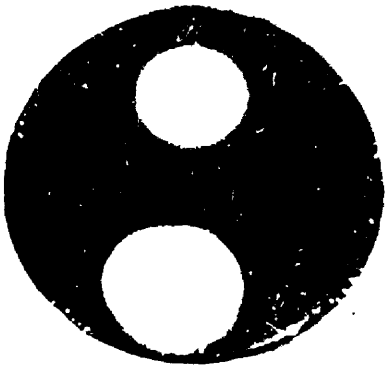

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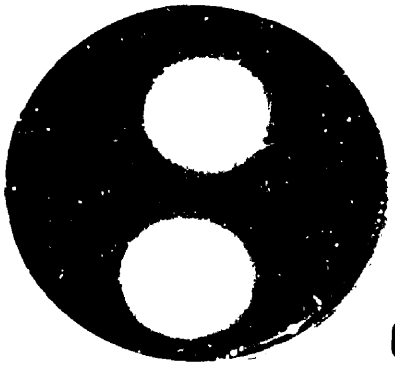

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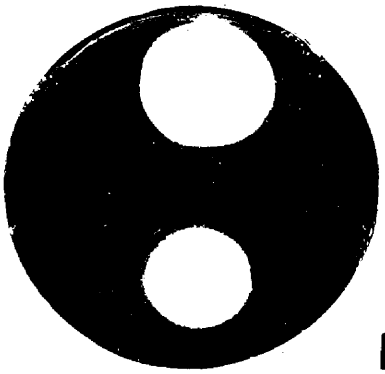

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IV. ELECTROPHORESIS

The moving boundary method of electrophoretic analysis reveals a detailed and reliable representation of the serum proteins. It may be considered to be of greater value than any other chemical or physical technique for the determination of serum proteins available at this time. The procedure yields quantitative information on a whole series of well-defined serum and plasma protein components which cannot be differentiated as readily by other methods. Two excellent features of this technique are its requirement of small amounts of material and that the conditions of analysis are relatively non-drastic to the protein.

The principle of electrophoresis is that a sharp boundary between protein solution and its solvent (usually buffer) is formed and observed as the charged protein migrates in an electric field. Should the solution contain only one species of protein, the boundary remains symmetrical and single. If there are different species of charged proteins in the solution they move at different speeds, so that the initial sharp boundary separates into several boundaries, each representing a different protein fraction of different charge.

It was earlier noted that a diagnostic tool to distinguish between resistant and susceptible animals might evolve from one or a combination of the methods employed. The

delicate procedures and extensive time required for electrophoretic analysis might be reduced and adapted for diagnostic purposes, if a simple distinguishing feature of the pattern results could be detected.

The usefulness of this procedure is the fundamental information regarding serum protein differences it can yield. It was for this purpose that it was used in the investigation.

Among the many papers available on the general subject of serum electrophoretic analysis, those of Stern and Reiner (64), Henley and Stern (26), Leutscher (38), Longsworth (40), and Moore and White (49) are pertinent.

Literature on electrophoresis related to the subject of dental caries could not be found.

Procedure

Blood was obtained from the anesthetized animals by cardiac puncture. The whole blood was pooled (usually from two or three animals of each group) and serum obtained by standard techniques. The serum was separated from the clot by means of a suction pipette and diluted with two volumes of buffer. The buffer used in this study was a barbiturate (veronal and sodium veronal) solution of pH 8.6, 0.1 M ionic strength made up at 24° C. It is stated by Longsworth that this buffer produces better resolution of the delta and epsilon from the gamma globulin boundaries.

The buffer-serum sample was placed in a Visking[1] casing and allowed to dialyze against 100 ml of buffer for two hours at which time 100 ml of fresh buffer was substituted. Three hours later this buffer was again changed for 300 ml of fresh buffer for overnight equilibration against the protein solution. Constant agitation of the membrane-filled with solution was maintained at all times to insure rapid equilibrium. The final dialysis time was carried out in a cold room at 4° C. The equilibrated serum was clarified, if necessary, by centrifugation for 15 minutes at 2000 rpm in the cold room.

The buffer-serum sample was then placed in the electrophoresis aparatus[2] and allowed to operate for 7200 seconds at 7.5 milliamperes which appears to give the best resolution of the serum proteins.

Resistance of buffer solution and protein solution was measured with a conductivity bridge (Model RC-18)[3] and conductivity cell with cell constant 0.4893 to obtain specific conductivity data.

The resolution of the serum proteins produced the electrophoretic patterns.

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[3] Perkin-Elmer Model 38 Tiselius Electrophoresis apparatus.

[2] Industrial Instruments, Inc.

[1] Membrane of seamless regenerated viscose process cellulose.

Each serum protein component mobility was determined as recommended by Longsworth and MacInnes (39), from the measured distance in centimeters between the initial boundary and the ordinate dividing the area of the component in half. This migration distance (d) was substituted into the following formula of Longsworth along with other pertinent data for the determination of mobility.

$$u = \frac{d\ q\ K_{sp}}{i\ t}$$

The potential gradients were evaluated by supplying data into the following formula:

$$\text{Potential gradient} = \frac{i}{q \cdot K_{sp}}$$

where:

d = distance migrated in centimeters
q = cross-sectional area of the cell
t = time of migration in seconds
i = current in amperes
K_{sp} = specific conductance of protein
= $\frac{\text{conductivity cell constant}}{\text{resistance of protein in ohms}}$

The area under each protein component was computed in the manner suggested by Greenberg (41). The negative containing the descending boundaries was placed on centimeter ruled graph paper. A strong light source overlaid with a glass plate was backed with graph paper. The boxes in each area were counted and a per cent of total concentration for each component was obtained.

Results

The patterns are listed in Figs. 3, 4, 5, and 6. The information obtained from the boundary patterns are listed in tabular form (Table 4). This table is, in part, reproduced in the form of line graphs. These line graphs, Figs. 7, 8, 9, and 10, indicate little, if any, differences in proteins composition exists in the sera of the animals studied.

A minimum difference of 10 per cent between any of the component fractions was arbitrarily established as a basis for considering the results significant. With this criterion it became apparent that the albumin fraction offered no point of difference.

Close scrutiny of the pattern from susceptible animals of the caries developmental period reveals a discontinuity in the boundary of the alpha-globulins and a needle-like projection rising out of it. This effect may be due to imperfections in the apparatus. Ice not made of distilled water may carry entrapped material that might interfere with the light going to the camera lens. This explanation is suspected to be a more correct interpretation of this pattern.

The remaining beta globulins and gamma globulins clearly do not indicate the 10 per cent difference as there is a factor of experimental error to be considered. It is

believed that 10 per cent is not an excessive figure to establish as a standard.

This procedure apparently does not reveal any unusual or unique patterns that might shed light on the factor of difference between the two animal strains.

Table 4. Mobilities and per cent total area of serum protein fractions of Hunt-Hoppert rat sera at different stages of caries activity.

	Stage of caries activity	Albumin		Alpha-Globulin		Beta Globulin		Gamma Globulin		Albumin/Globulin ratio
		Mobility	% of total area	Mobility	% of total area	Mobility	% of total area	Mobility	% of total area	
Resistants	Pre-caries level	4.53*	55	6.86	18	1.99	17	1.84	7.0	1.30
	Developmental	5.63	48	8.64	28	6.01	18	1.74	4.0	1.20
	Advanced caries	5.97	57	4.73	23	2.97	13	-	4.0	1.42
	Appearance of caries in resistants	7.24	48	6.59	28	8.69	18	2.17	4.0	1.20
Susceptibles	Pre-caries level	4.83	53	7.19	19	2.30	16	-	8.0	1.23
	Developmental	6.30	44	10.60	18	3.85	12	3.40	5.0	1.22
	Advanced caries	6.00	57	5.25	20	2.17	18	1.74	8.0	1.23
	Appearance of caries in resistants	6.93	45	11.37	25	8.20	17	2.49	12.0	0.83

*$x10^{-5}$ cm^2 $volt^{-1}$ Sec^{-1}

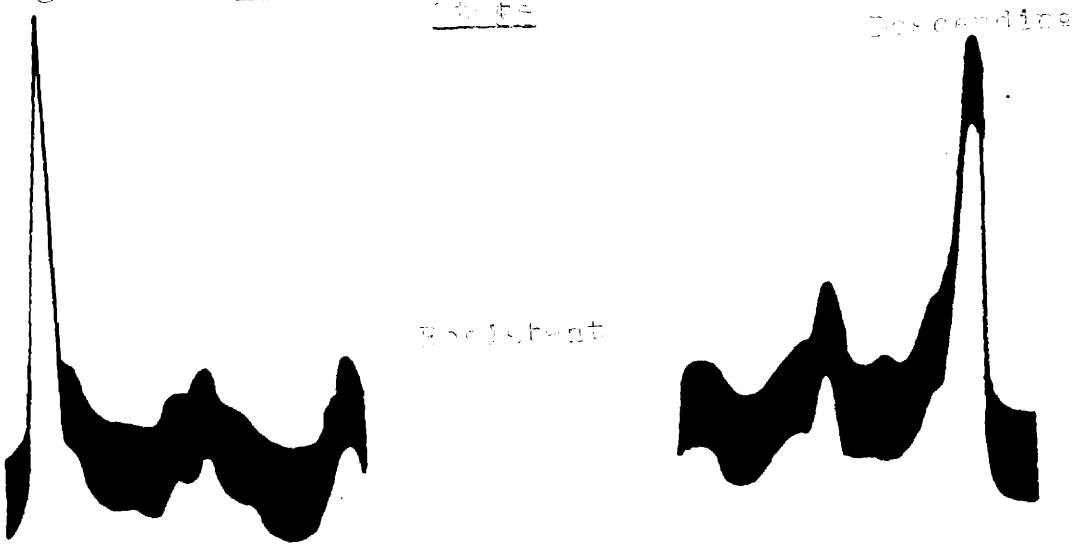
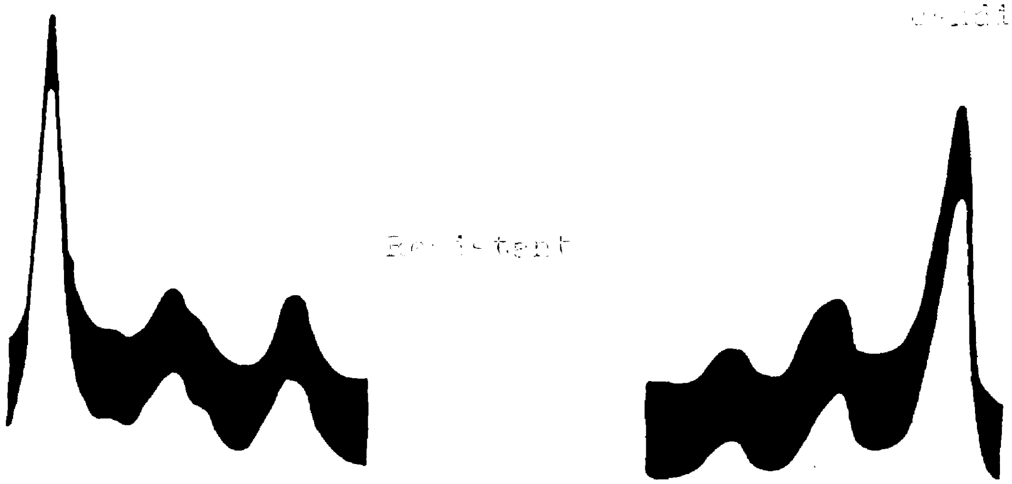


Figure [illegible] Electrophoretic patterns of [illegible] resistant and complement [illegible] rat sera.

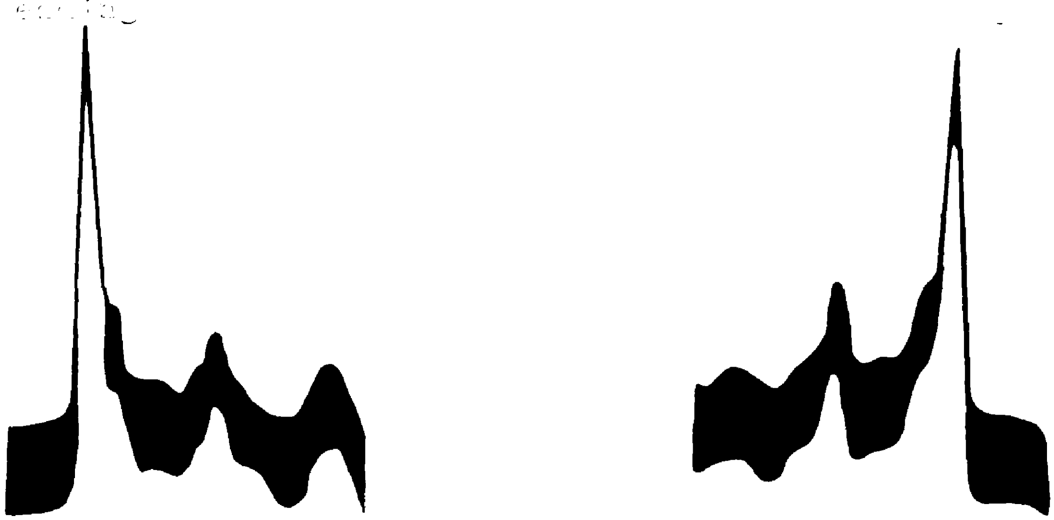
<u>Advanced [illegible] stage</u>

Ascending [illegible] Descending



pH 8.6; Veronal Buffer, μ = 0.[illegible]; 7200 secs, 9.78 v[illegible] $^{-1}$.
Serum diluted with t[illegible] volumes of buffer.

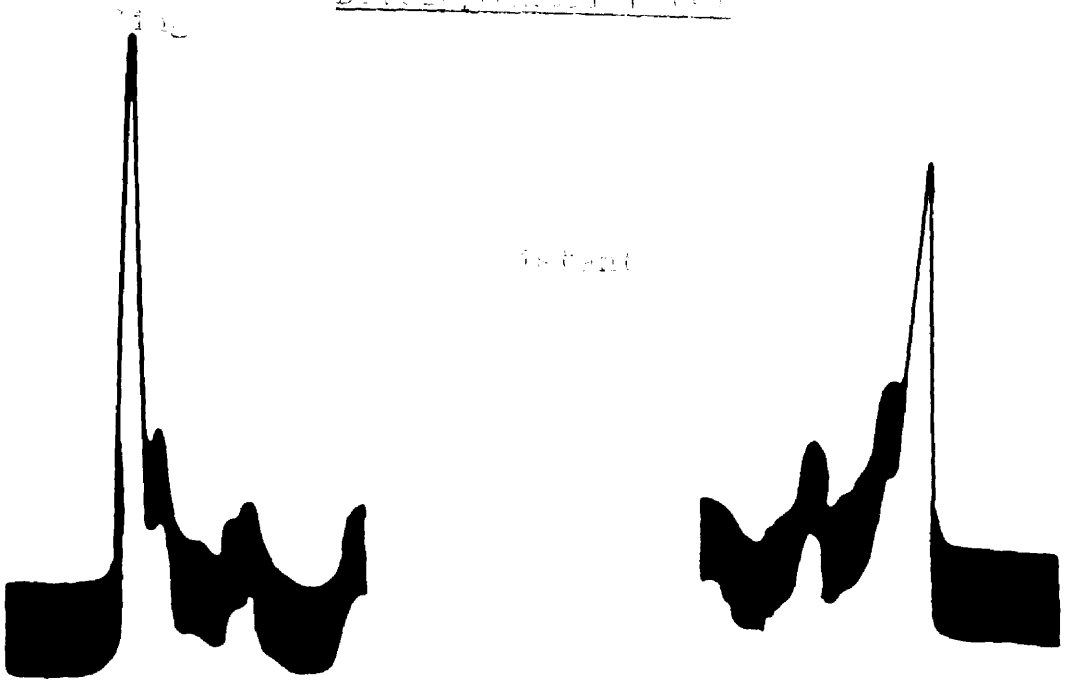
[illegible]



[illegible] μ = [illegible]

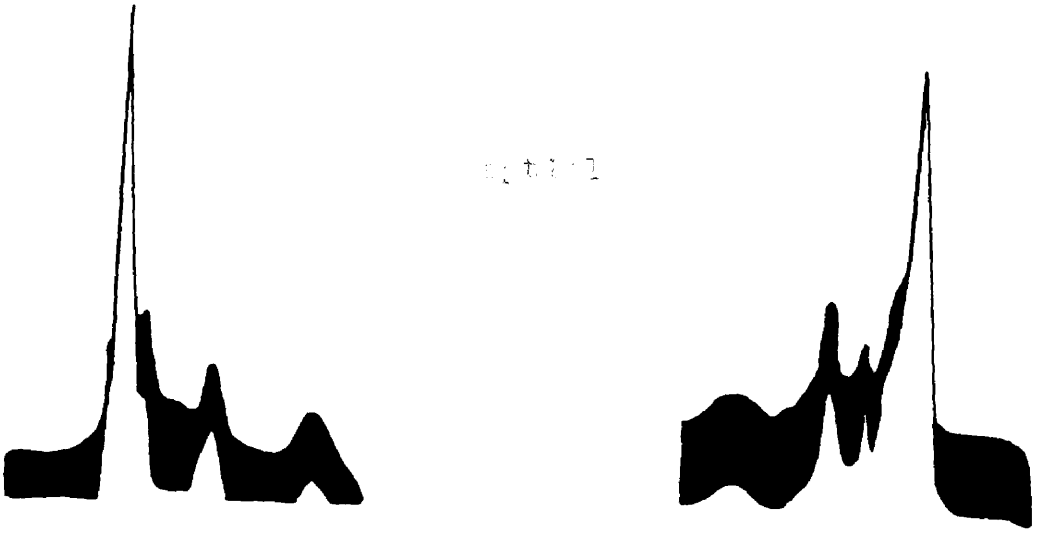
Figure [illegible] Electrophoretic [illegible] of oral caries susceptible [illegible]

Developmental [illegible]



[illegible] μ = [illegible]

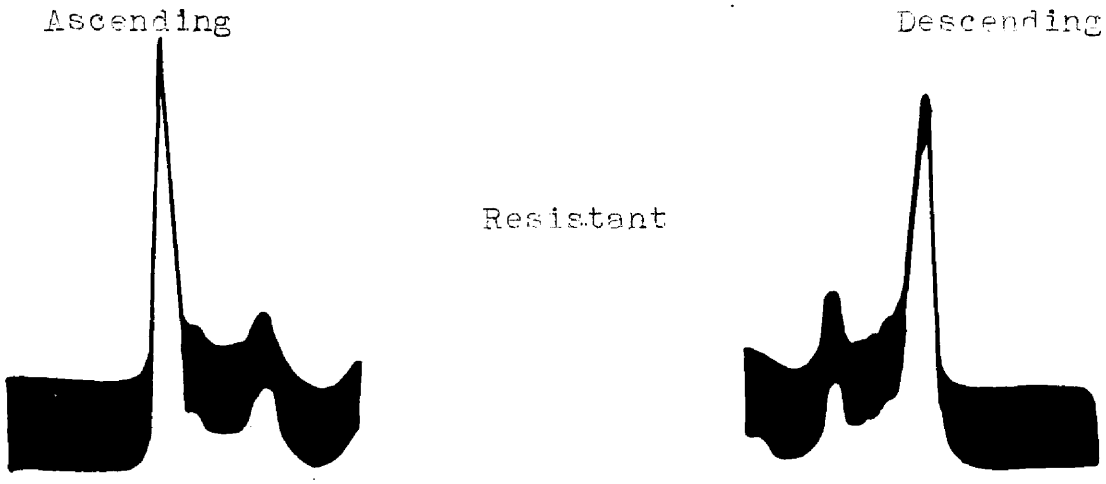
Precipitin



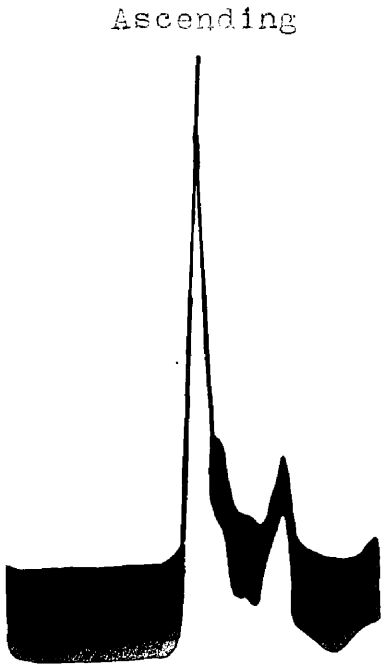
[illegible] μ

Figure 6. Electrophoretic patterns of caries resistent and caries susceptible rat sera.

Pre-caries Stage



pH 8.6; Veronal Buffer, μ = 0.10; 7200 secs; 6.64 volts cm^{-1}
Serum diluted with two volumes of Buffer.



pH 8.6; Veronal Buffer, μ = 0.10; 7200 secs; 6.64 volts cm^{-1}
Serum diluted with two volumes of Buffer.

THE RELATION OF ALBUMIN AREA TO CARIES EXPERIENCE

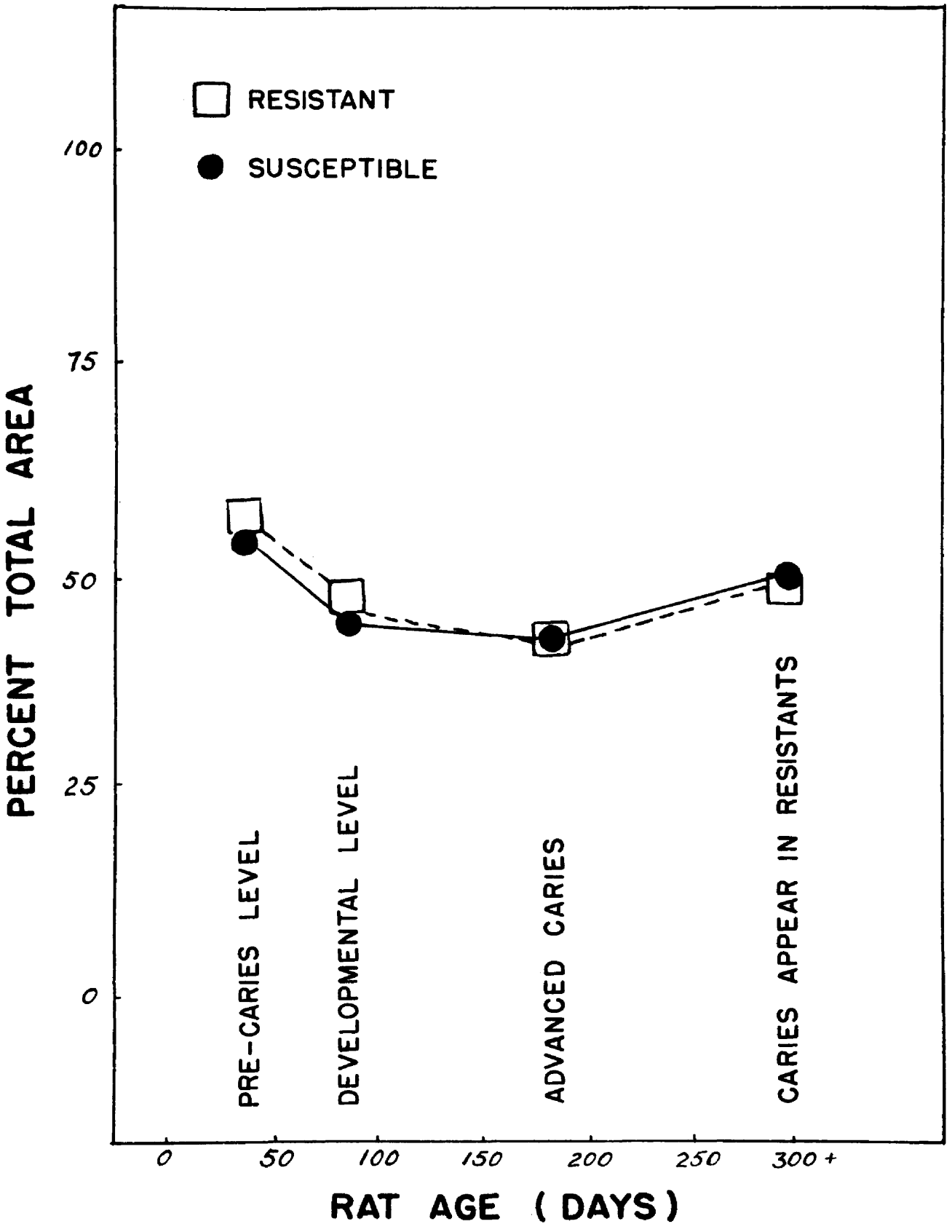


FIGURE 10

THE RELATION OF ALPHA GLOBULIN AREA TO CARIES EXPERIENCE

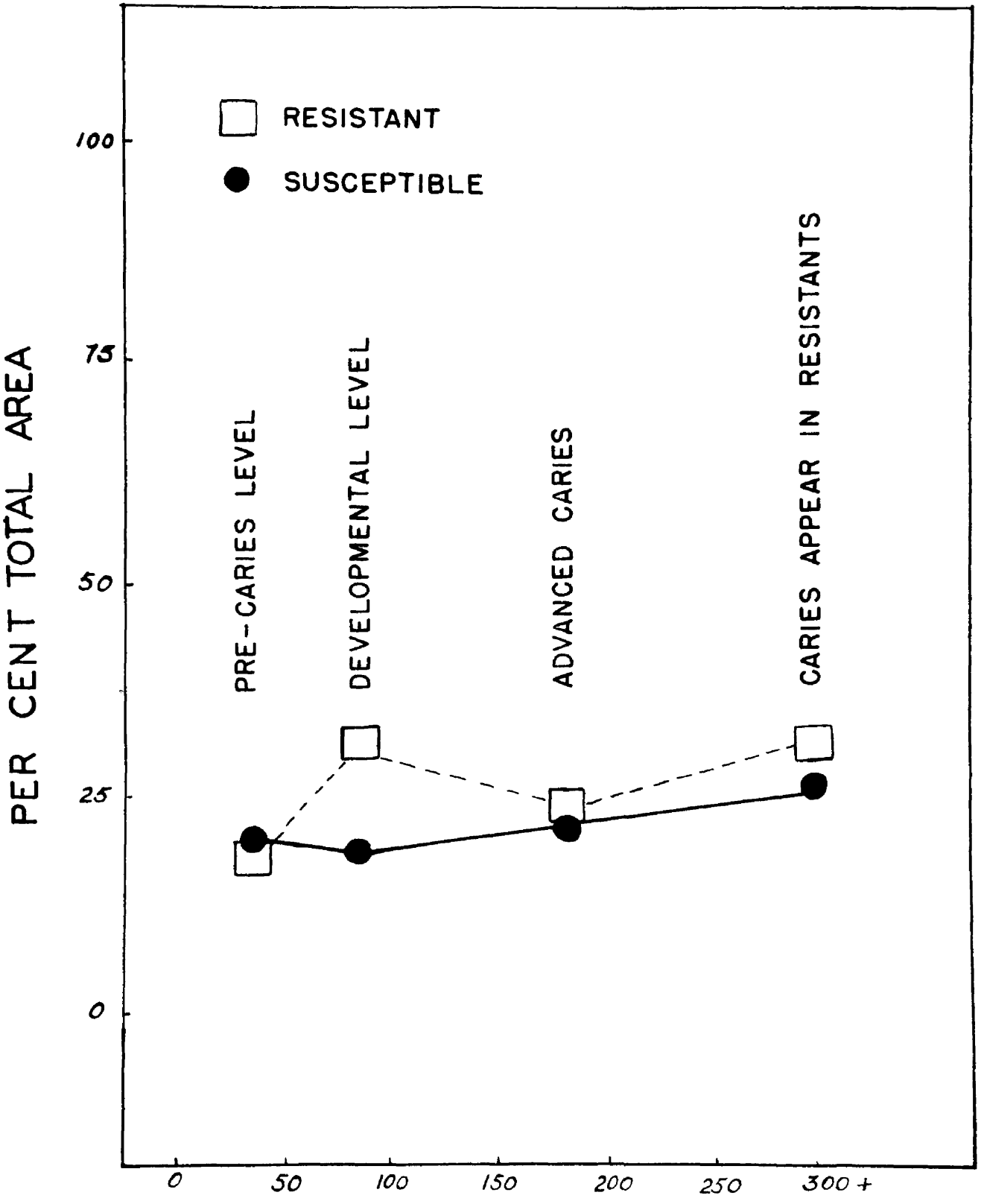


FIGURE 7

THE RELATION OF BETA GLOBULIN AREA TO CARIES EXPERIENCE

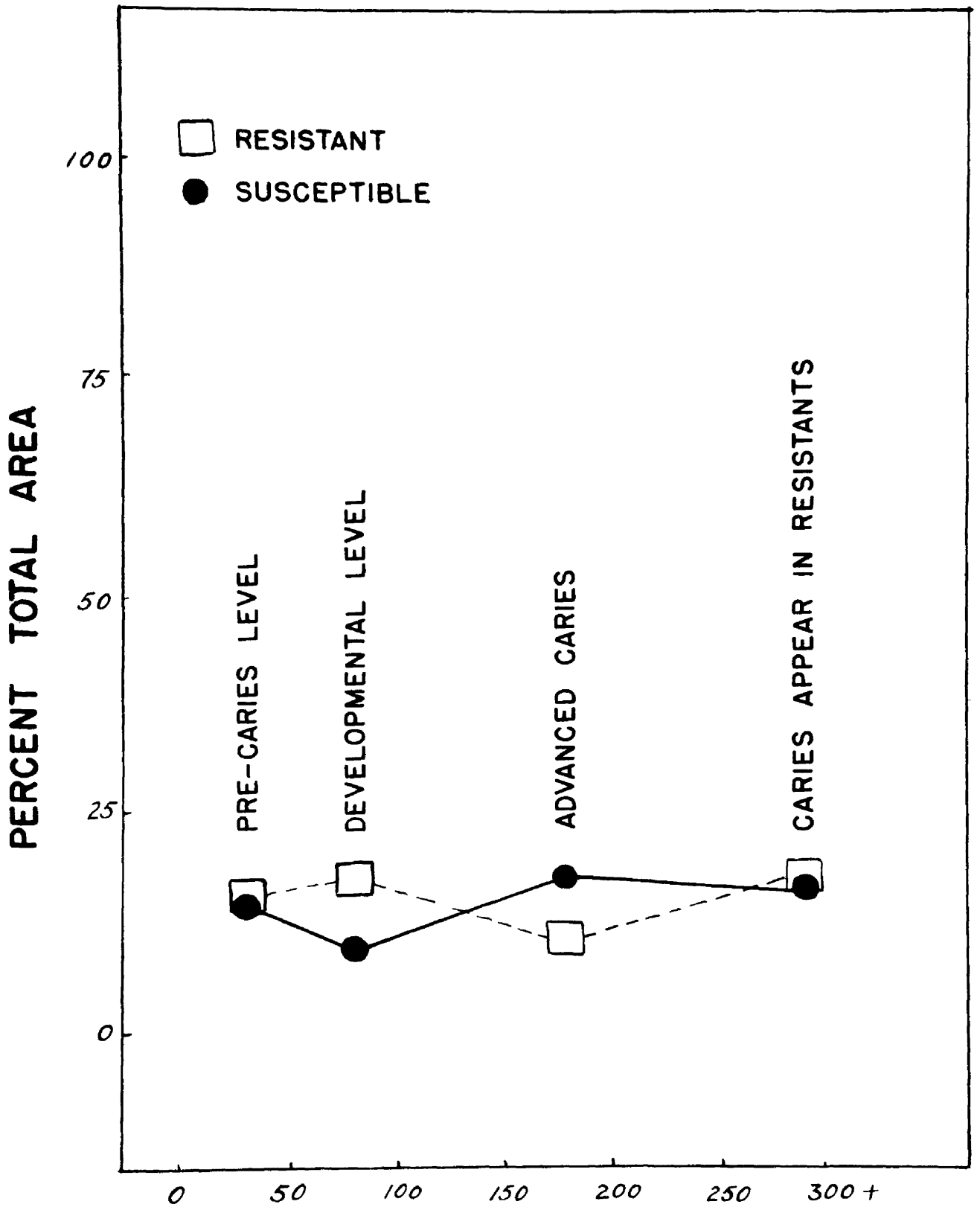


FIGURE 8

THE RELATION OF GAMMA GLOBULIN AREA TO CARIES EXPERIENCE

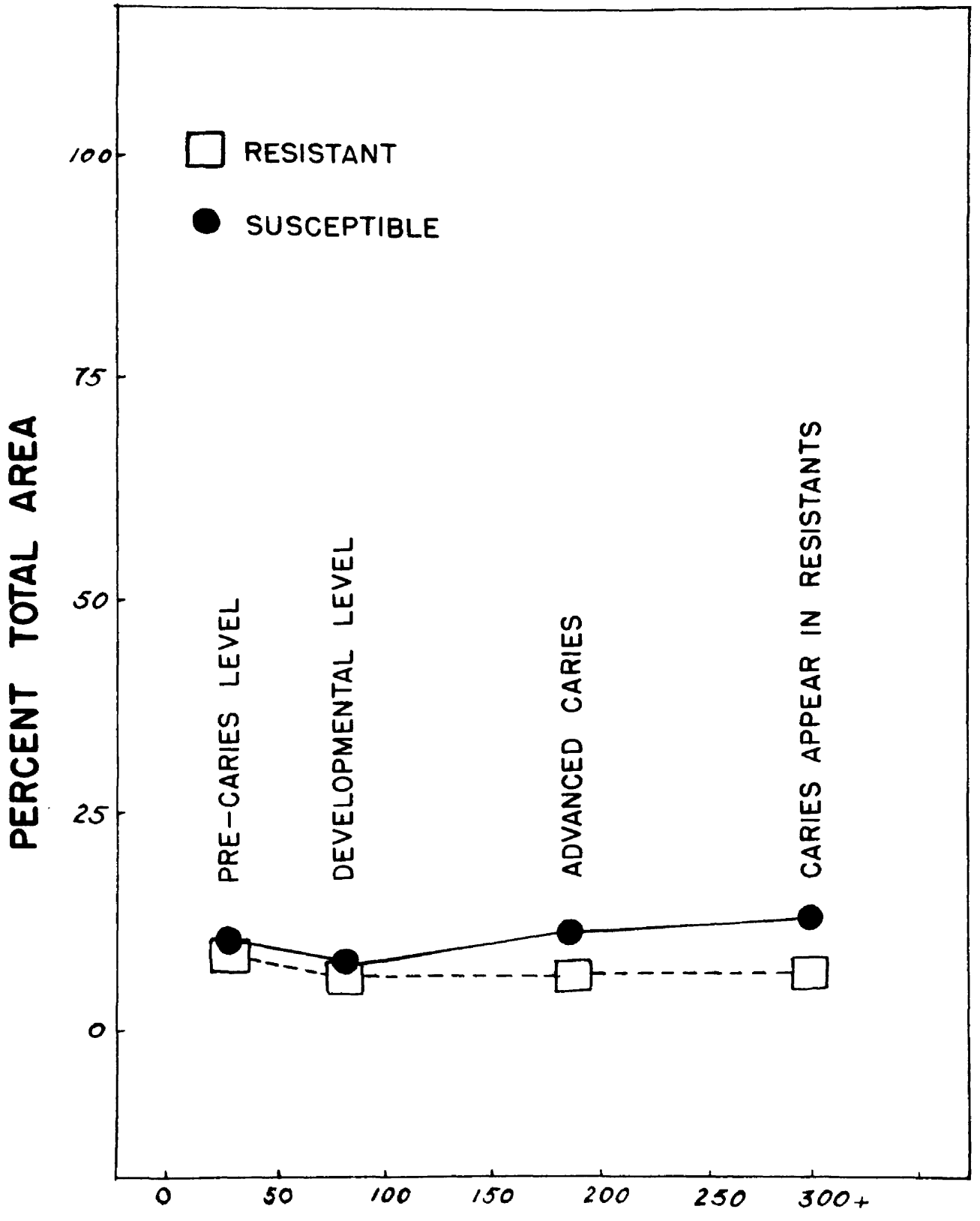


FIGURE 9

V. DETERMINATION OF AGGLUTININS IN SALIVA AND SERUM

To elucidate further differences that might influence the rate of caries development, an investigation into the presence of antibodies (humoral agents that may offer some measure of protection) in the serum and saliva of these rats was undertaken.

It might be well to note here that among the reasons for the performance of each of the procedures undertaken was the desire to obtain a tool for the ready diagnosis of incipient caries.

The immunity implied may be defined as a state of resistance to the development of a disease. Should the disease be initiated by an infectious agent then the immunity may be due to substances in the blood or in the tissues which assist in eliminating the organisms.

Dental caries cannot be strictly termed an infectious disease with the concomitant symptom complex as seen in connective or epithelial tissue infections and therefore one would not expect to find immune bodies protecting directly against caries. However, caries resistant individuals exhibit an immunity which is unexplainable but is suggestive of an immunological "principle" which may be active through either the blood or the saliva. In an attempt to procure information relative to the two objectives mentioned, the tube agglutination test was employed.

Extensive as the literature is on dental caries, relatively few reports document the findings of agglutinins in serum or saliva against the lactobacilli.

McIntosh, James and Lazarus-Barlow (43) reported finding agglutinins against L. acidophilus odontolyticus in the sera of seven out of ten persons to a titer of 1:160. In 1943, Dietz, et al (18), in a study of agglutinins for lactobacilli in 15 caries susceptible and 15 caries free men, concluded that the highest titers of oral lactobacillus agglutinins are accompanied by a low incidence of lactobacilli, but not necessarily a low caries experience. He obtained titers of 1:13-1:88 in non-caries subjects and 0-1:72 in subjects with caries.

Further observations with serum agglutinins are concerned with attempts to induce titers by vaccines of lactobacilli and correlate them with caries experience.

In 1927, Ross, Krasnow and Samet (60) reported agglutinins to a titer of 1:8 with 14 or 18 rabbits inoculated with vaccines prepared from two strains of L. acidophilus.

The following year Jay and his group (33) were unable to produce agglutinins in rats by vaccines of mixed types of organisms. His attempts to use autogenus vaccines in children resulted in abcesses ascribed to the rough colony type of L. acidophilus.

Canby and Bernier (12) vaccinated twenty caries-susceptible men with vaccines prepared from strains of

L. acidophilus isolated from carious dentin. The average count of lactobacilli in the saliva was reduced in 19 subjects and increased in one. The agglutinin titer of serum was increased in ten, unchanged in three and undetermined in seven.

Williams (71) reported in 1944 on an investigation employing 23 volunteers who were inoculated subcutaneously four times at weekly intervals. They each received equal mixtures of two widely cross-agglutinating organisms with both live and heat-killed vaccines. He observed (72) that salivary counts for lactobacilli are not appreciably affected by increased blood agglutinin titers after vaccination.

Procedures

The following organisms were used to prepare the antigen for all the agglutination tests to be described for both serum and saliva:

TS 109
TS 116
LS 110
LS 149

These organisms are described by Rosen et al (57) as satisfying the genus Lactobacillus. All four organisms were isolated from the mouths of susceptible rats. TS 109 and TS116 were isolated on tomato juice agar special and LS 110 and 149 were isolated on LBS medium. These organisms were maintained on micro assay culture agar (Difco).

When an agglutination test was to be performed, 24-hour micro inoculum broth (Difco) cultures of each of the above isolates was used as antigen. The broth cultures were aggregated by centrifugation and the broth removed.

The cells were resuspended in 0.85 per cent saline diluted 1:1000. This dilution was used in order to minimize auto-agglutination since it has been shown that lactobacilli generally agglutinate spontaneously in 0.85 per cent salt (70, 69, 51). Despite this procedure, one of the isolates, TS 109, agglutinated spontaneously and was not used. Therefore, only three of the four isolates previously described were used to prepare antigens.

Five-tenth per cent phenol was added to the bacterial suspension as a preservative. These suspensions were diluted to a turbidity, equal to that of tube #3.0 of McFarland's nephalometer.

Seven Wasserman tubes were set up containing 1.0 ml of distilled water in which the saliva or serum dilutions were made. It is apparent that the final saline concentration is quite small, but sufficient to manifest the second stage of antigen-antibody combination (69). Subsequent procedure is standard and need not be described here.

Results

The results shown in Tables 5, 6, and 7 indicate that agglutinating antibodies were present in the saliva of these animals. These agglutinins were present at all levels of caries activity in both resistant and susceptible animal strains to some degree.

In the pre-caries stage greater agglutination was exhibited by the resistant animals. This was of a variable nature differing between the three antigens which was not unexpected. These isolates were not the same in respect to biochemical definition and their antigenic characteristics may differ also. The variable titers obtained from trial to trial with a particular antigen was not anticipated. This will be discussed presently.

No difference in antibody content was apparent between the groups during the period of caries development. The same occasional variability was evidenced here.

At the advanced caries level all three antigens of the resistant group exhibit marked increases over the susceptible group with less variation within any single antigen over four trials.

In the final group, where caries were seen in the resistant animals, isolate TS 116, generally the strongest agglutinating strain, appeared to have combined with four times as much agglutinin in the resistant saliva as compared

with the susceptible. When all antigens were taken together, the difference still existed but was less obvious. In this group the variation within the antigens was also found but to a lesser degree.

It should be noted that the saliva of the resistant group exhibited titers of 1:32 or greater, 53.3 per cent of the time as compared with 15.1 per cent for the susceptibles. However, the variability within a single antigen suppressed an attempt to regard this as significant.

For the most part pooled saliva was examined. At least three animals were used for a pool. The same animals of any specific group were not used repeatedly in gathering data. This use of different animals may be one factor responsible for the occasional variation of titer with a single antigen. Three resistant animals were used twice. These animals were those in the period of caries development. They were tested 34 days apart (trials 1 and 3, Table 5) and this interval might explain the increase in titer exhibited between these two trials.

The antibody present in the saliva may represent leakage from the blood serum. Fluctuations in the serum may be reflected in the saliva. Another factor that may well be responsible for the trial to trial titer variations are the bacterial isolates themselves. It has been reported that lactobacilli can undergo changes while being transferred through ordinary media. As these isolates were

transferred with regularity in order to prevent their dying out, antigenic changes may have occurred. (16, 24). Orland (51) writes that "the apparent suppression or loss, at least temporarily, of a major somatic antigen among lactobacilli constitutes a rather interesting immunological phenomenon, though the mechanism involved is highly uncertain at this time."

There is a further possibility that the agglutination obtained is of a non-specific nature. It remains for further experiments to prove that the lactobacilli are either agglutinated by a substance with the characteristics of an immune body and produced in response to the presence of antigen in the tissues or that the clumping is only the non-specific manifestation of the presence of salts or proteins capable of educing bacterial aggregation.

Table 5. Agglutinin titers* of pooled caries resistant and caries susceptible Hunt-Hoppert rat saliva at different stages of caries activity.

	Antigen	Titer					
		Resistant			Susceptible		
		Trials			Trials		
		1	2	3	1	2	3
Pre-caries level	TS 116	32	-*	128	8	8	32
	LS 110	0	-	16	0	0	0
	LS 149	0	-	16	0	0	0
Developmental period	TS 116	32	64	256	32	32	256
	LS 110	0	-	16	0	-	0
	LS 149	8	-	0	0	-	0
Advanced caries	TS 116	64	128	64	16	8	64
	LS 110	64	32	32	-	32	8
	LS 149	64	32	16	0	0	16
Caries appear in resistants	TS 116	64	64	64	16	8	8
	LS 110	0	0	8	0	0	0
	LS 149	0	64	16	0	16	0

*Number of antibody units per unit volume (1.0 ml) of the undiluted serum.
(-) indicates lack of data.

Table 6. Per cent of total occurrences per unit of titer.

Resistant			Susceptible	
Titer	Number of occurrences	Per cent of total occurrences	Number of occurrences	Titer
0	7	23.3	51.5	17	0
8	2	6.6	18.1	6	8
16	5	16.6	15.1	5	16
32	4	13.3	12.1	4	32
64	9	30.0	0.0	0	64
128	2	6.6	0.0	0	128
256	1	3.3	3.0	1	256
	30			33	

Table 7. Per cent of total occurrences showing antibody titer of 32 or greater.

Resistant		Susceptible	
Number of titers	Titers of 32 or greater	Number of titers	Titers of 32 or greater
30	16	33	5
	Per cent		Per cent
	53.3		15.1

Procedure with Serum

Blood was obtained from lightly etherized animals by means of carbiac puncture. Two to four ml of blood from each of three to four animals were pooled and serum obtained by standard procedure. The agglutination tests performed have been previously described.

Results

As indicated in Table 8, the titers obtained from pre-caries animals with isolate TS 116, indicated a slightly greater antibody content for the resistant group. Although the resistant titer was never below 640 with this antigen, the susceptible strain exhibited a titer of 1280 in one instance. The resistant titers of 1280, obtained with isolates LS 110 and LS 149 on the first trial, were probably not characteristic of this group.

The periods of caries development, advanced caries and caries in resistant strains exhibits little difference in titers between strains. In general, the resistant strains showed a slightly higher titer but the difference did not appear to be significant. This observation diminishes the possibility that a protective mechanism in the form of serum agglutinins was at work in some of the strains of rats. This procedure, therefore, did not appear to be useful as a diagnostic tool.

In an attempt to discover if the titers obtained were unique for the Hunt-Hoppert rats, other animal species were examined.

Rabbit, human and other rat sera were obtained. The rats were obtained from two different areas. Rats I were from the Department of Physiology and had recently arrived from Carworth Farms. Rats II were from the Vitamin and Amino Acid Assay Laboratory of the Chemistry Department. They were born on this campus.

Subjecting these sera to the same agglutination tests brought to light significant observations. Table 11 shows that rats, in general, yield titers equal to, if not greater than, the Hunt-Hoppert strains. Possibly rodents in general yield this type of pattern. Rabbit and human sera, on the other hand, yield very low titers. This was anticipated as many species of bacteria are clumped in low dilutions by "normal" sera.

Table 8. Titers of pooled caries resistant and caries susceptible Hunt-Hoppert rat sera at different stages of caries activity.

	Antigen	Resistant					Susceptible			
		Trials					Trials			
		1	2	3	4	5	1	2	3	4
Pre-caries level	TS116	1280	640	1280	1280	640	640	320	320	1280
	LS110	1280	0	0	0	0	0	0	160	640
	LS149	1280	0	0	0	0	640	0	320	320
Developmental period	TS116	2560	1280				320	2560	-	
	LS110	640	640				320	640	160	
	LS149	1280	1280				320	2560	640	
Advanced caries	TS116	1280	2560	1280			2560	2560	2560	
	LS110	1280	1280	1280			640	1280	1280	
	LS149	1280	1280	1280			1280	640	1280	
Caries appear in resistant strain	TS116	2560	2560				2560	1280		
	LS110	1280	1280				1280	1280		
	LS149	2560	1280				2560	640		

Table 9. Per cent total occurrences per unit of titer.

Rat Sera

Resistant				Susceptible	
Titer	Number of occurrences	Per cent of total occurrences		Number of occurrences	Titer
0	8	22.2	8.8	3	0
160	0	0.0	5.7	2	160
320	0	0.0	20.0	7	320
640	4	11.1	22.8	8	640
1280	19	52.8	22.8	8	1280
2560	5	13.8	20.0	___	2560
	36			35	

Table 10. Per cent of total occurrences of antibody titer of 1280 or greater.

Resistant		Susceptible	
Number of titers	Titers of 1280 or greater	Number of titers	Titers of 1280 or greater
35	24	36	15
Per cent		Per cent	
66.6		42.8	

Table 11. Comparison of serum titers of Hunt-Hoppert rats with other rats and mammels.

Antigen	Rabbit		Human		Rats I		Rats II		Hunt-Hoppert rats	
	1	2	1	2	1	2	1	2	R*	S
TS116	8	0	16	8	2048	4096	1024	1024	1792	1120
LS110	0	0	8	0	256	256	256	256	1024	720
LS149	0	0	0	0	256	256	128	256	700	1120

*R = Resistant
S = Susceptible

VI. SALIVARY LEUCOCYTES

The presence of leucocytes in the blood and their role as phagocytic agents have been recognized since the work of Metchnikoff. The literature is replete with studies of these cellular elements. But this is not so in the case of leucocytes present in saliva. Despite the fact that certain leucocytes are known to exhibit a phagocytic action toward many bacteria, virtually no work has been done to establish the function of these salivary corpuscles.

Appleton (2) stated that polymorphonuclear leucocytes were constantly passing through the epithelium of the oral mucosa to the free surface. He suggested that these cells were potentially phagocytic and may contribute to the diminution of bacteria present in the mouth.

Bibby, Hine and Clough (7) shared the opinion of Appleton that leucocytes were phagocytic and reduced the population of oral bacteria.

From their work, Isaacs and Daniclian (32) concluded that saliva, as it appeared at the opening of the duct of the salivary glands, was free of leucocytes. They believed that leucocytes present in the saliva had wandered through the oral mucous membrane.

Orban and Weinmann (50) reported finding fewer leucocytes in stained preparations of caries free individuals than in caries susceptible persons.

The leucocytes from the mouths of susceptible individuals were in better condition than those from resistants' mouths.

Wright and Jenkins (73) found no difference in the total number of leucocytes in caries free and caries susceptible individuals. They found that the number of intact cells were four times greater in the caries free subjects. Many cells were found to be in various stages of disintegration.

The sparse literature dealing specifically with numbers of leucocytes present in the saliva of caries free and caries susceptible individuals is not conclusive. Agreement is also lacking as to the meaning of leucocytes in saliva.

The possibility presented itself that salivary leucocytes might be instrumental in reducing the oral lactobacilli population. Rosen (59) observed that these bacteria in the saliva of the Hunt-Hoppert rats were a permanent flora of the susceptible rats and were transient in the resistant strain.

In keeping with the idea of a large screening program that would point the way for future investigations, a study of the saliva with respect to leucocytes was incorporated into the program. Two objectives were sought: to demonstrate the presence of leucocytes in the saliva of these animals and to quantitatively describe the difference in

numbers between them; and secondly, if possible, obtain a diagnostic tool whereby a leucocyte count would indicate the advent of the caries condition.

Procedure

Glass slides were scrupulously cleaned in soap solution, followed by immersion in a dichromate bath. With the aid of a tungsten-carbide pencil, a 7/8 inch (22 x 22 sq. mm) square was etched into the slides.

Saliva, obtained in the manner previously described, was pooled and 0.01 ml was placed in the center of the etched square from a 0.2 ml pipette. The glass jar containing the pooled saliva was vigorously rotated immediately prior to removing the 0.01 ml sample to distribute the leucocytes evenly throughout the saliva. This portion was evenly spread to the edges of the square and quickly fixed in a current of warm air which was obtained by placing a bunsen flame behind a fan rotating at moderate speed. Both a susceptible and a resistant smear were made in rapid succession. These were then ready for staining. Wright's stain proved most satisfactory for this purpose. Cover slip preparations did not prove as effective as the slide preparations.

Staining Procedure

Wright's stain was made up from the powder as follows:

Wright's powder	-	0.3 gm.
Glycerin	-	3.0 cc.
Methyl alcohol	-	97.0 cc.

The powder and glycerin were ground in a mortar and the methyl alcohol added. The stain was then placed in a dark brown bottle and allowed to incubate at 37° C for at least two weeks.

Buffer

Monopotassium phosphate	- 6.63 gm.
Disodium phosphate anhydrous	- 2.56 gm.
Distilled water	- one liter

(1) Ten drops of dye were placed on the prepared slide for two minutes.

(2) Ten drops of buffer were added and allowed to stand for seven more minutes after which a bright metallic sheen appeared.

(3) The preparation was washed with distilled water in a manner calculated to lift off the dye.

(4) The stain was then dried between blotting paper.

The cytoplasm of the leucocytes appeared blue-purple and the nuclei stained mauve. Bacteria stained deep blue-purple. This preparation was not examined under the oil immersion lens of the microscope.

Haemacytometer preparations, including those stained with Randolph's stain, in an Ac Spencer Bright-Line Haemacytometer did not yield the fine results obtained with dry mounts, and, of course, the dry mounts are a permanent record. The high magnification that can be used with the dry mounts allow differential counts to be made easily.

All counts were translated to numbers present in 1.0 ml of saliva. The calculation is made according to the following formula:

$$\text{No. of cells counted} \times \frac{484}{\text{area covered (sq. mm)}} \times 100 = \text{No. of cells per ml}$$

484 = the total area of the etched square

Area covered = Area of one field under oil immersion multiplied by the number of fields observed.

The formula may be simplified further after the area of one field is calculated. In this case the area of an oil immersion field was 0.08 mm^2. The formula then becomes:

$$\text{No. of cells counted} \times \frac{6,050}{\text{No. of fields counted}} \times 100 = \text{No. of cells per ml}$$

Only intact dells were counted. Fragments of cells that could be identified were not included in the total count. This procedure did not impose difficulty as only a very small number of the total cells appeared to be in some degree of disintegration.

Results

A glance at Table 12 reveals that leucocytes are present in the mouths of Hunt-Hoppert rats. The data show that there is a distinct difference between the numbers of leucocytes present in the resistant and susceptible strains.

It is to be noted that in only the pre-caries stage are leucocytes present in larger numbers in the resistant animals. There is an approximately three-fold higher leucocyte count among the resistant animals. But the figures represent averages of several trials and in this instance may not indicate the true condition prevailing. Three separate trials on susceptible saliva yielded consistent counts averaging 12,165 cells per ml. On the other hand, the trials with the resistant animals did not reveal this type of consistency. One of the trials showed a zero count and another trial a very high count. The average in this case, although a higher figure than the susceptible, may lead to false interpretation.

Each successive stage thereafter exhibits a marked difference in leucocyte numbers with the susceptible animals showing far higher counts. A three-fold increment is noted in the developmental period and four-fold increases are seen in both the advanced caries and caries in resistant groups.

The type of leucocytes found is noteworthy. Polymorphonuclear leucocytes predominate in the susceptible strain with monocytes second. Lymphocytes, on the other hand, predominate in the resistant strain with monocytes again second and polymorphonuclear leucocytes being relatively few in number. Table 13 presents a tabulation of this differential count.

Table 12. Leucocytes per ml of Hunt-Hoppert rat saliva at different stages of caries activity.

	Trials	Pre-caries level	Developmental period	Advanced caries	Caries appear in resistants
Resistant	1	67,222	26,000	45,783	8,344
	2	0	13,511	65,283	10,453
	3		30,250	20,116	68,381
	4	-	-	43,559	-
	Avg.	33,611	23,253	43,635	29,059
Susceptible	1	13,865	43,900	173,522	135,159
	2	12,112	129,066	95,647	129,066
	3	10,520	94,380	174,148	123,794
	Avg.	12,165	89,115	217,929	129,339

0 = none
- = not determined

Table 13. Differential count of salivary leucocytes as found in stained preparations of saliva of Hunt-Hoppert rats at different stages of caries activity.

	Trial	Resistant			Susceptible		
		Mono-cytes	Lympho-cytes	Polymorpho-nuclear leucocytes	Mono-cytes	Lympho-cytes	Polymorpho-nuclear leucocytes
Pre-caries	1	27*	22	1	3	4	0
	2	0	0	0	3	5	0
	3				4	2	4
Developmental period	1	3	6	0	18	36	24
	2	7	6	7	3	8	1
	3	3	7	5	23	9	24
Advanced caries	1	4	11	8	58	54	46
	2	5	10	0	35	23	59
	3	16	31	11	40	16	27
	4	16	15	4	33	24	61
Caries in resistents	1	12	22	18	33	18	55
	2	2	6	0	20	22	58

*These numbers represent the actual count as seen in the stained preparation.

Figure 11. Photomicrograph of salivary leucocytes (Each space represents 10 micron.)

A. Non-filamentous polymorphonuclear leucocyte and small lymphocyte.

B. Monocyte, upper right; lymphocyte, lower left.

C. Group of small lymphocytes; monocyte off to right.

D. Clump of small lymphocytes; monocyte, lower right.

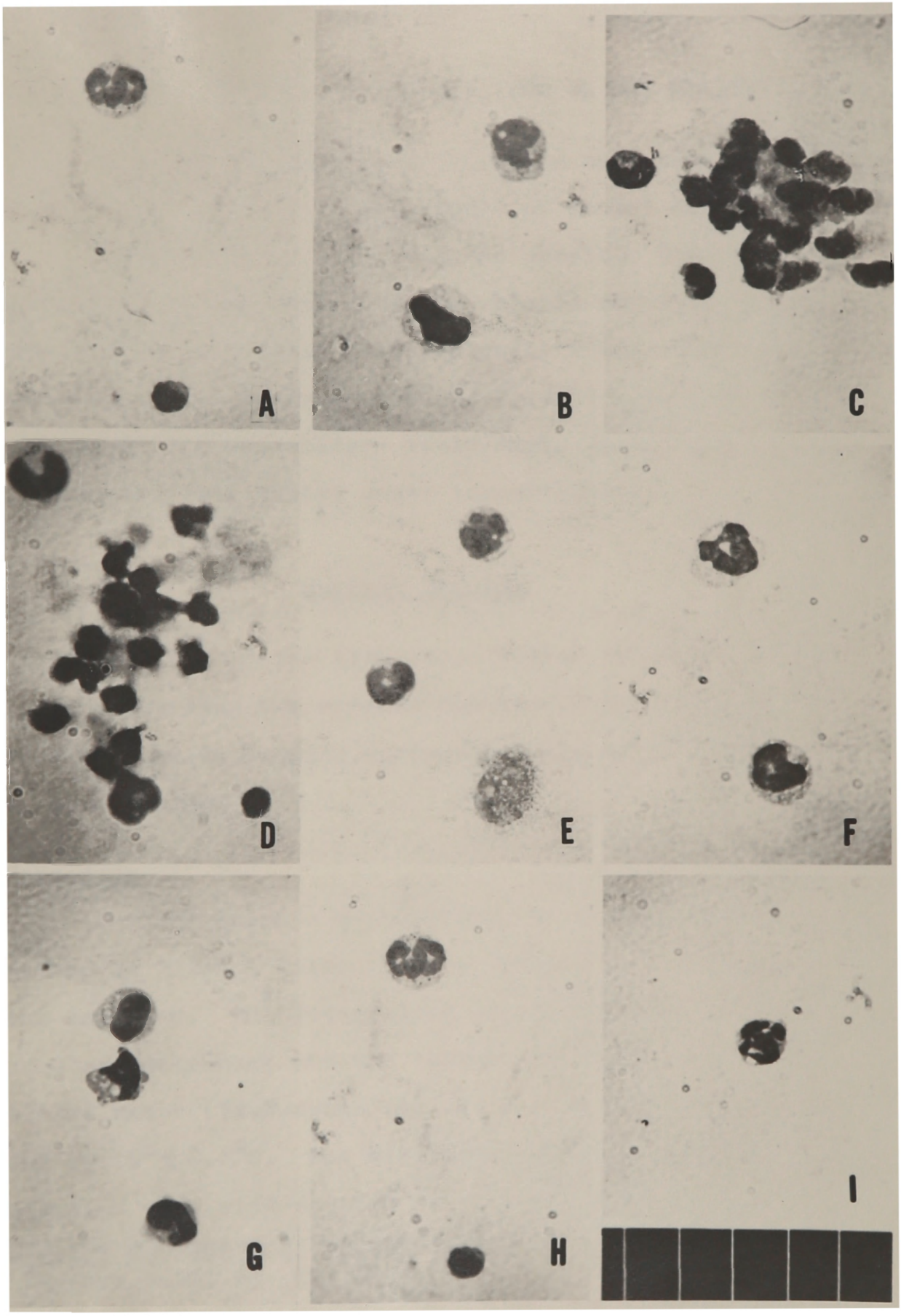
E. Nuclei of epithelial cell, upper left; polymorphonuclear leucocyte, center right; monocyte, center left.

F. Monocytes

G. Monocytes

H. Non-filamentous polymorphonuclear leucocyte and small lymphocyte.

I. Non-filamentous polymorphonuclear leucocyte.


A
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VII. PHYSICAL DETERMINATIONS ON RAT SALIVA

Very few physical determinations have been performed on saliva in pursuit of the riddle of dental caries.

To describe as completely as possible the conditions prevailing in the mouths of the animals studied, the physical nature of saliva required definition. The four measurements;-- surface tension, specific gravity, refractive index and viscosity-- would amply describe the physical character of the saliva under investigation.

Surface Tension

A search of the literature failed to uncover observations concerning the surface tension of rat saliva or observations concerning surface tension of saliva from carious mouths.

Procedure

Surface tension was measured by a torsion balance method in which a Cenco Du Nouy Interfacial Tensiometer* was employed. The interfacial tensiometer is a direct reading instrument and was calibrated against boiled distilled water (72.0 dynes/cm) and C.P. Benzene (28.2 dynes/cm) at $23^{\circ} \pm 0.5^{\circ}C$. One milliliter samples of saliva were pipetted onto acid cleaned watch glasses for measurement.

*Serial No. 890, No. 70542 Platinum Ring - Mean circumference 5.995 cm. R/r 53.6.

Results

Table 14 presents the figures obtained. It is obvious that little difference exists between the two groups of animals. Four-tenths of a dyne per cm difference between the animals of the pre-caries level and 0.9 dynes per cm difference in the period of caries development are well within the limits of experimental error.

The slightly higher differences obtained with the remaining two groups, 1.8 dynes per cm and 2.7 dynes per cm in that order, likewise were within the limits of experimental error. Preliminary tests had shown that variations from one to four dynes per sample could be anticipated.

Although these figures do not indicate differences between the two strains, the very nature of the figures themselves are interesting. The low surface tension, (average of 42.9 for the resistants and 43.7 for the susceptibles), is quite prpvoking considering its high water content. When the values for saliva are compared with a surface tension depressant such as 0.1 per cent sodium oleate solution which has a surface tension of 42.2 dynes per cm or to water with a surface tension of 72.0 dynes per cm, one cannot escape the conclusion that saliva has great wetting characteristics. This may account for the efficiency of saliva in moistening feed materials.

It was observed as early as 1925, by Albus (1), that L. acidophilus could grow well at a surface tension of 36 dynes per cm. This low surface tension vitiates any anti-lactobacillus effects due to surface tension. Since both strains exhibit the same surface tension, it is doubtful that the difference in bacterial population can be attributed to this factor of saliva.

It is of more than passing interest to note that LBS medium selective for lactobacilli contains 0.1 per cent Tween 80, a surfactant, as an integral part of its formula. The object of this medium is to grow lactobacilli selectively to the exclusion of all other microbial forms and a low surface tension is a contributing factor. The incorporation of a surface tension reducing reagent obtains a surface tension of approximately 45 dynes per centimeter for this medium.

It may be reasonable to speculate that an equally low order of surface tension present in the saliva of the rats limits the types of microflora present.

Table 14. Surface tension* of pooled Hunt-Hoppert rat saliva at different stages of caries activity.

	Trial	Pre-caries level	Developmental level	Advanced caries	Caries appear in resistant
Resistant	1	43.1	43.6	43.1	43.7
	2	42.8	42.8	41.9	42.0
	3			42.1	44.3
	Avg.	42.9	43.2	42.3	43.3
Susceptible	1	42.6	42.5	45.2	46.3
	2	43.0	42.2	45.0	43.2
	3	42.2		44.8	46.0
	Avg.	42.5	42.3	45.0	45.1

*Measured in dynes per centimeter

Refractive Index

Careful searching of the literature on dental caries failed to reveal papers concerned with the refractive index of saliva. Since differences in the intrinsic concentration of saliva could be revealed, this procedure might prove of value in characterizing the saliva.

Procedure

For the determinations to be made an AO Spencer Refractometer (series 424) was employed. The scales of this instrument are calibrated directly in refractive index as measured with the "D" line of the sodium spectrum. The scale may be read directly to the third decimal place and the fourth may be estimated with an accuracy of $\pm$ 0.0002.

A drop of saliva was spread evenly on the ground surface of the auxiliary prism. The lever arm was moved until a dividing line was observed through the telescope. The refractive index $\left[N_D^{22} \right]$ was read directly from the Alidade scale.

Results

The information obtained from this procedure is presented in Table 15. The magnitude of difference among any of the groups, or between resistants and susceptibles of

any one group is negligible. The index of refraction obtained tends to indicate that the saliva contains a low concentration of dissolved solids. But it is sufficiently high to affect the saliva such that detection of differences can be accomplished with other procedures.

Although this study presents new information that can be applied to Hunt-Hoppert rat saliva, it does not appear to yield data, the nature of which can shed light on the caries problem.

Table 15. Refractive index of pooled Hunt-Hoppert rat saliva at different stages of caries activity.

	Trial	Pre-caries level	Developmental	Advanced caries	Caries appear in resistant
Resistant	1	1.3341	1.3339	1.3350	1.3358
	2	1.3340	1.3340	1.3340	1.3357
	3	1.3339	1.3340	1.3340	1.3357
	Avg.	1.3340	1.3340	1.3343	1.3354
Susceptible	1	1.3340	1.3343	1.3351	1.3360
	2	1.3339	1.3343	1.3347	1.3361
	3	1.3340	1.3341	1.3347	1.3359
	Avg.	1.3340	1.3342	1.3348	1.3360

Viscosity

Viscosity measurements of saliva, both animal and human are conspicuous by their absence from the dental literature.

Hewat (28), in one of the very few reports available, observed that of the children he studied those exhibiting caries had salivas with a relative viscosity of 1.23. Saliva from caries free mouths had a relative viscosity of 1.02. He believes that the more viscous saliva tends to bind particles of food (especially sugar) to the teeth.

Procedure

Viscosity measurements were carried out on the saliva of the Hunt-Hoppert rats in an attempt to ascertain the condition of difference between the two groups as a function of the internal triction of the liquid. The viscosity then, would reflect the resistance experienced by the molecules in moving around in the interior of the liquid due to inter-molecular forces.

One milliliter volumetric pipettes were softened in a Bunsen flame and drawn out to a length, such that distilled water, falling between two points, yielded dropping times of twenty seconds. Standard viscometers were not available for the small volumes required by this study.

After filtration through a Swinney apparatus, the

saliva was drawn into a pipette by suction bulb. Dropping time was measured with the aid of a sweep hand stop watch. All measurements are relative to distilled water with a dropping time of twenty seconds.

Results

The figures in Table 16 are most interesting. In every instance the susceptible groups exhibit a lower viscosity than the resistant group. Table 17 indicates that the relative viscosities are on the average 9.0 per cent higher for the resistant animals as compared with the susceptible animals. Considering the technique employed, the data presented are well beyond the limits of experimental error. This nine per cent difference in relative viscosity may be indicative of smaller, more mobile molecules in the interior of the susceptible saliva as compared with larger molecules in the resistant saliva. These smaller molecules may be the result of enzyme action on carbohydrates. If this is true, then it might be reasoned that the susceptible strain of animals would be the recipient of greater acid production in their mouths with subsequent caries activity. The mechanism of carbohydrate degradation with resultant acid formation is presented in the section on Amylase determinations.

The viscosity data suggested that amylase determinations be incorporated into the body of the investigation. The enzymatic destruction of large molecules to smaller fragments might reasonably yield the viscosity data presented in the tables.

Table 16. Dropping times* of pooled Hunt-Hoppert rat saliva at different stages of caries activity.

	Trial	Pre-caries level	Developmental level	Advanced caries	Caries appear in resistant
Resistants	1	24.02	24.12	22.60	25.50
	2	24.16	23.90	23.84	23.06
	3		23.43	23.72	23.52
	4		23.16		
	Avg.	24.14	23.65	23.38	24.02
Susceptibles	1	22.35	22.64	21.32	23.33
	2	22.24	22.72	21.30	21.40
	3		21.40	21.26	21.16
	4		21.60		
	Avg.	22.29	22.09	21.29	21.96

*Measured in seconds.

Table 17. Relative Viscosity of Hunt-Hoppert rat saliva.

	Resistant	Susceptible
Pre-caries level	1.20	1.11
Developmental period	1.18	1.10
Advanced caries	1.17	1.06
Caries appear in resistants	1.20	1.10

Specific Gravity

Specific gravity measurements are a convenient aid in characterizing liquids. They have been proven to be valuable in differentiating between similar compounds.

Specific gravity, d_t^t, which is defined as the mass, m, of a substance at t^o C. relative to the mass, m_o, of an equal volume of water at t^o C, is therefore a dimensionless number.

The most common method of specific gravity determination consists in finding the weight of liquid occupying a known volume defined by the shape of a given vessel. The vessel is calibrated in terms of the weight of pure water which it holds. Pycnometers are the usual vessels employed.

There is a paucity of information on determinations of specific gravity performed on salivas of persons, or animals, with and without caries. Hewat (28), who obtained saliva from children with and without dental caries, reported that specific gravity of saliva from the non-carious children was 0.9937. The figure presented for the saliva from carious mouths was 0.9918.

The incorporation of specific gravity measurements into this study was to further characterize the saliva obtained from the Hunt-Hoppert rats. As no specific gravity determinations have ever been performed on the saliva

of these animals, such a determination was needed and might indicate a factor of difference between the animals.

Procedure

Since only small volumes of saliva were available, a 1.0 ml Lipkin-type* pycnometer was employed. This is a bi-capillary vessel with one of the capillary arms bent at an angle of 140° so as to allow self-filling. The liquid is first drawn into the pycnometer by capillary action and the pycnometer then fills by siphoning. This hook is also convenient for hanging the pycnometer on the Ainsworth precision balance for the weighing operation. Four place accuracy can be obtained with this procedure.

After filtration through a Swinney apparatus, the filtered saliva was placed in a glass tube (1/2-inch tall by 1/2-inch external diameter) and the hook end of the pycnometer immersed below the surface of the saliva. The saliva was allowed to rise to any arbitrary level on the pycnometer scale. The filled vessel was then hung on a precision balance and weighed. After flushing and rinsing with distilled water the same procedure was followed using distilled water, except that the water was brought to the same level as the saliva. From the weights of equal volumes of saliva and water, relative specific gravity measurements were obtained.

*Available commercially from the H. S. Mastin Co., Evanston, Indiana.

Results

Table 18 lists the data obtained. It is generally seen that little or no difference in specific gravity was the rule.

The salivas of both animal groups exhibited no differences at the pre-caries level. This was also true at the developmental level. An average difference of 0.0053 units was obtained between the two strains at the advanced caries level. This represented a reduction in density from the developmental period. But this was not reflected in the older animals as would be anticipated since caries activity was present in both strains. As the specific gravity in this group was similar to that in the developmental period, the reduction obtained at the advanced caries level in the susceptible saliva appeared not to be a consequence of the carious condition.

Although the data collected in this study had never been obtained previously, it does not appear to delineate the problem of differences between the two strains of animals.

Table 18. Specific gravity of pooled Hunt-Hoppert rat saliva at different stages of caries activity.

	Trial	Pre-caries level	Developmental period	Advanced caries	Caries appear in resistant
Resistant	1	1.0008	1.0003	1.0010	1.0003
	2	1.0005	1.0004	1.0001	1.0004
	3		1.0003	1.0006	1.0006
	Avg.	1.0006	1.0005	1.0008	1.0006
Susceptibles	1	1.0008	1.0003	0.9958	1.0004
	2	1.0006	1.0003	0.9948	1.0003
	3		1.0002	0.9950	1.0004
	Avg.	1.0007	1.0002	0.9952	1.0003

DISCUSSION

Of the various tests employed in an attempt to reveal differences between the two strains of rats, several showed promise of being effective.

In the area of physical measurements only viscosity determinations offered significant differences. The resistant strain had a nine per cent higher viscosity which may be correlated with the higher amylase content present in the saliva of the susceptible animals.

Contemporary with these findings was the further observation that larger numbers of bacteria were recovered from the saliva of susceptible animals. Not only was the total bacterial population larger, but the acidogenic bacteria represented by the lactobacilli and streptococci, were also present in larger numbers.

The existence of three potentially interrelated conditions may offer an avenue for speculation regarding the initiation of caries in the susceptible strain of rats. Particles of the carbohydrate containing diets may probably lodge in the crevices and fissures of molar teeth. Contained in the saliva bathing these teeth are starch-hydrolyzing enzymes (amylases) that may aid in the degradation of the larger polysaccharide molecules to smaller disaccharide and monosaccharide sugars. This reduction of large molecules to smaller ones may be mirrored in the lower

viscosity of the saliva. The amylases may be either salivary or bacterial in origin, or a combination of the two. The large numbers of oral bacteria present could conceivably produce quantities of this enzyme such that the resulting hydrolysates, disaccharide and monosaccharide sugars, would be available for bacterial fermentation with subsequent production of a sustained acid action on the enamel of the teeth.

This synthesis, though in agreement with the currently popular theory of caries initiation, may in no way contribute to actual caries formation, but may be the result of the genetic differences between the two strains. Nevertheless, the observations suggest the direction future investigations should take. If the results of this study are further verified with larger numbers of animals the mechanism of caries development presented here will assume greater meaning.

It was mentioned earlier that dental caries is a unique disease. Because of the nature of the tissue involved, dental lesions do not lend themselves to strict definition of infection with its symptom complex. Particularly lacking is the condition of tissue repair and replacement. This study establishes the presence of leucocytes in the saliva of these rats. These cellular elements are present in a manner suggestive of a positive attraction. The numbers of leucocytes, particularly polymorphonuclears

and monocytes present in higher numbers at each stage of caries activity in the caries susceptible animals indicates some type of stimulative mechanism in operation. The large numbers present may be in response to the dental infection. The possibility that a chemical "trigger" liberated in the diseased area sets the mechanism in motion, is in accord with the vitalistic theory promulgated recently. On the other hand, the presence of these leucocytes may be in response to a chemotactic attraction toward the large numbers of bacteria present. The higher polymorph and monocyte count obtained with susceptible saliva as compared with the high lymphocytes of the resistant saliva suggests the possibility that phagocytosis is being attempted by the macrophage system.

The aspects of chemotaxis and phagocytosis could not be studied at this time.

SUMMARY

A screening study was made of the saliva and blood of the Hunt-Hoppert caries susceptible and resistant rats. Of the eight tests applied to these body fluids, only four showed promise of helping to differentiate between the two strains.

Viscosity measurements appear promising as a means of elucidating the caries problem. A nine per cent difference between the two strains was obtained. The susceptible animals had the lower viscosity. The other physical tests employed, viz., specific gravity, refractive index, and surface tension, showed no significant difference between the two strains of rats.

The numbers of lactic acid bacteria and "total" numbers of bacteria recovered from susceptible saliva was substantially greater at all levels of caries activity as compared with the numbers recovered from the resistant saliva.

The amylase content of caries susceptible rat saliva was consistently higher at all levels of caries activity than is the saliva of non-carious rats.

Salivary leucocytes were present in the saliva of both strains of Hunt-Hoppert rats. However, there was a marked difference in numbers and types of leucocytes present in the two strains of rats. The susceptible animals

had the greater numbers in their saliva.

Agglutination tests run on the saliva and blood sera on both strains of the Hunt-Hoppert rats showed no significant differences between the two strains.

The electrophoretic pattern of the serum proteins of these animals did not yield significant differences between the two strains.

Saliva collected from both strains of rats and tested for its antibacterial action against organisms normally present in the mouth of these rats showed no activity against these organisms.

CONCLUSION

The results of this work would indicate that the larger bacterial population, the lower viscosity measurements, the higher amylase content and the greater numbers of salivary leucocytes appear to offer a means of differentiating between the two strains of the Hunt-Hoppert rats. These differences may lead to greater knowledge of the mechanism of caries formation.

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