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The serological specificity of the lectin from *Lens culinaris*

by

Deborah Ward Heritage

Thesis

submitted in partial fulfillment of the requirements for the Degree of Master of Science in the Department of Genetics at the Medical College of Virginia

Health Sciences Division, Virginia Commonwealth University
Richmond, Virginia

August, 1973

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This thesis by Deborah Ward Heritage is accepted in its present form as satisfying the thesis requirement for the degree of Master of Science.

Date:

Aug. 22, 1973 ....

Advisor, Chairman of Graduate Committee

Aug. 22, 1973 ....

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Aug. 22, 1973 ....

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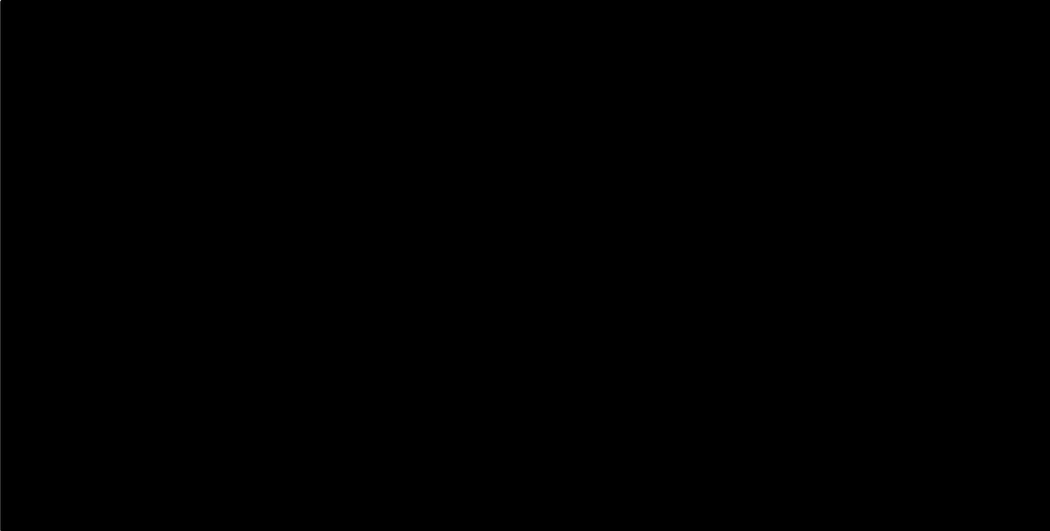
Approved: ....

Dean of the School of Basic Sciences and Graduate Studies



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INTRODUCTION

Lectins are blood antigen specific plant agglutinins which are also specific precipitins (Boyd, 1963). The lectin from the common lentil, _Lens culinaris_ (synonymous with _Lens esculenta_ and _Ervum Lens_) agglutinates human erythrocytes of all ABO types equally well (Mäkela, 1957). It has also been reported to agglutinate the erythrocytes of some horses, cows, sheep, goats, hogs and mice but not erythrocytes of geese, ducks and chickens (Mäkela, 1957; Vetter, 1965; Křupe and Ensgraber, 1966; Howard and Sage, 1969). Hemagglutination is thought to occur by lectin molecules binding with erythrocyte surface receptors. There are approximately 5.5×10^5 receptors for the lectin per erythrocyte (Kornfeld et al., 1971).

Sharon and Lis (1972) and Tomita et al. (1970) have reported the differential agglutination of mammalian tumor cells from Yoshida sarcoma in rats and Ehrlich ascites tumor and L1210 leukemia in mice by _L. culinaris_ lectin. Such agglutination mayprove important in understanding the nature of cellular changes in cancer.

L. culinaris lectin is capable of agglutinating and stimulating mitosis in cultured lymphocytes (Downing et al., 1968; Toyoshima et al., 1970; Young et al., 1971). Kornfeld et al. (1971) have estimated 2.4×10^5 surface receptors for _L. culinaris_ lectin per lymphocyte. This mitogenic property of some lectins has been extremely important for cytogenetic analysis of cultured peripheral lymphocytes and may become a valuable tool in determining the nature of lymphocyte transformation from a resting cell to a blast-like cell (Sharon and Lis, 1972).

The lectin molecule, stable to temperatures of 65° C, can be denatured by heating at 75° C for 30 minutes (Howard and Sage, 1969). L. culinaris lectin separates into two fractions during extended electrophoresis (Entlicher et al., 1969), and these two fractions have identical hemagglutination titers and precipitin reactions against antisera to the lectin (Howard et al., 1971). The lectin molecule has a molecular weight which has been estimated in the range of 42,000 to 69,000 (Sharon and Lis, 1972) and is composed of two identical subunits (Howard et al., 1971).

Each globular protein molecule of L. culinaris lectin contains 1.5% glucose and 0.05% glucosamine (Ticha et al., 1970; Toyoshima et al., 1970). Aspartic acid and threonine are the most abundant amino acids contained in the lectin; there is little or none of the sulfur-containing amino acids (Ticha et al., 1970; Toyoshima et al., 1970; Howard et al., 1971). Paulova et al. (1971) have reported the presence of 0.13% Mn^{2+}, 0.37% Ca^{2+}, and trace amounts of Mg^{2+} in the lectin molecule. The addition of Mn^{2+} to the lectin extracts promotes agglutinations titers and Ca^{2+} enhances the precipitin reaction (Paulova et al., 1971).

The specificity of the lectin for human antigens has not been explored extensively. It is known that the hemagglutination reaction of the lectin is inhibited by sugars of Group III of Mäkela (1957). All these sugars have hydroxyl groups on carbons 3 and 4 arranged sterically like those of D-glucose. Of these, D-mannose and N-acetyl-D-glucosamine are considered its determinant sugars (Kornfeld et al., 1971).

Previous experiments involving sugar inhibition of L. culinaris

lectin have been limited to the hemagglutination reaction (Mäkela, 1957; Entlicher et al., 1969; Kornfeld et al., 1971; Toyoshima et al., 1970) ; the coprecipitation reaction of the lectin with a phosphomannan and an enriched glycoprotein serum fraction monitored turbidimetrically; the stimulation of mitosis in cultured lymphocytes (Young et al., 1971) and the agglutination of mammalian tumor cells (Tomita et al., 1970).

Because their reactivity is inhibited by D-mannose, Entlicher et al. (1969) have concluded that the lectins of L. culinaris, Pisum sativum and Canavalia ensiformis belong to the same class. Lectins from L. culinaris and P. sativum, however, were found to migrate in different directions when electrophoresed with borate buffer (Entlicher et al., 1969).

Mäkela (1957) has reported the inhibition of L. culinaris lectin hemagglutination by saliva from ABH secretors and nonsecretors. No further research concerning the nature of the inhibitory saliva antigen has been published. At present, antigens of A, B, H, Le^a, Le^b, I and Sd^a blood group substances have been identified in saliva (Mollison, 1972). The allele (Se) for the secretion of A, B and H blood group substances in the saliva is dominant over its recessive allele (se); and they are inherited independently of the ABO blood group system (Race and Sanger, 1968). Secretor status is an influencing factor in the Lewis blood group system. A nonsecretor individual possessing a Le gene secretes Le^a substance in his saliva; and a secretor individual of the same genotype secretes Le^a and Le^b substance in his saliva. Individuals who possess only the le gene secrete no Lewis substance in their saliva (Mollison, 1972). Secretor

status has not been found to have an effect on either blood group I (Dzierzkowa-Borodej et al., 1970) or Sd^a (Macvie et al., 1967).

Using Ouchterlony immunodiffusion methods, Young et al. (1971) have reported precipitation of L. culinaris lectin with antisera to human α_2-macroglobulin, IgM, GC protein, α-glycoprotein and traces of IgA and IgG. Precipitation did not occur with antisera to transferrin, ceruloplasmin, hemopexin, α_1-acid-glycoprotein and α_1-lipoprotein. Electrophoresis of serum developed with L. culinaris lectin has been reported to produce a strong band in the α_1 region and two or more weaker bands in the β region. The lectin, when electrophoresed and developed with human serum, resulted in a band in the cathode region (Young et al., 1971).

The present project had the aim to characterize the components of serum and saliva in precipitin reactions with L. culinaris lectin. In addition, saliva inhibition of the lectin and causes of variation in saliva inhibition titers according to age, sex, race, secretor status, ABO blood type and quantitative genetic factors were investigated.

METHODS AND MATERIALS

Extracts

Dried *L. culinaris* seeds (purchased at a local grocery store) were ground in a blender and soaked overnight at 4° C in 5 volumes of saline (w:v). The suspension was cleared by centrifugation at 1000g for 15 minutes and stored frozen at -15° C. This crude extract, used in hemagglutination inhibition studies, was prepared in one large batch, diluted 1/2 with saline and frozen in small aliquots. These samples were thawed only once and diluted to 1/8 the original concentration with saline at the time of testing. For the precipitin reactions, concentrated extract was prepared by precipitating crude extract with saturated ammonium sulfate followed by dialysis against saline, according to the method of Grundbacher (1973). Concentrated seed extracts from *Ulex europaeus*, *Canavalia ensiformis*, *Pisum sativum* and *Lotus tetragonolobus* were provided by Dr. Grundbacher.

Samples

Unstimulated whole saliva samples for routine testing were boiled for 5 minutes, cooled and centrifuged at 1000g for 5 minutes. "Unboiled" saliva samples were heated for 30 minutes at 56° C, cooled and centrifuged at 1000 g for 5 minutes. Lyophilized saliva was fractionated by the method of Kabat (1956) to yield a phenol insoluble Fraction I. Further fractionation was carried out on the phenol supernatant to produce a phenol soluble, ethanol insoluble Fraction II (Napier, 1972). All saliva samples and fractions were stored frozen at -15° C. Precipitation of a human serum sample with increasing concentrations of ammonium sulfate (1.8 M, 2.0 M, 2.4 M, 2.8 M and 3.2 M) was carried out. Total protein determinations for saliva

and seed extracts were carried out using the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Immunodiffusion

Immunodiffusion slides were prepared from 1.5% Special Agar-Noble (Difco) and 0.05% sodium azide in 0.05% Veronal buffer, pH 7.3 (Kabat and Mayer, 1961). Slides were refrigerated at 4° C and checked at 24 hour intervals for the formation of precipitin bands. Immunodiffusion slides were photographed over indirect incandescent lighting.

Sugars used in tests for direct inhibition of precipitin reactions included D-mannose, D-glucose, N-acetyl-D-glucosamine and α-methyl D-mannoside (Sigma Chemical Company). One-half ml each of sugar solution and lectin extract were added to small tubes, shaken and incubated at room temperature for 1 hour to allow thesugar to bind to the lectin. This mixture was added to immunodiffusion wells for precipitin reactions against saliva and serum. Well sizes were 75 µl for saliva and 25 µl for lectin-sugar mixtures and serum; all wells were filled twice. Slides were observed after 24 hours for precipitin band formation. The anti-Le^a serum used in immunodiffusion tests with L. culinaris lectin was prepared in a rabbit. Antisera to IgG, IgM and IgA were provided by Dr. Grundbacher.

Electrophoresis and Immunoelectrophoresis

Electrophoresis and immunoelectrophoresis were carried out in 0.8% Ionagar (Colab) in 0.025 M Veronal buffer, pH 8.6 (Buchler Instruments, Inc.). Slides of about 1 mm thickness were prepared by pouring 2 ml agar onto 1 X 3 inch microscope slides and 3 ml onto 1-1/2 X 3 inch slides. Wells were cut and filled with the appropriate antigen or antibody. Runs were carried out at 110

v (15 v/cm and 4 ma/slide) for approximately 1-1/2 hours for saliva and serum, using a Buchler Electrophoresis Apparatus with 0.05 M Veronal buffer, pH 8.6, in the tanks. Albumin was stained with bromophenol blue in order to determine the extent of serum migration. Afterward, troughs were removed and filled with the appropriate antigen or antibody. Slides were incubated at 4° C for saliva and at room temperature for serum and checked at 24 hour intervals for precipitin bands. Antisera to human whole serum, α_1-glycoprotein and α-lipoprotein (Miles Laboratories) were used in immunoelectrophoresis.

Immunoelectrophoresis slides to be stained were washed at room temperature for 24 hours each in saline and distilled water to remove all unreacted material, dried under filter paper and stained with Sudan Black B (Allied Chemicals) for lipoprotein (Cawley, 1960) or phenhyene-diamine (Fisher Scientific Company) for ceruloplasmin (Uriel, 1964). Slides subjected to simple electrophoresis were fixed for 3 hours in 2% acetic acid in 50% ethanol and then dried under filter paper. Periodic acid with an equimolar mixture of p-phenylenediamine and α-naphthol (NADI reagent) were used for staining glycoprotein (Uriel, 1964).

Erythrocyte Agglutination

Direct hemagglutination titrations and hemagglutination inhibition titrations of boiled saliva and human serum with L. culinaris lectin were carried out in doubling dilutions according to modifications of the methods of Zmijewski (1968). Briefly, the hemagglutination technique consisted of incubating at room temperature 1 drop of lectin (approximately 0.05 ml) with 1 drop 2% erythrocyte suspension for 40 minutes, followed by centrifugation at 75 g for 60 seconds and reading macroscopically for agglutination. For hemagglutination

inhibition testing, 1 drop of lectin was incubated with 1 drop of saliva (or serum) for 10 minutes, followed by the addition of 1 drop 2% erythrocyte suspension, incubation for 40 minutes at room temperature, centrifugation and reading for agglutination. The lectin or saliva (or serum) dilution of the first tube showing hemagglutination of 2 or more on a scale of 0 to 4 was used to establish titer. Saliva inhibition was scored as follows: 0 for no inhibition with undiluted saliva, 1 for no inhibition with saliva diluted 1/2, 2 for no inhibition with saliva diluted 1/4, etc., for the entire 2-fold dilution series. Saline controls and one saliva sample from the same individual were included in each set of hemagglutination inhibition titrations.

Saliva samples from 52 Richmond area families previously described (Grundbacher, 1972) were utilized for genetic study. In order that experimental error would not obscure family variation, the samples were tested at random. All mothers except one were blood type O; 25 fathers were blood type O, 19 were blood type A and 8 were blood type B. Illegitimate individuals, determined by blood group analysis, were excluded from the population. Simultaneous hemagglutination inhibition tests using Ulex extract (0.8 mg/ml total protein), a lectin specific for H substance, and L. culinaris extract (1.5 mg/ml total protein) were carried out on all parents and children of blood type O families.

L. culinaris inhibition titers were determined from averages of 3 tests for parents and 2 tests for children, except in 8 cases in which saliva samples were too small. The Ulex inhibition titers were based on the average of 2 tests for parents and 1 test for children.

Computer analysis for the mean values and correlations of

the titer of the saliva antigen specific for _L. culinaris_ with H antigen titer, secretor status, age, sex, race and ABO blood type were performed on the entire population. Mean titers for H antigen were determined by secretor status, race and ABO blood type. Intrafamily means, correlations and regressions were determined for all _L. culinaris_ titers and for H antigen for secretors.

RESULTS

Immunodiffusion

Concentrated *L. culinaris* extract used for precipitin reactions in agar contained 42.9 mg/ml total protein. *L. culinaris* extract precipitated with an antigen present in both boiled saliva (0.22 mg/ml total protein) and "unboiled" saliva (0.51 mg/ml total protein) from the same individual, indicating that this reaction is not merely an artifact of boiling the saliva. Precipitin bands, however, were achieved more readily with boiled saliva.

Figure 1 presents precipitin patterns of saliva and serum with *L. culinaris* extract. Saliva from secretor and nonsecretor individuals formed a continuous precipitin band against *L. culinaris* extract, indicating that the lectin precipitates the same components of both salivas (Fig. 1a). This patterns shows that *L. culinaris* extract does not precipitate A, B or H saliva antigens since it formed bands against nonsecretor saliva. The antigen precipitated by *L. culinaris* is a component of saliva Fraction II (Fig. 1b), showing that the antigen is soluble in phenol. The extract formed precipitin bands with both nonsecretor saliva and antiserum to Le^a, indicating that saliva and serum contain antigens that react specifically with the extract (Fig. 1c). The band which formed between Le^a antiserum and nonsecretor saliva intercepted these bands in a pattern that could indicate partial identity or nonidentity of the Le^a, saliva and serum antigens. Because all three bands met in the same area of the slide, the pattern was difficult to interpret. Attempts to remove the precipitin band between *L. culinaris* extract and anti-Le^a serum were made by

incubating the antiserum with the extract. These resulted in an additional band between the anti-Le^a serum and saliva, presumably because of the presence of extract with the antiserum, before the band between the extract and the antiserum is removed.

Human saliva and serum also possess antigens which formed precipitin bands of identity with the lectin (Fig. 1d), indicating that they possess the identical antigenic sites. The precipitin band between saliva and lectin did not stain with Sudan Black B, but the band between lectin and serum stained slightly. This suggests that the saliva antigen is not lipoprotein and the serum antigen is lipoprotein. Some difficulty was encountered in washing all unreacted lipoprotein from the slide, evidenced by the staining of a ring around the well which contained serum.

Precipitation of a human serum sample with increasing concentrations of ammonium sulfate was carried out. A description of the serum protein components of each of the fractions can be found in Schultze and Heremann (1966). A band formed between the L. culinaris extract and the fractions from 2.0 M and 2.4 M ammonium sulfate precipitation. No precipitin band formed between the extract and the fraction collected by precipitation of serum with 1.8 M ammonium sulfate (Fig. 1e) which contains IgG, IgM, IgA and α_2-macroglobulin. Ouchterlony diffusion testing comfirmed the presence of the immunoglobulins in this fraction (Fig. 1f, g, h).

A comparison of the precipitin patterns of L. culinaris extract and other extracts is given in Figure 2. L. culinaris and Pisum sativum extracts formed precipitin bands of identity against saliva and serum antigens(Fig. 2a, b). L. culinaris and Canavalia

ensiformis extracts precipitated in a pattern of identity against saliva, indicating that they are specific for the same saliva antigen (Fig. 2c). In precipitin reactions against serum, however, L. culinaris extract formed only one band, and C. ensiformis formed 2 broader bands. Only the precipitin band closer to the serum well was continuous with the L. culinaris band. This suggests that L. culinaris is more selective in its precipitation of serum antigens than C. ensiformis, even though they appear to share specificity for one serum component, or that C. ensiformis may also contain more than one lectin.

L. culinaris extract and Lotus non-H lectin formed precipitin bands indicating partial identity against nonsecretor saliva (Fig. 2e). This implies that the extracts are specific for cross reacting antigens. The direction of the spur in the pattern indicates that L. culinaris extract contains a lectin not present in Lotus non-H lectin. The precipitin pattern of nonidentity for L. culinaris and Lotus extracts against blood group O secretor saliva is due to the precipitation of the Lotus non-H lectin and the precipitation of a second Lotus lectin with the H antigen of secretor saliva (Fig. 2f). This pattern shows that L. culinaris extract does not contain a lectin specific for H substance. The precipitin patterns of L. culinaris and Ulex extracts against saliva from a blood group O secretor and human serum showed a distinct spur made by the L. culinaris precipitin band (Fig 2g, h). This suggests that L. culinaris is not specific for the H antigen in saliva or serum. It is not apparent from these slides whether or not the Ulex precipitin band is partially identical or nonidentical with the

L. culinaris precipitin band because the *Ulex* band is characteristically so short.

D-mannose, D-glucose and N-acetyl-D-glucosamine were capable of inhibiting direct precipitin reactions between *L. culinaris* extract and saliva from secretor and nonsecretor individuals of blood type O. The levels of inhibition for single sugars and combinations of sugars tested over a range of 2-fold dilutions from 0.5 M to 0.0078 M are given in Table 1. The concentration of D-mannose inhibiting precipitin band formation with saliva from the nonsecretor individual was much higher than for the secretor individual. D-glucose and N-acetyl-D-glucosamine inhibited precipitin reactions of the extract against secretor and nonsecretor saliva at approximately the same concentrations. When combinations of 2 or 3 sugars were used with the extract, no additive or synergystic effects were present between D-mannose, D-glucose and N-acetyl-D-glucosamine for precipitin inhibition.

Both D-mannose and α-methyl-D-mannoside were capable of inhibiting the direct precipitin reaction between *L. culinaris* extract and human serum. The precipitin reaction with serum was inhibited with 0.75 M D-mannose but not with 0.5 M D-mannose. α-methyl-D-mannoside was a somewhat stronger inhibitor, preventing the precipitin reaction at a molarity of 0.5 but not at 0.25. Inhibition of the precipitin reaction between *L. culinaris* extract and serum was not achieved with 1.0 M D-glucose or 0.5 M N-acetyl-D-glucosamine. The results of saliva and serum inhibition studies indicate that sugars are present in the immunodeterminants of both of these antigens with which the extract precipitates.

Electrophoresis and Immunoelectrophoresis

Figure 3 gives the results of electrophoresis experiments using saliva, serum and *L. culinaris* extract. When whole saliva or saliva Fraction II was electrophoresed and developed with *L. culinaris* extract, a band formed in the cathode region (Fig. 3a). Simple electrophoresis of whole saliva stained by the periodic acid-NADI reaction produced both an anode and a cathode migrating glycoprotein fraction. Fraction II of saliva, after identical electrophoresis and staining, showed only a cathode migrating fraction (Fig. 3b). These results indicate that glycoprotein migrates in the region of the precipitin band formed when electrophoresed saliva or saliva Fraction II were developed with *L. culinaris* extract. Electrophoresis of *L. culinaris* extract developed with saliva produced a cathode migrating band (not shown). Similar results have been reported using serum with electrophoresed *L. culinaris* extract (Young et al., 1971).

Electrophoresis of whole serum developed with *L. culinaris* extract resulted in a strong band and two other smaller bands in the anode region (Fig. 3c). These bands were stained with Sudan Black B a lipoprotein specific stain (Fig. 3e). and did not stain with phenylene-diamine for ceruloplasmin. Electrophoresis of serum developed with antiserum to whole human serum from one side and the *L. culinaris* extract from the other side as suggested by Osserman (1960) was carried out in order to determine the component reacting with *L. culinaris*. This procedure indicated that *L. culinaris* extract forms precipitin bands with an antigen migrating in the α_2 region and the β region (Fig. 3d). Staining also indicated that these bands are lipoprotein

in nature (Fig. 3e).

Simultaneous development of electrophoresed serum with extract and anti-human-α-lipoprotein serum revealed that the extract precipitated with a lipoprotein migrating in the region of α_2-lipoprotein, but not with α_1-lipoprotein (Fig. 3f). Staining procedures indicated that the bands formed with the antiserum contained lipoprotein (Fig. 3g). Similar slides were prepared using anti-human-α_1-glycoprotein serum. These slides indicated that the precipitin bands between the lectin and electrophoresed serum do not migrate as far as the α_1 region. This photograph (Fig. 3h), in particular, shows clearly the 3 precipitin bands formed between L. culinaris lectin and electrophoresed serum.

Erythrocyte Agglutination

Erythrocytes from individuals of A, B, O and Bombay (provided by Ms. M. Gellerman, Philip Levine Laboratories of Ortho Research Foundation, Raritan, New Jersey) blood types gave similar direct agglutination titers with L. culinaris extract (Table 2). Table 3 gives the extract hemagglutination inhibition levels with serum from individuals of various ABO blood types. None of the serum was capable of completely inhibiting hemagglutination by the extract; however, the serum from the blood group A and B individuals studied exhibited inhibition titers higher than those from blood type AB or O individuals. The testing of a much larger population would be necessary to establish these results as a general trend.

Fluctuation of an individual's saliva inhibition titer for L. culinaris extract was determined by three kinds of tests. (a) Five

saliva samples taken from an individual throughout a single day revealed that the sample taken immediately after waking has a lower inhibition titer than those which persist through the rest of the day (Table 4). Similar results have been reported for daily changes in H antigen titer (Sturgeon et al., 1973) and in the level of the SC_1 saliva component (Niswander et al., 1964). (b) Saliva samples taken from 6 individuals at weekly intervals at the same time of day, over a period of 4 weeks, exhibited only minor changes in inhibition titer (Table 5). (c) Members of one family which were retested 3 years after initial saliva samples were taken showed no appreciable changes in inhibition titer over that period of time. These tests indicated that one saliva sample from an individual is sufficient to determine his inhibition titer for *L. culinaris* extract, if the sample is not taken immediately after waking.

The frequency distribution of the titers for the saliva antigen specific for *L. culinaris* lectin within the population studied is given in Figure 4. Such a distribution is suggestive of a quantitative mode of inheritance (Falconer, 1960). The range of inhibition titers was from 0 to 7.7. The mean titer for the population was 2.86 ± 0.08 corresponding to a saliva dilution of approximately 1/8 in the hemagglutination inhibition test.

The saliva inhibition titers for *L. culinaris* were not significantly correlated with age ($r = 0.008$, $P > 0.05$). The mean inhibition titers for males of 2.98 ± 0.12 and for females of 2.77 ± 0.10 were not significantly different. Table 6 gives the mean saliva inhibition titers for the different ABO blood groups. No significant differences were found between the mean titers for blood group A,

B or O individuals.

The mean saliva inhibition titer for ABH secretors was significantly higher than that for nonsecretors ($P < 0.001$) (Table 7). Among secretor individuals, a positive correlation of 0.178 ($P < 0.05$) was found between saliva inhibition titers for L. culinaris and Ulex. This is possibly the result of a general increase in most substances secreted in saliva along with the increased H substance.

Initially, Blacks exhibited a significantly higher mean titer for saliva inhibition than Whites. When the races were further divided by secretor status, it bacame evident that the higher mean titer in Blacks was the result of a higher frequency of nonsecretors in the Black population compared with the White population (Table 7). The Black population contained 36% nonsecretors and the White population contained 22% nonsecretors.

In order to minimize the effect of secretor status in intrafamily analyses of the saliva inhibition titers for L. culinaris, the difference between the mean titers of secretors and nonsecretors was added to the saliva inhibition titers of secretor individuals. Black secretor individuals' titers were increased by 0.78 and those of White secretor individuals were increased by 0.54.

Table 8 gives the mean saliva inhibition titers for family members in the population studied. Fathers showed higher mean titers than mothers or children and Blacks showed higher mean titers than Whites in all family classes, but these differences were not statistically significant ($P > 0.05$).

Regressions of children on parents and correlation coefficients between siblings were used to estimate the heritability values

(Falconer, 1960). These heritability estimates are given in Table 9. The difference in the heritability estimates derived from child-father and child-mother regressions may have been, in part, the result of the consistent O blood type among mothers which was not present in fathers. Even though no important difference in the inhibition titers was found for the ABO blood groups, this is a possible source of variation. The upper limit of heritability, determined by full sibling correlations, was 0.80. The child midparent regression gave a heritability estimate of 0.34, falling between that for child-mother and child-father estimates. This midparent determination is probably the most meaningful of all the determinations.

The mean saliva inhibition titers of *Ulex* for secretors and nonsecretors of blood group O according to race are given in Table 10. These mean titers for the Black and White populations did not differ significantly ($P > 0.05$). The Black population, however, exhibited a higher percentage of nonsecretors compared with the White population, 35% compared with 17%. *Ulex* inhibition titers were of the same magnitude in males and females (Table 11).

Table 12 gives the mean *Ulex* inhibition titers of saliva for secretors and nonsecretors of the different ABO blood types. The mean inhibition titer for blood type O secretors was higher than that for individuals of blood types A or B, as has been shown in other studies (Clarke et al., 1960).

Estimates of the heritability of *Ulex* inhibition titer in the saliva of blood type O secretors are presented in Table 13. These estimates ranged from 0.12 for child-mother regressions to 0.74 for child-father regressions. Such a wide variation in estimates

can probably be attributed to chance because of the low numbers of parents involved. Heritability estimates for child-midparent regressions of 0.33 and for full sibling correlations of 0.42 are in close agreement. These two latter determinations are probably the most reliable of the heritability estimates.

DISCUSSION

The foregoing results indicate that *L. culinaris* extracts contain a lectin specific for a glycoprotein antigen of saliva which has been detected previously but had not been characterized or quantitated. This lectin also precipitates with a serum glycolipoprotein.

The saliva antigen reacting with *L. culinaris* extract was not destroyed by boiling the saliva and was found to be a constituent of saliva Fraction II (soluble in phenol and water, insoluble in ethanol). D-mannose, D-glucose and N-acetyl-D-glucosamine were capable of inhibiting the direct precipitin reaction between *L. culinaris* lectin and saliva, indicating that the saliva antigen specific for the lectin contains some form of D-mannose and/or D-glucose as part of its immunodeterminant. D-mannose was the most potent inhibitor of the lectin, a characteristic probably related to its higher affinity for the lectin (Stein et al., 1971). The absence of an additive or synergystic effect between the sugars for lectin inhibition suggests that the sugars bind to different sites on the lectin molecule. These results of fractionation, heat stability and sugar inhibition experiments as well as staining with periodic acid-NADI reagent indicate that the saliva antigen specific for *L. culinaris* lectin is glycoprotein in nature.

Precipitin bands which formed between the lectin and nonsecretor saliva showed that the lectin is not specific for A, B or H blood group antigens. The possibility of the lectin being specific for Le^a antigen in saliva, because of similar effects of secretor status, was partially eliminated by Ouchterlony immunodiffusion testing. Although direct testing for blood group I (Dzierzkowa-Borodej et al., 1970) or

blood group Sd^a (Macvie et al., 1967) were not carried out, neither of these has ever been shown to be affected by secretor status. The SC_1 saliva component (Niswander et al., 1964) could be excluded since it migrated to the anode and the saliva antigen precipitated by *L. culinaris* migrated to the cathode on electrophoresis.

Genetic study of the saliva antigen indicated that it occurs over a wide range of titers from those undetected by this testing method to those detectable in saliva diluted 1:128. The Gaussian frequency distribution of inhibition titers supports the inheritance of saliva inhibition levels by quantitative genes. Based on child-midparent regressions, these saliva inhibition titers have a heritability estimate of 0.34. This indicated that about one-third of the total phenotypic variation in inhibition levels is accounted for by quantitative genetic variation. ABH secretors had a significantly lower mean inhibition titer than nonsecretors. This was further reflected by inhibition of the precipitin reaction between the lectin and secretor saliva with a lower concentration of D-mannose than between lectin and nonsecretor saliva. It appears that in the absence of H antigen in saliva, more of the saliva component inhibiting *L. culinaris* may be produced. Within the secretor individuals studied, as saliva inhibition titers for *Ulex* increased so did those for *L. culinaris*.

In all Ouchterlony immunodiffusion slides prepared, a single band formed between the lectin and serum, indicating the presence of a minimum of one antigen in serum with which the lectin precipitates. Electrophoresis of serum developed with the lectin produced one strong α migrating band and two additional bands in the β region. Both the immunodiffusion band and the immunoelectrophoresis bands stained

with Sudan Black B, showing their lipoprotein composition. The immunoelectrophoresis bands, however, stained much more readily than the immunodiffusion bands. Fractions collected from precipitation of serum with 2.0 M and 2.4 M ammonium sulfate, according to Schultze and Heremann (1966), are those containing α_2- and β-lipoproteins. L. culinaris lectin precipitated only with these two fractions. It was shown that the lectin is not specific for the immunoglobulins and probably not for α_2-macroglobulin as has previously been reported based on results of immunodiffusion testing with those specific antisera (Young et al., 1971). In Ouchterlony double diffusion studies attempted with goat antiserum to α_1-glycoprotein and α-lipoprotein, interfering bands of cross reactivity between the antiserum and the lectin, obscured the results. Monosaccharide inhibition of the direct precipitin reaction between the lectin and serum showed that D-mannose is present in the determinant of the serum antigen. Therefore, L. culinaris lectin precipitates with a serum glycolipoprotein, most likely the α_2 and β-lipoproteins.

Immunodiffusion testing indicated that P. sativum and C. ensiformis lectins precipitate the saliva component precipitated by L. culinaris lectin. The non-H lectin of Lotus precipitated a cross reacting saliva antigen, even though the Lotus lectin is inhibited by L-fucose instead of D-mannose (Napier, 1972). Precipitin reactions indicated that P. sativum and L. culinaris lectins are specific for the same serum antigen. C. ensiformis lectin precipitated a broader range of serum antigens, including that precipitated by L. culinaris. Unlike L. culinaris, Lotus lectin has not been found to precipitate with serum (Napier, 1972).

Family studies on blood type O secretor individuals indicated that *Ulex* inhibition titers in saliva have a heritability estimate of 0.33 based on child-midparent regressions. This suggests that about one-third of observed phenotypic variation is the result of quantitative genetic variation.

SUMMARY

Lens culinaris, the common lentil, contains a lectin which has been shown to be specific for a glycoprotein saliva antigen and a glycolipoprotein serum antigen. Both the saliva and serum precipitin reactions with the lectin are directly inhibited with saccharides, especially those related to D-mannose. Electrophoresis of the serum antigen showed that it migrates as three bands, while appearing as a single band in double diffusion precipitin patterns. Quantitative studies of the saliva antigen levels by hemagglutination inhibition titration indicated a polygenic, quantitative mode of inheritance with a minimum heritability of 0.34. Blood group ABH secretor individuals were found to have a significantly lower mean saliva antigen level than nonsecretor individuals.

The lectins from *Pisum sativum* and *Canavalia ensiformis* formed precipitin bands of identity with *L. culinaris* lectin against saliva. *C. ensiformis* and *L. culinaris* lectins exhibited precipitin bands of partial identity against serum; and *P. sativum* and *L. culinaris* lectins exhibited a pattern of identity against serum. In addition, precipitin patterns of partial identity with the non-H lectin from *Lotus tetragonolobus* has been demonstrated.

Using *Ulex europaeus* lectin in hemagglutination inhibition experiments with saliva from blood group O secretor individuals, a minimum heritability of approximately 0.40 for H antigen levels was found. A higher frequency of nonsecretor individuals was observed in the Black population compared with the White population.

Table 1: Maximum concentrations of simple sugars and combinations of simple sugars permitting precipitin band formation between *L. culinaris* extract and saliva from blood group O secretor and nonsecretor individuals.

Sugars	Secretor[1]	Nonsecretor[2]
Single sugars:		
D-mannose	0.015 M	0.25 M
D-glucose	0.25 M	0.5 M
N-acetyl-D-glucosamine	0.25 M	0.25 M
Combinations of two sugars:		
D-mannose D-glucose	0.015 M 0.25 M	0.25 M 0.5 M
D-mannose N-acetyl-D-glucosamine	0.015 M 0.25 M	0.25 M 0.25 M
D-glucose N-acetyl-D-glucosamine	0.25 M 0.25 M	0.5 M 0.25 M
Combination of three sugars:		
D-mannose D-glucose N-acetyl-D-glucosamine	0.015 M 0.25 M 0.25 M	0.25 M 0.5 M 0.25 M

[1]Total protein : 0.46 mg/ml
[2]Total protein : 0.97 mg/ml

Table 2: Titration of concentrated *L. culinaris* extract with 2% erythrocyte suspensions of different ABO blood types. Hemagglutination was read after 40 minutes incubation at room temperature followed by centrifugation.

ABO blood type	Lectin dilution						
and donor	1/10	1/20	1/40	1/80	1/160	1/320	1/640
O (GT)	4	4	4	4	4	1	1
O (FG)	4	4	4	4	4	2	0
O_h (Bombay)	4	4	4	4	4	2	1
A (DW)	4	4	4	4	4	2	1
A (CJ)	4	4	4	4	4	2	1
B (WC)	4	4	4	4	4	1	0

Table 3: Hemagglutination inhibition of *L. culinaris* extract by serum from individuals of different ABO blood types. Agglutination was read after 40 minutes incubation at room temperature followed by centrifugation.

ABO blood type	Serum dilution						
and donor	1	1/2	1/4	1/8	1/16	1/32	1/64
O (FG)	4	4	4	4	4	4	4
O (SL)	2	2	4	4	4	4	4
O (GT)	1	2	2	3	4	4	4
A (KR)	1	1	1	4	4	4	4
A (DW)	1	1	1	3	4	4	4
B (WC)	1	1	2	4	4	4	4
AB (SS)	2	4	4	4	4	4	4

Saline control: 4

Table 4: Hemagglutination inhibition of *L. culinaris* extract by saliva[1] from a blood group A nonsecretor individual at various times during a single day. Hemagglutination was read after 40 minutes incubation at room temperature followed by centrifugation.

Time of day	Saliva dilution				
	1/2	1/4	1/8	1/16	1/32
8:00 a.m.*	3	3	4	4	4
10:30 a.m.**	0	1	3	4	4
2:00 p.m.	0	1	3	4	4
4:30 p.m.	0	1	3	4	4
10:00 p.m.	0	1	3	4	4

Saline control: 4

[1] Saliva samples were boiled immediately after collection and stored frozen until the time of testing.
*Total protein: 0.33 mg/ml
** Total protein: 0.22 mg/ml

Table 5: Hemagglutination inhibition of L. culinaris extract by saliva from individuals of various ABO blood groups. Samples were taken at weekly intervals over a 4 week period. Hemagglutination was read after 40 minutes incubation at room temperature followed by centrifugation.

ABO blood type and donor	Saliva dilution 1	1/2	1/4	1/8	1/16	1/32
O (FG) #1		0	0	3	3	3
O (FG) #2		0	0	2	4	3
O (FG) #3		0	0	2	3	4
O (FG) #4		0	0	2	2	3
O (SL) #1		0	3	3	4	4
O (SL) #2		0	3	3	4	4
O (SL) #3		0	2	3	4	4
O (SL) #4		0	3	4	4	4
O (GT) #1		0	0	1	3	4
O (GT) #2		0	0	1	2	3
O (GT) #3		0	0	0	2	3
O (GT) #4		0	0	0	2	4
A (DW) #1		1	2	3	4	4
A (DW) #2		0	2	3	4	4
A (DW) #3		0	3	3	4	4
A (DW) #4		0	2	4	4	4
A (CJ) #1	1	3	4	4	4	
A (CJ) #2	0	3	4	4	4	
A (CJ) #3	1	3	4	4	4	
A (CJ) #4	0	2	3	4	4	
B (WC) #1		0	0	2	2	3
B (WC) #2		0	0	2	3	3
B (WC) #3		0	1	2	3	4
B (WC) #4		0	0	2	2	3

Table 6: Mean titers for hemagglutination inhibition of *L. culinaris* extract by saliva from individuals of different ABO blood types. The number of individuals in each population is given in parenthesis.

Blood type	Secretor	Nonsecretor	Combined
O	2.63 ± 0.09 (188)	3.24 ± 0.18 (71)	2.80 ± 0.09 (259)
A	2.94 ± 0.22 (40)	3.94 ± 0.31 (19)	3.25 ± 0.18 (58)
B	2.06 ± 0.28 (14)	3.36 ± 0.38 (12)	2.66 ± 0.23 (26)

Table 7: Mean saliva inhibition titers for *L. culinaris* extract, according to race and secretor status. The number of individuals in each population is given in parenthesis.

	Secretor	Nonsecretor	Difference
Whites	2.55 ± 0.11 (199)	3.09 ± 0.28 (33)	0.54
Blacks	2.74 ± 0.12 (123)	3.52 ± 0.17 (68)	0.78**
Races combined	2.65 ± 0.08 (242)	3.38 ± 0.15 (101)	0.73**

** $P<0.001$ for difference between secretors and nonsecretors

Table 8: Mean saliva inhibition titers of L. *culinaris* extract for different family members. Data have been corrected for secretor status. The number of individuals in each population is given in parenthesis.

	Blacks	Whites	Combined
Fathers	4.03 ± 0.13 (27)	3.39 ± 0.15 (25)	3.75 ± 0.10 (52)
Mothers	3.02 ± 0.10 (27)	2.99 ± 0.12 (25)	3.10 ± 0.07 (52)
Midparent	3.52 ± 0.07 (27)	3.19 ± 0.11 (25)	3.38 ± 0.06 (52)
Children	3.49 ± 0.09 (137)	3.06 ± 0.11 (102)	3.31 ± 0.13 (239)

Table 9: Heritability estimates for levels of saliva inhibition of L. culinaris extract determined from 52 families, 27 Black and 25 White.

Subject	Race			Combined
	Blacks	Whites	Combined	
	Regression (b) or correlation coefficient (r)			Heritability
Child-father	b = -0.09 ± 0.07	b = 0.31 ± 0.08**	b = 0.10 ± 0.05	0.20
Child-mother	b = 0.31 ± 0.10*	b = 0.38 ± 0.11**	b = 0.34 ± 0.07**	0.68
Child-midparent	b = 0.11 ± 0.13	b = 0.51 ± 0.11**	b = 0.34 ± 0.08**	0.34
Sibling-sibling	r = 0.34**	r = 0.48**	r = 0.40**	0.80

* $P<0.01$ for difference from zero
** $P<0.001$ for difference from zero

Table 10: Mean saliva inhibition titers of *Ulex* extract, according to race and secretor status. Numbers in parenthesis indicate population size.

Race	Secretor status	
	Secretor	Nonsecretor
Black	7.32 ± 0.12 (79)	1.19 ± 0.10 (42)
White	7.28 ± 0.10 (83)	1.18 ± 0.13 (17)
Combined	7.25 ± 0.08 (162)	1.19 ± 0.08 (59)

Table 11: Mean saliva inhibition titers of *Ulex* extract, according to sex and secretor status. Numbers in parenthesis indicate the size of each population.

Sex	Secretor status	
	Secretor	Nonsecretor
Male	7.30 ± 0.11 (83)	1.30 ± 0.16 (28)
Female	7.19 ± 0.11 (79)	1.08 ± 0.05 (31)

Table 12: Mean saliva inhibition titers of <u>Ulex</u> extract, according to ABO blood type and secretor status. The number of individuals in each population is given in parenthesis.

Blood type	Secretor	
	Secretor	Nonsecretor
O	7.33 ± 0.08 (144)	1.14 ± 0.05 (51)
A	6.85 ± 0.34 (13)	2.00 ± 1.00 (4)
B	6.00 ± 0.63 (5)	1.00 ± 0 (4)

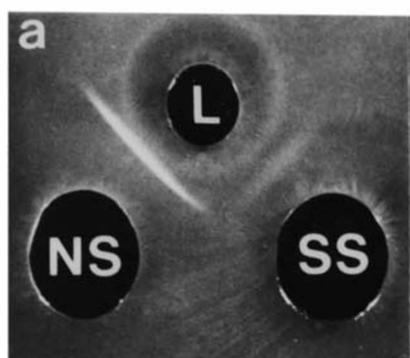
Table 13: Heritability estimates of inhibition titers for *Ulex* extract by saliva from blood group O secretor individuals. Numbers in parenthesis indicate the size of each population.

Subject	Race			
	Blacks	Whites	Combined	Combined
	Regression (b) or correlation coefficient (r)			Heritability
Child-father	b = 0.35 ± 0.13** (34)	b = 0.45 ± 0.20* (43)	b = 0.37 ± 0.11** (77)	0.74
Child-mother	b = 0.22 ± 0.18 (35)	b = 0.15 ± 0.12 (41)	b = 0.06 ± 0.09 (76)	0.12
Child-midparent	b = 0.50 ± 0.21* (24)	b = 0.36 ± 0.19 (40)	b = 0.33 ± 0.14* (64)	0.33
Sibling-sibling	r = 0.22* (86)	r = 0.20 (82)	r = 0.21** (168)	0.42

* $P<0.05$ for difference from zero
** $P<0.01$ for difference from zero

Figure 1: Immunodiffusion precipitation patterns of *L. culinaris* extract with whole saliva and serum.

L:	*L. culinaris* extract (concentrated)
NS:	Nonsecretor whole saliva
SS:	Secretor whole saliva
S:	Serum
Le^a:	Anti-Le^a serum, prepared in rabbit
I:	Saliva Fraction I
II:	Saliva Fraction II
Sf:	Serum fraction from precipitation with 1.8 M ammonium sulfate
A:	Anti-IgA serum
G:	Anti-IgG serum
M:	Anti-IgM serum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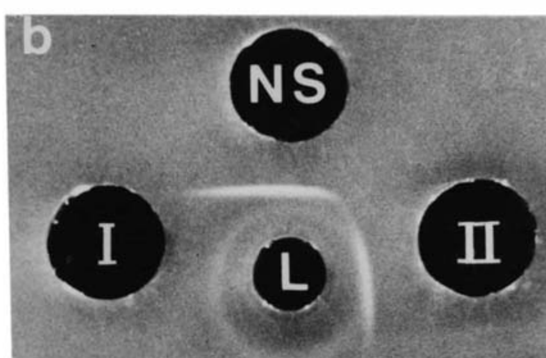
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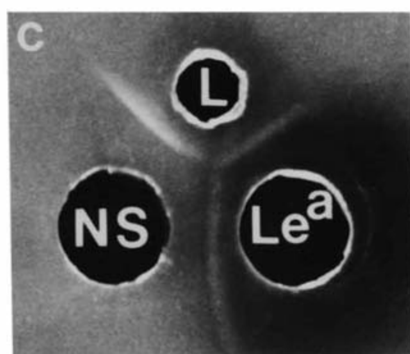
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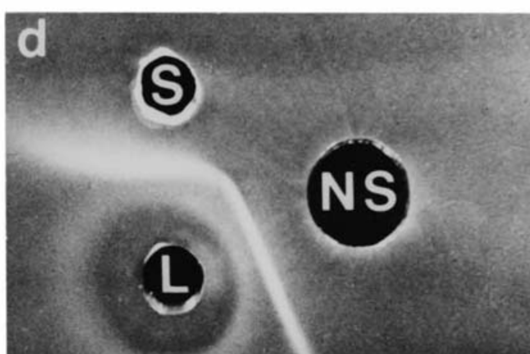
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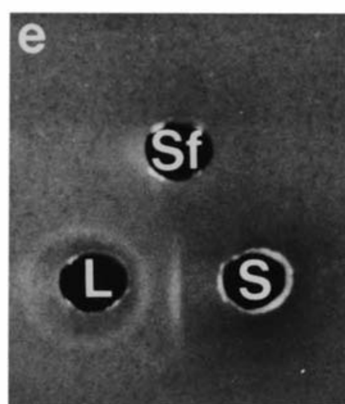
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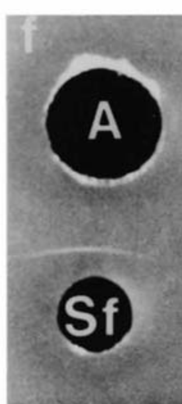
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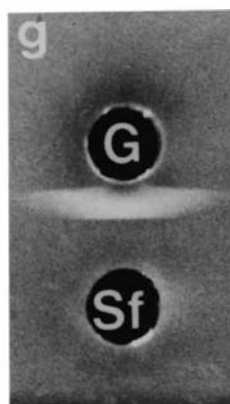
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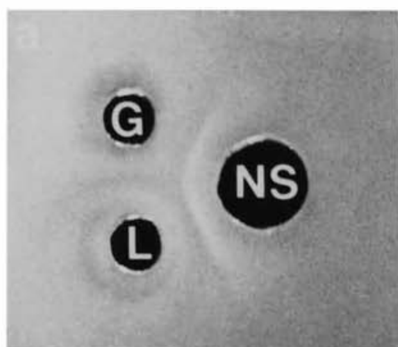
Figure 2: Immunodiffusion precipitation patterns of *L. culinaris* extract and other seed extracts with human saliva and serum.

L: *L. culinaris* extract (concentrated)
P: *P. sativum* extract
C: *C. ensiformis* extract
Lt: *Lotus* extract
U: *Ulex* extract
NS: Nonsecretor whole saliva
SS: Secretor whole saliva
S: Serum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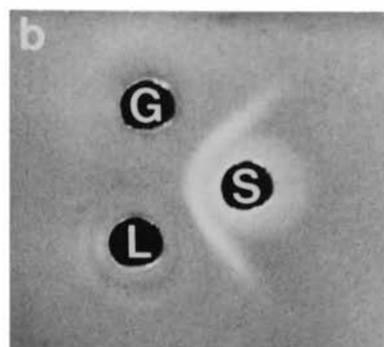
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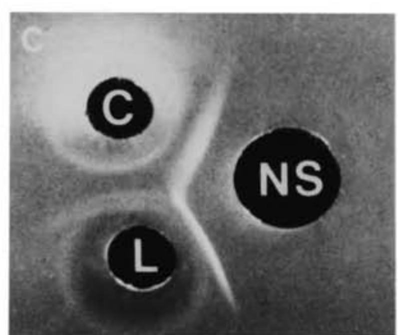
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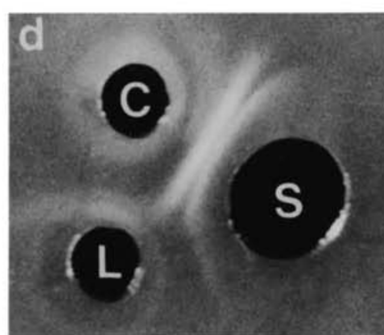
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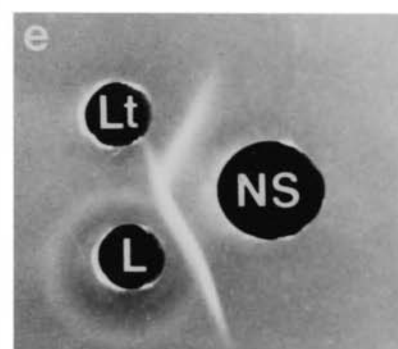
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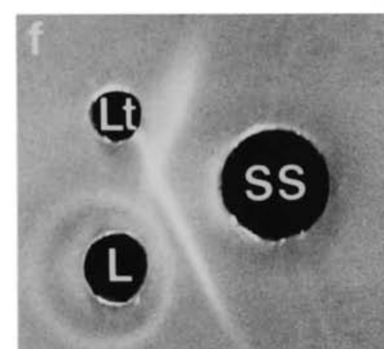
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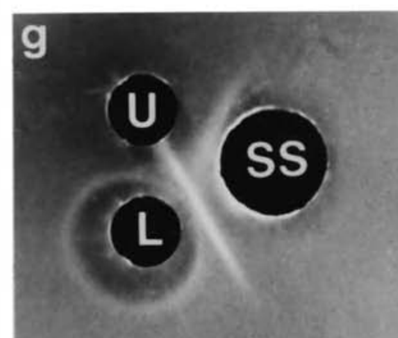
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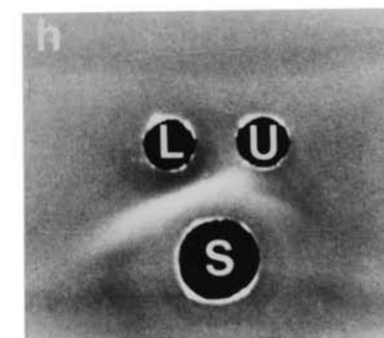
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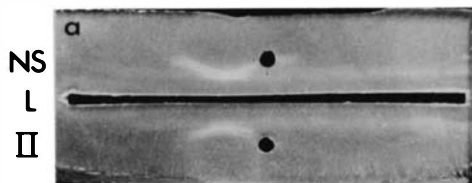
Figure 3: Immunoelectrophoresis patterns of L. culinaris extract with human saliva and serum. The anode is to the right.

L: L. culinaris extract
NS: Nonsecretor whole saliva
II: Saliva Fraction II
S: Serum
A-S: Anti-whole human serum, prepared in goat
A-G: Anti-α_1-glycoprotein serum, prepared in goat
A-L: Anti-α-lipoprotein serum, prepared in goat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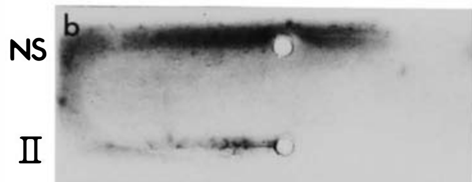
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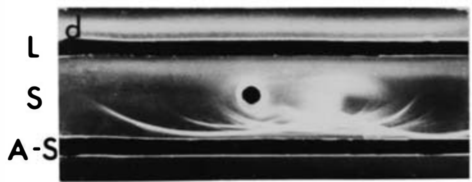
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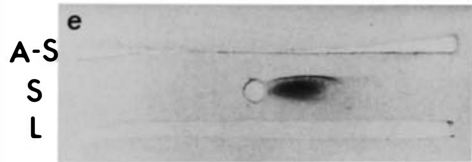
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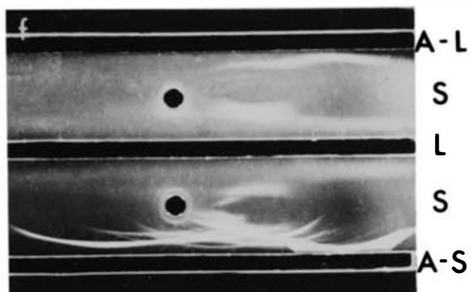
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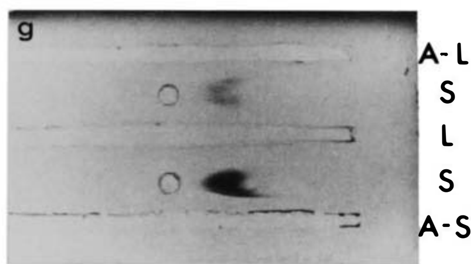
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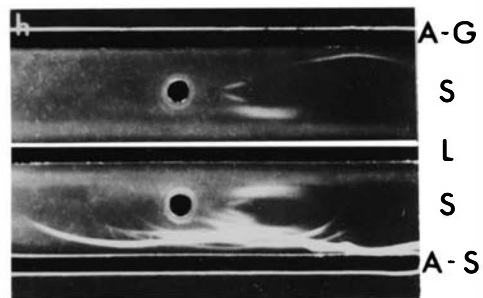
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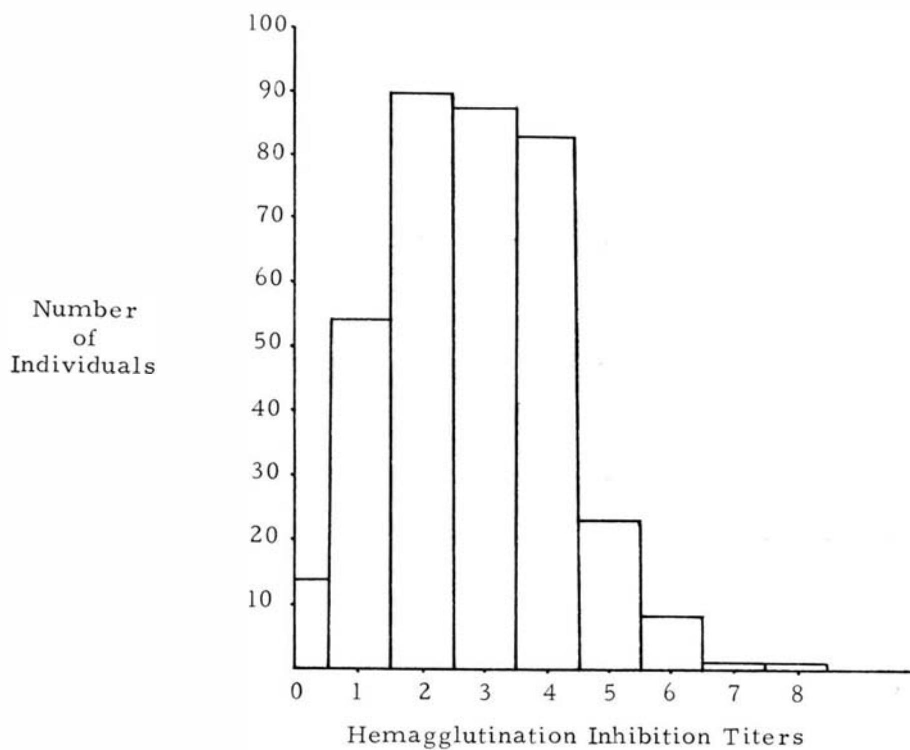

Figure 4: The frequency distribution of titers for saliva inhibition of *L. culinaris* extract in a population of 343 individuals. For an explanation of the method of scoring titers, see text.

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