Lectins in Precipitin Reactions with Soluble H Substance of Human Saliva and Serum

Abstract. *Lectins that precipitate the soluble H substances of saliva were produced for the first time from the four commonly used sources of seed. In Ouchterlony precipitin reactions, identity was observed between extracts of* Cytisus sessilifolius *and* Laburnum alpinum, *but these extracts displayed incomplete identity with extracts from* Ulex europaeus. *These three sources of the precipitating lectin formed bands when tested with saliva from all secretors and none with saliva from nonsecretors. Extracts from* Lotus tetragonolobus, *however, formed two bands with saliva from secretors and one strong band with all saliva from nonsecretors. This new antigen, which reacts with* Lotus, *is present in all saliva and on red cells of all normal individuals, but it is absent in the* O_h *(Bombay) type. The lectins formed bands with serums of secretor and nonsecretor individuals, but these bands were weaker than those with saliva, and revealed incomplete identity between H substance of serum and saliva. The precipitin reaction of the lectins is an efficient method for demonstrating relations among lectins and among antigens and has led to the discovery of a new human antigen.*

In testing for the presence of H substance in saliva, lectins that react with H substance (anti-H lectins) have been widely used in the determination of secretor status. The hemagglutination inhibition test, although it has provided useful information on lectins and soluble substances, lacks the precision of precipitin reactions for establishing the degree of identity between lectins or between antigens. No precipitating anti-H extracts from seeds have to my knowledge been prepared previously. With the aim of developing a direct test for determination of secretor status, seed extracts which were strong enough to form visible bands in Ouchterlony precipitin reactions against soluble H substance were prepared. Methods of preparation of the precipitating lectins are described in this report, together with some results on precipitin reactions of the commonly used lectins from *Ulex europaeus, Laburnum alpinum, Cytisus sessilifolius,* and *Lotus tetragonolobus* [synonym *Tetragonolobus purpureus* (*1*)].

Ulex and *Laburnum* seed was obtained from Herbst Brothers, Brewster, N.Y.; *Cytisus* from H. E. Saier, Dimondale, Mich.; and *Lotus* from Thompson and Morgan, Ipswich, England.

The extracts were prepared as follows. The seed was finely ground in a blender, suspended in 5 parts of saline to 1 part (by weight) of seed, extracted for 24 hours at 4°C with repeated stirring, followed by centrifugation for 20 minutes at 3000*g* in a refrigerated centrifuge. The supernatant was heated for 10 minutes at 56°C, cooled in tap water, and centrifuged for 20 minutes at 3000*g*; the supernatant was then decanted. Two parts of saturated ammonium sulfate solution were slowly added with stirring to 1 part (by volume) of supernatant, followed by centrifugation for 20 minutes at 3000*g* in the refrigerated centrifuge. The ammonium sulfate precipitate was suspended in saline and centrifuged; the resulting supernatant was precipitated with ammonium sulfate as before, and centrifuged. The solid precipitate was transferred to a dialysis bag and dialyzed until all solid material had dissolved. The heating at 56°C, mentioned above, had the effect of precipitating large amounts of nonspecific proteins, thus permitting a higher concentration of the specific anti-H lectins. Also the strong nonspecific rings formed in the agar around the wells of unheated extracts were eliminated in most samples by heating. However, reactivity of the lectins was not noticeably reduced in the heating process.

Extracts strong enough to form visible precipitin bands against saliva were prepared from *Laburnum* seeds and from one lot of *Ulex* seeds. From another lot of *Ulex* seed, however, the extract after dialysis had to be heated a second time for 10 minutes at 56°C in order to obtain a solution that did not form a cloudy precipitate around the lectin wells in agar. *Lotus* extracts always had to be heated twice and the *Cytisus* extract had to be adjusted to *p*H 7.0 with sodium hydroxide after the addition of ammonium sulfate in order to obtain a usable precipitating lectin. The extracts were stored at − 50°C until used.

Salivas were collected and boiled (*2*). Agar gels were prepared with barbital buffer at *p*H 7.3 (*3*), 0.05 percent sodium azide, and 1.5 percent special agar-Noble (Difco). Wells of 6 mm for saliva and serum and 3.5 mm for lectins were punched and filled as evenly as possible. After the wells were filled, the agar plates were kept open at room temperature until all fluid was diffused, and then they were incubated in a moist box at 4°C. The plates were checked daily for band formation; the first bands had developed after 18 to 24 hours. The gels shown in Fig. 1 had been incubated for 4 to 7 days.

The patterns of Fig. 1a show the precipitin bands obtained with the four anti-H lectins and saliva from a secretor (left) and a nonsecretor (right), both of blood group O. All lectins formed a single precipitin band against the saliva from the secretor with the exception of the extract from *Lotus* which formed two bands. Only the weaker band shows continuity with the bands against *Ulex* and *Cytisus*, suggesting that only this band resulted from a reaction against the H antigen of saliva. After incubation for several weeks, the weaker band intersected the band against *Cytisus* but not that against *Ulex*, indicating that the anti-H lectin of *Lotus* is identical to one in *Ulex*, but that the anti-H specificities of *Lotus* and *Cytisus* are different.

Precipitin bands against *Cytisus* and against *Laburnum* extracts show continuity, indicating identity of the anti-H from the two species of plant. However, the band against *Ulex* extract forms a spur with *Laburnum* and *Cytisus* extracts, revealing incomplete identity between these lectins. The direactions of the spurs show that *Ulex* extract contains a type of anti-H lectin which is not present in *Laburnum* and *Cytisus* extracts, but that the lectin present in the *Laburnum* and *Cytisus* is also present in *Ulex*. These observations are supported by reports on sugar inhibitions revealing that the lectin from *Lotus* is most strongly inhibited by L-fucose, the lectins from *Cytisus* and *Laburnum* are inhibited by chitobiose, and *Ulex* extract contains both types of anti-H lectins (*4*).

None of the seed extracts, except that from *Lotus tetragonolobus*, developed a precipitin band with saliva from the nonsecretor person. The *Lotus* extract formed a strong precipitin band with saliva from all nonsecretor individuals tested thus far. Red blood cell samples from various persons absorbed both lectins from the *Lotus* extract, an indication that red cells have a specificity at least cross-reactive with the mate-

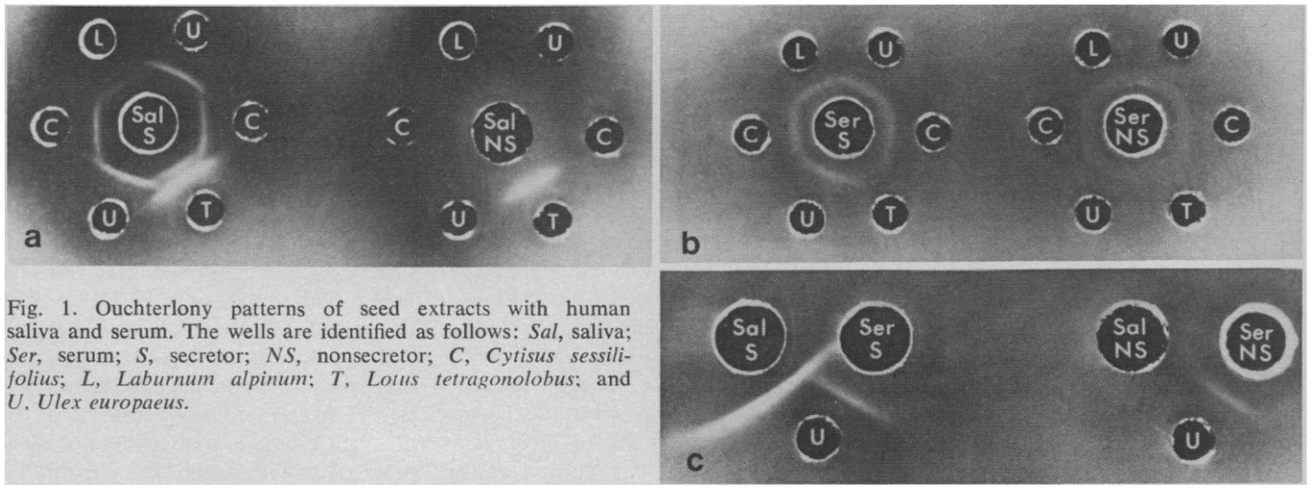

Fig. 1. Ouchterlony patterns of seed extracts with human saliva and serum. The wells are identified as follows: *Sal*, saliva; *Ser*, serum; *S*, secretor; *NS*, nonsecretor; *C*, *Cytisus sessilifolius*; *L*, *Laburnum alpinum*; *T*, *Lotus tetragonolobus*; and *U*, *Ulex europaeus*.

rial found in salivas. The unknown antigen may thus be present in all salivas and red cells of normal individuals, but it is absent in saliva and red cells of the Bombay type (*5*). The Bombay or O_h type of person is very rare, but studies of O_h have contributed much to the understanding of the synthesis of the A and B antigens (*6*). The O_h individuals lack the H gene, are unable to synthesize the H antigen, and, consequently, are unable to synthesize the A or B antigens, even though the genes for the A or B antigen are inferred to be present. The fact that this new antigen is absent in the Bombay type reveals that synthesis of this antigen is also under control of the H gene. It has been proposed (*7*) that this new antigen be called the L antigen because the extract from *Lotus* made its detection possible. This antigen showed nonidentity with the other known antigens; however, sugar inhibition studies revealed that it is closely related to the H and Le^a antigens (*7*).

Figure 1b shows the precipitin reactions of anti-H lectins with serum. The serums were from the same individuals as the saliva. Weak precipitin bands were formed with the serums of the secretors and the nonsecretors. The bands were continuous from one seed extract to the next, revealing identity of H antigen in the serum reacting with the anti-H lectins. Thus, the anti-H lectin from the different plants displays differences against the soluble substance of saliva, but not against the soluble substance of serum. The unusual band formed by *Lotus* extract with saliva of nonsecretors is absent with serum, and the band formed by *Lotus* extract with serum is very weak.

In Fig. 1c the saliva and serum of the same person were placed side by side against *Ulex* extract. Saliva from the secretor formed a strong band with *Ulex* extract, while serum formed a relatively weak band. The band with saliva does not terminate at the junction with the serum band, revealing only partial identity of the H substance of saliva and serum. The saliva thus seems to contain a class of H substance not present in the serum. The pattern on the right shows a weak band with serum but no band with saliva. All serums from blood group O nonsecretor individuals tested thus far developed a band against *Ulex* extract.

The foregoing results have demonstrated for the first time that anti-H lectins can form precipitin bands against soluble H substance. Figure 1 provides critical information on the similarities and differences among the anti-H lectins. An essential step in the preparation of the precipitating lectins was the heating by which large amounts of nonspecific proteins were removed, thus permitting a much higher concentration of the specific anti-H lectins.

The results have shown identity of the anti-H lectins from *Cytisus sessilifolius* and *Laburnum alpinum* but incomplete identity between the lectins from *Ulex* and those from *Cytisus* and *Laburnum*. These findings are in agreement with results from sugar inhibition tests (*4*, *8*) and saliva inhibition tests (*9*), and thus furnish direct evidence for some conclusions previously reached indirectly by inhibition studies.

The finding of strong reactivity other than anti-H in extracts from *Lotus tetragonolobus* may explain previous inconsistent results when extracts from these seeds were used. For example, McNeil *et al.* (*10*) reported the finding of individuals of blood group A or B who secrete H, but not A or B. Race and Sanger (*11*), using *Ulex* extracts as anti-H, have not found such an individual.

The precipitin reactions have demonstrated only partial identity between the H antigen of saliva and serum, and that H substance in saliva of secretors may be considerably more concentrated than in serum. These findings support the view that the H substance secreted in the saliva is synthesized in the salivary glands and is not merely a filtrate from the blood. Furthermore, the presence in the serum of nonsecretors of a material that reacts with the anti-H lectins indicates that the H specificity, like the A and B specificities, can be borne by molecules whose release does not require the presence of the secretor allele.

The precipitin test with extract from *Ulex* was applied in a study of a population consisting of more than 300 persons of blood group O whose secretor status had previously been established by the conventional hemagglutination inhibition test. The results of the two tests agreed, except for four persons who had been typed as secretors but whose saliva formed no bands. Retesting with both methods revealed that all four persons were nonsecretors who had been tested the same day in the hemagglutination inhibition test and for unknown reasons were not detected. The fact that all secretor saliva formed precipitin bands against *Ulex* provides strong evidence that the band formed is against the H antigen and not against the Le^b antigen, because about 10 percent of the secretor individuals (more than 24 in the above population) are expected to lack Le^b in their saliva.

Absorption and neutralization studies revealed that the precipitating lectin is absorbed by red cells of blood group O both from secretor and nonsecretor

individuals, but not by cells of the Bombay type, and saliva from secretors but not from nonsecretors neutralized the precipitating lectin. Furthermore, seed extracts showed a high correlation between hemagglutination titer against red cells of blood group O and the ability to form precipitin bands against saliva of secretors. The results thus leave little doubt that precipitin bands shown between seed extracts and saliva of secretors result from a precipitin reaction between the anti-H lectin and H substance, except for the extract from *Lotus* which was found to contain a lectin other than anti-H in addition to anti-H.

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Chlorinated Urban Water: A Cause of Dialysis-Induced Hemolytic Anemia

Abstract. *Unexplained acute hemolytic anemia is sometimes seen in uremic patients undergoing hemodialysis. Chloramines, which are oxidant compounds made up of chlorine and ammonia and are widely used as bactericidal agents in urban water supplies, have been found responsible for two recent epidemics, in dialyzed uremic patients, of acute hemolytic anemia characterized by Heinz bodies. Chloramines produce denaturation of hemoglobin, both by their direct oxidizing capacity and their ability to inhibit red cell reductive (hexose monophosphate shunt) metabolism.*

Hemolytic anemia is frequently a serious problem among patients undergoing long-term hemodialysis. A few incidents of hemolytic anemia among dialyzed patients have been caused by contaminants such as copper (*1*) and nitrates (*2*) in the dialyzing fluid. We have investigated two renal dialysis facilities where patients manifested obvious hemolytic anemia. The patients' red cells contained large numbers of Heinz bodies, which usually consist of oxidatively denatured hemoglobin (*3*); these inclusions are associated with a decreased survival of red cells (*3, 4*). Reflecting the oxidative denaturation, these patients' red cells also showed increased concentrations of methemoglobin, especially during and immediately after dialysis. Both dialysis units had recently adopted the reverse osmosis (RO) technique of purifying dialysis water. In the reverse osmosis technique a semipermeable membrane is used through which water is sieved at high pressure. This technique removes particulate matter and trace metals such as copper.

The oxidant responsible for this hemolytic anemia was identified primarily through in vitro studies in which small volumes of red cells were exposed to large volumes of test waters made isotonic with NaCl. Methemoglobin concentration and red cell hexose monophosphate shunt (HMPS) metabolism (measured by the conversion of ^{14}C-labeled glucose to $^{14}CO_2$) were assayed by known techniques (*5, 6*). Chlorine and chloramine were assayed by the *o*-tolidine method (*7*) which detects total chlorine (that is, OCl^-, HOCl, NH_2Cl, $NHCl_2$, and NCl_3). Reference values for the *o*-tolidine test were obtained by titration of standard solutions of calcium or sodium hypochlorite with iodine and thiosulfate (*8*). In most of the experiments, chlorinated water was prepared by adding previously assayed sodium hypochlorite alone or with ammonium hydroxide (to form chloramines) in a 1 : 2 molar ratio to distilled water containing 0.15*M* NaCl (*9*). During hemodialysis, relatively small volumes of blood are exposed to large volumes of dialyzing solution. Therefore, in most experiments, 0.5 ml of packed, saline-washed red cells were added to 50 ml of test water made isotonic through the addition of dry NaCl.

Hemodialyzed patients from three University of Minnesota hospitals were studied. Significant methemoglobinemia (> 5 percent) and Heinz body inclusions in red cells were regularly observed in patients in two hospitals, but not in a third. Unpurified tap water and RO water were utilized for dialysis baths in the former two, whereas charcoal-filtered water was used in the third. Analogously, red cells briefly incubated in Minneapolis tap water before or after

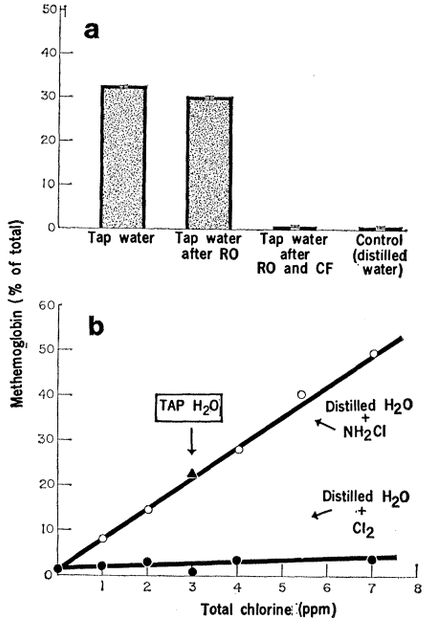

Fig. 1. (a) Production of methemoglobin in washed red cells by water treated in various ways. One volume of red cells was incubated for 15 minutes at 37°C with 100 volumes of isotonic NaCl solution made from the indicated types of water. Red cells were subsequently packed and assayed for methemoglobin. Means ± 1 standard deviation are shown in the center of each bar. (b) Effects of chlorination and chloramination on methemoglobin production in washed red cells. Note the lack of effect of chlorine (added as sodium hypochlorite) relative to that of chloramines. Chloramines were made by mixing molar concentrations of sodium hypochlorite and ammonium hydroxide (1 : 2). The major product of this mixture (at our incubation *p*H of about 7.6) is assumed to be in the form of NH_2Cl. Methemoglobin accumulation in red cells incubated in Minneapolis tap water is depicted by the black triangle and coincides with the value obtained with chloraminated water of the same total chlorine content. Conditions of incubation are the same as in (a).