Crepitin, a Phytohemagglutinin from *Hura crepitans*

The toxic properties of the sap of the 'jabillo' tree *Hura crepitans* were described in 1825 by BOUSSINGAULT and RIVERO (for the older literature see [1]). In 1909, RICHET[2] obtained a precipitate showing antigenic properties by fractionating the crude sap with alcohol. The active precipitate agglutinated red blood cells. He proposed the name crepitin for this fraction and considered it to be a toxic phytohemagglutinin similar to ricin from castor beans. No reports have been published on this subject since RICHET's papers.

Recently, phytohemagglutinins or lectins have found considerable interest because of their multiple biological activities[3]. Their existence in some edible legumes have been related to the toxic properties exhibited by the corresponding seeds, unless inactivated by heat. In the following, some observations on chemical and biological properties of crepitin will be reported, which permit certain comparisons to be made with the more important legume lectins.

Materials and method. The sap of the tree, *Hura crepitans*, was obtained by collecting the outflow from incisions in the bark. It was dialyzed for 48 h at 4 °C against distilled water, centrifuged, brought to pH 4.5 by the addition of acetate buffer and fractionated at 4 °C with acetone. The fraction precipitating at an acetone concentration between 50 and 75% was centrifuged off and freeze dryed. This material was further purified by column electrophoresis in a Porath column LKB Model 5801A. The buffer of pH 8.6 contained 0.2*M Tris*, 0.0084*M* EDTA and 0.03*M* boric acid. The results are presented in Figure 1.

Hemagglutinating activity was determined with rabbit blood cells[4] and found only in peak I, while the milk clotting action[5] in peaks II–IV indicated the localization of the proteolytic enzymes[6].

The dialyzed and lyophilyzed material from peak I gave a strong main line and 2 faint lines in electrophoresis on cellulose acetate strips. In the immunoelectrophoresis test the strong line produced could be stained with the specific lipid stain, sudan black. Comparison has been made with the proteins from the unfractioned sap, which produced 6 lines, 2 of which were stained by sudan black (Figure 2). The N content of fraction I was 14.7%. The following sugars were identified in hydrolizates of fraction I by their Rf values in paper and thin layer chromatography with different solvent systems and staining methods: rhamnose, fucose, xylose, mannose, galactose, and glucosamine.

Hemagglutinating activity was tested with washed erythrocytes of the following species: rabbit, rat, mouse, cow, guinea-pig and human group A, B and O. Only rabbit blood was agglutinated, the lowest active concentration being about 30 ug/ml. The results of the toxicological tests are summarized in the Table and show that the LD_{50} for mice of our fraction I is about 187 mg/kg.

The crepitin preparation was tested for mitogenic activity on human lymphocyte cultures by the technique of SAINT-PAUL et al.[7]. No activity could be detected.

It can be concluded from the chemical and biological properties of our product that it is probably a glyco-

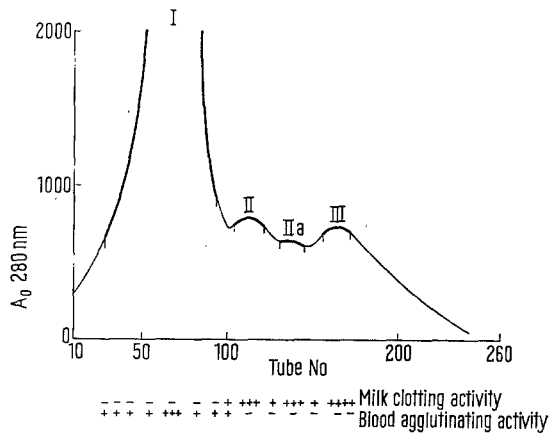

Fig. 1. UV-absorption, blood agglutinating activity and milk clotting action of the effluents of acetone fractionated *Hura crepitans* sap after column electrophoresis.

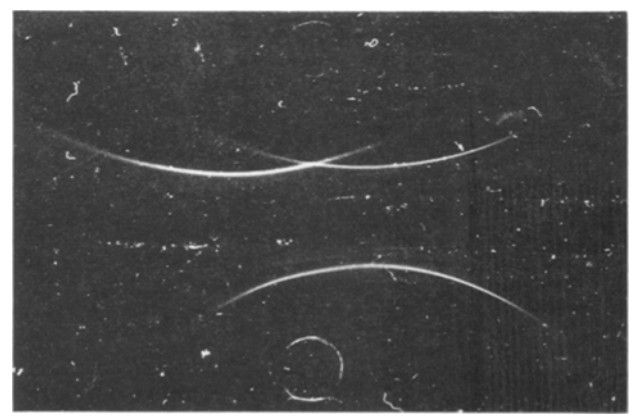

Fig. 2. Immunoelectropherogram of the lipoproteins from *Hura crepitans* acetone powder and of purified crepitin. Coloration: Sudan black.

Table. Mortality of mice injected with crepitin

Dose/animal (mg)	No. of animals dead within 1 week
1.25	1/6
2.50	2/6
3.75	3/6
5.00	6/6

The weight of the mice was 20 ± 2 g.

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lipoproteid with hemagglutinating and toxic activities. This is of interest, as the glyco-lipoproteid nature of the agglutinins form castor beans[8] and from kidney beans[9] has been suggested.

The older statements about a toxic phytohemagglutinin in the sap of *Hura crepitans* referred to crude preparations from a material in which several toxic factors are known to exist[1]. The proteolytic enzyme, hurain, existing in the sap[6] was probably not separated in this work and could have contributed to the formerly described physiological actions. The present results confirm the existence of a toxic lectin in this plant, for which the name crepitin will be conserved.

Crepitin is less toxic than the hemagglutinin from soybeans with a LD_{50} of about 50 mg/kg[10] and of black beans which is similar to that of soybeans[4], while ricin is much more toxic[8]. The hemagglutinating power of our crepitin preparation was also inferior to that of the bean lectin and of ricin. In its specific hemagglutinating action on rabbit blood, it is similar to that of soybeans.

Zusammenfassung. Aus dem Saft des Baumes *Hura crepitans* wurde eine Fraktion gewonnen, die wahrscheinlich ein Lipoglykoprotein ist und hämagglutinierende und toxische Eigenschaften besitzt.

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[11] The efficient technical assistance of Miss Ursula Zentgraf is gratefully acknowledged. The work was supported by a grant of the Consejo del Desarrollo Científico of the Central University of Venezuela.

PRO EXPERIMENTIS

Rivanol-Ethanol Fractionation of Parotid Fluid and Colostrum

The rivanol-ethanol method has been used extensively for fractionation of human serum and colostral proteins and for purification of γG-globulin[1,2]. Rivanol (6,9-diamino-2-ethoxy acridine lactate) precipitates all serum proteins with the exception of immunoglobulins and transferrin.

In the present study, treatment by rivanol with further fractionation by cold ethanol was applied to human salivary proteins. The precipitability of amylase, the main protein constituent of saliva, seemed of particular interest, since bacterial amylase has been successfully purified with the aid of rivanol[3].

Materials and methods. Parotid secretion was collected by capping the parotid duct with Curby caps[4] and stimulating salivary flow with lemon drops. 10 g of the pooled lyophilized fluid containing about 2 g protein was reconstituted in 58 ml distilled water and dialyzed overnight against 0.14 *M* NaCl before fractionation. Colostrum was collected within 3 days after delivery, was defatted by repeated centrifugation, and was then pooled.

Either body fluid was added slowly, and with continuous stirring, to a threefold volume of 0.4% rivanol (K and K Laboratories, Inc.) in distilled water at room temperature. After precipitation, the mixture was centrifuged for 1 h at 8000 *g* in the cold. Activated charcoal was stirred into the supernatant fluid and into the redissolved precipitate. The charcoal including the adsorbed rivanol was removed by filtering through Hyflo Super Cell. The filtrates were then precipitated at 0 to −7 °C with 95% ethanol to a 25% (v/v) concentration and centrifuged in a precooled centrifuge. The ethanol sediment from each rivanol fraction was dissolved in physiologic saline. The supernatant fluids, as well as the redissolved sediments, were gel-filtered on a Sephadex G-200 column equilibrated with 1% ammonium bicarbonate saturated with CO_2, which permits lyophilization of the eluate without intervening dialysis.

Antiserum to colostral IgA was prepared by repeated multiportal immunization of rabbits with colostral IgA in complete Freund's adjuvant. The IgA was purified by gel filtration on Sephadex G-200, ion exchange chromatography on DEAE cellulose[5], and refiltration on Sephadex G-200. Secretory IgA differs from serum IgA by an extra antigenic determinant, the 'secretory piece' (SP). Anti-SP serum was obtained by adsorbing anticolostral IgA serum with normal human serum (NHS)[5]. Antihuman-parotid serum was produced by repeated multiportal injection of rabbits with 10 mg lyophilized pooled parotid fluid dissolved in saline and mixed with complete Freund's adjuvant.

Amylase activity was measured by the diameter of a lytic zone in starch-agar gel[6]. Lysozyme activity was similarly estimated from the zone of lysis of a suspension of *Micrococcus lysodeikticus* in agarose[7].

Results and discussion. During the fractionation of parotid fluid, we followed the distributions of lysozyme, amylase, IgA and IgG globulins, and albumin. The level of enzyme activity in every fraction is shown in the Table. Most of the lysozyme activity was lost, since neces-

Distribution of lysozyme and amylase in rivanol-ethanol fractions

Enzyme	Reconstituted parotid fluid	Fractions R− E−	R− E+	R+ E−	R+ E+
Lysozyme	464,000[a]	Trace	>80,000	2,440	594
Amylase	>580,000	900	52,000	11,040	450,000

[a] Total activity = activity/ml × volume. Lysozyme activity expressed in μg/ml of egg white lysozyme (Sigma). Amylase activity in IU/ml.

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