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.

W. G. Jaffé and Dinah Seidl

Department of Biochemistry, Escuela de Biología, Facultad de Ciencias, Universidad Central, Caracas (Venezuela), 22 January 1969.

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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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sary precautions were not taken during dialysis. The greater part of the remaining lysozyme was not precipitated by rivanol but was precipitated by cold ethanol (R−E+). Amylase occurred in all fractions, but more than 75% was precipitated by rivanol and ethanol and was recovered in a single fraction (R+E+). Rivanol may be used to advantage in the purification not only of bacterial[3] but also of human salivary amylase.

Human parotid saliva contains small amounts of IgA of the secretory type[5]. Part of the salivary IgA was resistant to and part was precipitated by rivanol. The highest concentration was found in the R−E+ fraction, together with lysozyme and 10% of the recovered amylase. Colostrum contains much larger concentrations of IgA than does saliva; therefore, colostrum was fractionated for the purpose of comparison. The R−E+ fraction contained IgG, which was not detectable in parotid fluid.

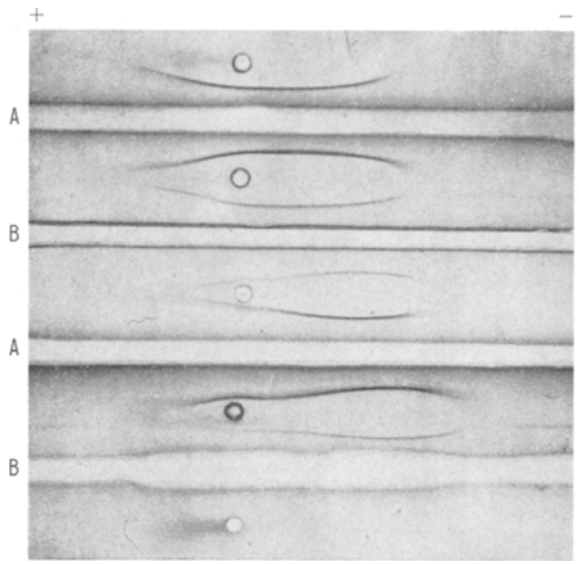

Fig. 1. Immunoelectrophoresis of the R+E+ fraction of human colostrum and the R−E+ fractions of colostrum and parotid fluid gel-filtered through Sephadex G-200. 1, NHS; 2, R+E+ (colostrum); 3, R−E+ (colostrum); 4, R−E+ (parotid). Antisera: A, anti-α chain (Hyland); B, anti-SP.

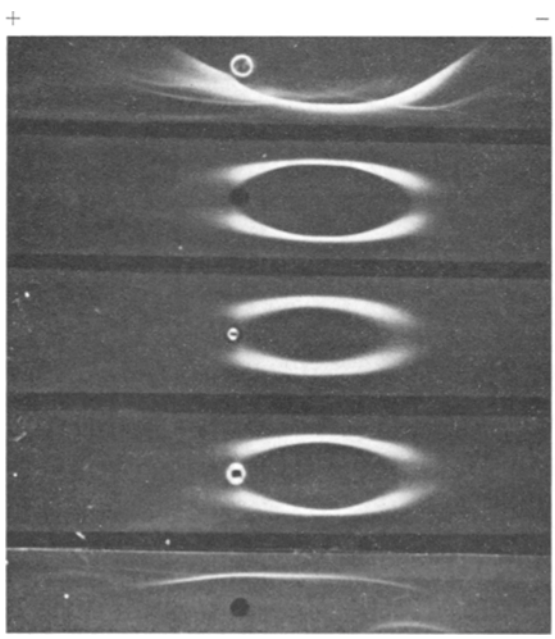

Fig. 2. Immunoelectrophoresis of human parotid fluid and its fractions. 1, concentrated parotid fluid; 2, R+E+ fraction; 3, R+E+ fraction gel-filtered through Sephadex G-200; 4, R+E+ fraction chromatographed on DEAE cellulose; 5, NHS. Antiserum: anti-human parotid.

The colostral R+E+ fraction and the colostral, as well as parotid, R−E+ fractions were filtered through Sephadex G-200. The high-molecular portions with IgA were clearly separated from the low-molecular portions containing amylase and, in the R+E+ fraction, a trace of albumin. By ultracentrifugation, all IgA molecules in these rivanol-ethanol fractions were polymeric, i.e. predominantly of 11S velocity with a smaller admixture of 16S material. Rivanol did not dissociate the secretory IgA. Immunoelectrophoretic analysis of the gel-filtered fractions (Figure 1) showed that the 2 IgA fractions differed in electrophoretic mobility. The rivanol-precipitable (R+E+) IgA moved as fast as serum IgA, whereas the rivanol-resistant (R−E+) IgA moved more slowly. The mobilities of the R−E+ fractions of IgA from colostrum and from saliva were equal. (The salivary R+E+ IgA was not tested, because the amount was too small.) All 3 fractions precipitated with anti-SP serum; therefore, the SP content did not appear to influence the electrophoretic mobility, nor was it a distinguishing characteristic with respect to rivanol susceptibility.

The purity of amylase in the parotid R+E+ fraction was additionally examined by double-immunodiffusion – which showed a very small amount of IgA and a trace of albumin – and by immunoelectrophoresis (Figure 2). All the precipitin bands detectable by antihuman parotid serum with either parotid fluid or NHS were reduced to 1 band in the R+E+ fraction. Purification of this fraction by either Sephadex G-200 gel filtration, or ion exchange chromatography on DEAE, did not change the immunoelectrophoretic pattern. By polyacrylamide-gel electrophoresis[8], the trace impurities disappeared after Sephadex G-200 filtration. This additional step, then, is required when the highest attainable purity of amylase is desired. The preparatory rivanol-ethanol fractionation of parotid fluid takes only 6 h for a 75% yield of amylase, the purity of which should be satisfactory for most applications[9].

Zusammenfassung. Die Reihenfraktionierung durch Rivanol und Äthylalkohol wird mit Vorteil für die Trennung von Speicheleiweisskörpern verwendet: Immunglobulin A des Speichels und der Vormilch erscheint in zwei Formen, von denen die eine gegenüber Rivanol widerstandsfähig ist, während die andere durch Rivanol gefällt wird. Von der ursprünglich angesetzten Speichelamylase erscheinen 75% in hohem Reingehalt in der durch Rivanol und Äthylalkohol fällbaren Phase. Das gesamte Verfahren beansprucht 6 Stunden.

J. MESTECKY, F. W. KRAUS,
R. C. WILLIAMS and M. C. MIMS

Department of Microbiology and Institute of Dental Research, University of Alabama, Birmingham (Alabama 35233, USA), 27 March 1969.

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