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POLYSACCHARIDES INVOLVED IN SLIME-MOLD DEVELOPMENT

II. WATER-SOLUBLE ACID MUCOPOLYSACCHARIDE(S)

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SUMMARY

An acid mucopolysaccharide has been isolated from *Dictyostelium discoideum* and related species. It is trichloroacetic acid and water soluble, ethanol precipitable, and non-dialyzable. In purified preparations approx. 90 % of the dry weight is accounted for as galactose, galactosamine and galacturonic acid. The polysaccharide is antigenically reactive. Negligible levels are detected until the terminal stages of fruiting body construction at which time it accumulates to the extent of 1–2 % of the dry weight. It fails to appear in morphogenetically deficient mutants and its synthesis seems to be keyed to the overall morphogenetic rate. A novel method for the measurement of a specific polysaccharide antigen in crude cell sonicates is described.

INTRODUCTION

The life cycle of the cellular slime molds includes the following steps: (1) Germination of spores into vegetative amoebae. (2) Exponential growth. (3) Aggregation of the amoebae into organized multicellular assemblies after cessation of growth. (4) Development of the aggregates into complex fruiting bodies with terminal spore masses and cellulose-ensheathed supportive tissues.

The fates of at least three distinct polysaccharide fractions appear to be linked to this morphogenetic sequence. One is water, trichloroacetic acid, and alkali insoluble, containing glucose plus a trace of arabinose. It appears at the time of fruit construction and eventually comprises approx. 3–4 % of the dry mass[1]. It is probably cellulosic material known by cytochemical and X-ray analyses[2–4] to make up the outer sheath of the fruiting body stalk as well as the spore coats. The second is a water and trichloroacetic acid soluble, alcohol precipitable complex[1]. Glucose is the sole carbohydrate constituent and accounts for 95 % of the dry weight in the purest preparations. This fraction increases sharply by the factor of approx. 6-fold on a dry-weight basis, reaching a peak (1–2 % of the dry weight) during fruit construction, and thereafter disappears coincidentally with the synthesis of cellulose. The third fraction appears to be an acid mucopolysaccharide containing galactose, galactosamine, and galacturonic acid. It is synthesized during the last stages of fruit construction and is associated, perhaps uniquely, with the spores of the completed fruiting

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body. The composition, antigenic activity, and the developmental kinetics of this fraction are described below.

The patterns of synthesis of all three polysaccharide components are drastically altered or eliminated in mutant strains that display morphogenetic deficiencies.

METHODS

Organisms and cultivation

The organisms included *Dictyostelium discoideum* wild type and several mutants; *D. mucoroides* and *D. purpureum*; *Polysphondylium violaceum*. They were grown on SM-agar in association with *Aerobacter aerogenes*[5].

Morphogenetically synchronized cells

The myxamoebae were harvested from growth plates, washed three times by centrifugation and suspended in water. They were dispensed on washed agar plates at a density of $2 \cdot 10^8$ amoebae per plate (approx. 25–35 % of the density obtained on the growth plates). Plates were then harvested at intervals between the deposition of cells and the termination of the fruiting process. Under these conditions good morphogenetic synchrony is obtained[6].

Analytical procedures

Cell homogenates were obtained by sonic oscillation. Total protein was determined by the Folin reaction[7], total reducing power by the method of PARK AND JOHNSON[8], glucose by the glucostat microassay[9], total hexose by anthrone[10], indole[11], and secondary cysteine[12] reactions. Galactosamine was determined by the method of Elson and Morgan[13]. Immunochemical assays were carried out by complement fixation using the semi-micromethod of WASSERMAN AND LEVINE[14] and by the agar diffusion method of OUCHTERLONY[15]. Descending paper chromatography was conducted at room temperature with *n*-butanol–pyridine–water in proportions of 3:2:1.5 (ref. 16) and *n*-butanol–acetic acid–water in proportions of 4:1:5 (ref. 17) on Whatman No. 1 filter paper. The indicator sprays were aniline phthalate, ammoniacal silver nitrate, and ninhydrin.

RESULTS

Isolation of the mucopolysaccharide

Method 1: 100 SM-agar growth plates were incubated for 24–36 h subsequent to the appearance of fruiting bodies. The plates were harvested with water and the suspension (final volume 100 ml) sonicated for 40 min. The homogenate was deproteinized by treatment with $ZnSO_4$ and $Ba(OH)_2$ (see ref. 18). The supernatant was made slightly acid and concentrated *in vacuo* at 50° to 10 ml. Ethanol was added to a final concentration of 60 %, the precipitate was collected after incubation overnight at 4°, and was redissolved in water. The alcohol precipitation was repeated and the redissolved material was dialyzed against water overnight. A final yield of 30 mg was obtained (approx. 1 % of the starting material).

Normally the water-soluble glucose polysaccharide would appear in the same fraction[1]. However, two precautions reduced the level of this contaminant to a minimum. First, mature fruiting bodies were used as the source of material and at

this stage the amount of soluble glucose polysaccharide is quite low. Second, concentration *in vacuo* at 50° under slightly acidic conditions transformed practically all of the latter into dialyzable material, and thus it could be eliminated.

Method 2: In the presence of appreciable quantities of the soluble glucose polymer, the following procedure of separation was found useful. The sonicated material was incubated with 5 % trichloroacetic acid in the cold. The supernatant was extracted 5–6 times with equal volumes of ether to remove the trichloroacetic acid and then concentrated *in vacuo* at 50° at pH 7.8. The concentrate was clarified by centrifugation, made 0.5 N with NaOH, incubated at 100° for 15 min, and centrifuged again. Ethanol was added to the supernatant to a final concentration of 35 % and the precipitate collected after incubation overnight at 4°. Virtually all of the soluble glucose polymer and none of the mucopolysaccharide was in this fraction. The latter was then precipitated from the supernatant by increasing the ethanol concentration to 60 %. Repeated hot alkali treatment followed by ethanol precipitation gave mucopolysaccharide preparations which yielded less than 0.5 % Folin-reactive material. The disadvantage of this procedure lies in the fact that the harsh alkali treatment produces some degradation of the mucopolysaccharide which results in a change in antigenic activity (determined by equivalence point *vs.* dry weight).

The following data were obtained with a product purified by the first method.

(1) Approx. 90 % of the dry weight could be accounted for as reducing sugar after acid hydrolysis (1 N HCl at 95° for 1.5 h) by the method of PARK AND JOHNSON[8] using a galactose standard. Folin-reactive material accounted for less than 0.5 %.

(2) A sample was subjected to electrophoresis on Whatman 3MM paper at 600 V in 0.042 M NaOH–boric acid buffer at pH 9.2. The product, detected with periodic acid–Schiff reagent[19], moved toward the anode as a single spot. (When the alkali-treated product (Method 2) was used, only a portion migrated as above and the remainder stayed at the origin.)

(3) The purified product gave a single precipitin band with anti-spore serum in the agar diffusion assay (see below for details).

Composition

Three major components have been detected: galactose, galactosamine, and galacturonic acid. Together they constitute approx. 90 % of the dry weight. The data supporting these conclusions are summarized below.

The acid-hydrolyzed product was chromatographed on paper with *n*-butanol–pyridine–water and *n*-butanol–acetic acid–water as solvent systems and aniline phthalate and ammoniacal $AgNO_3$ as indicators. Both solvent systems and indicators yielded similar results. Four spots were detected in the hydrolyzate. Their positions corresponded to authentic samples of galactose, galactosamine·HCl, galacturonic acid, and glucose, chromatographed adjacent to aliquots of the unknown. The first appeared to be the major constituent, the last was present only in trace amounts. The second yielded a silver spot with ammoniacal $AgNO_3$ (to be expected as the result of the reaction between $AgNO_3$ and the free base of galactosamine·HCl) and also yielded color with ninhydrin. Its identification as galactosamine was confirmed by treatment of the hydrolyzate with hot ninhydrin and pyridine[20] to convert the amino sugar to the corresponding pentose, lyxose. The R_F of this derivative was identical to that obtained by corresponding treatment of an authentic sample of galactosamine.

The presence of galactose was indicated by the amount of color produced in the secondary cysteine reaction[11] in comparison with equivalent amounts of known sugars and by the carbazol reaction[11]. The ratios of the absorbancy values at 535 and 440 mμ in the latter assay were characteristic of those obtained with galactose and different from those obtained with glucose and mannose (Fig. 1). Finally, galactose specifically blocked the serological reaction between the mucopolysaccharide and anti-spore serum (see below).

The presence of galacturonic acid was further substantiated by the results of the secondary cysteine reaction for uronides[11]. In this assay, the absorption spectrum of galacturonic acid shows a marked rise in the region of 540–600 mμ whereas glucuronic acid, hexoses, and hexosamines do not. The hydrolyzed product yielded the expected rise in this region.

TABLE I

ANALYSIS OF TWO PREPARATIONS OF MUCOPOLYSACCHARIDE PURIFIED BY THE FIRST METHOD DESCRIBED IN THE TEXT

Components	*Assay*	*Preparation No. 1 (mg/ml)*	*Preparation No. 2 (mg/ml)*	*% dry weight*
Reducing sugars	PARK AND JOHNSON	1.32	1.48	90
Galactose plus galacturonic acid	Indole	0.92	0.99	63
	Tryptophan	0.97	1.03	
Galactose	α-Naphthol	0.80	0.88	54
	Primary cysteine	0.77	0.91	
Galactosamine	Elson–Morgan	0.38	0.41	25
Dry weight		1.51	1.61	

An estimate of the proportions of the three constituents could be obtained from the data shown in Table I. The indole and tryptophan reactions[11] measure both hexoses and hexuronic acids. The α-naphthol and primary cysteine reactions[11] measure hexoses only and thereby provided a measure of the galacturonic acid by comparison with the first two assays. The Elson–Morgan reaction[13] measures hexosamine. The data suggest the following composition. Galactose, approx. 54%; galactosamine, approx. 25%; galacturonic acid, approx. 9%. It was not possible to decide if the trace of glucose detected by chromatography is an integral component of the mucopolysaccharide or a contaminant.

Antigenic activity

A purified preparation of the mucopolysaccharide was examined for antigenic activity by the agar diffusion technique[15] using rabbit anti-spore serum[21]. A single band of precipitation was obtained which was eliminated in duplicate plates containing 1% galactose in the agar. The mucopolysaccharide also reacted with horse serum against Type VII *Diplococcus pneumoniae**. Simultaneous agar double diffusion assays with the two antisera and the purified mucopolysaccharide yielded precipitin bands that formed a sharp corner suggesting that both had reacted with a common antigen. The reaction of the mucopolysaccharide with anti-spore serum could also

* Kindly supplied by Dr. L. LEVINE of Brandeis University.

be followed by complement fixation. Fig. 2 shows data for preparations purified by the two different methods described above. Note that the antigenic activity was considerably altered by treatment with hot alkali. However, both purified products appeared to have reacted with a common antibody since each preparation when present in antigen excess inhibited fixation of complement by the other. Fig. 2 also demonstrates the ability of galactose to block the serological reaction. Lactose was found to be 50 % as effective at equimolar concentrations. Glucose, trehalose, maltose, melibiose, cellobiose, raffinose, glucosamine, galactosamine, *N*-acetylgalactosamine, glucuronic and galacturonic acids were completely ineffective.

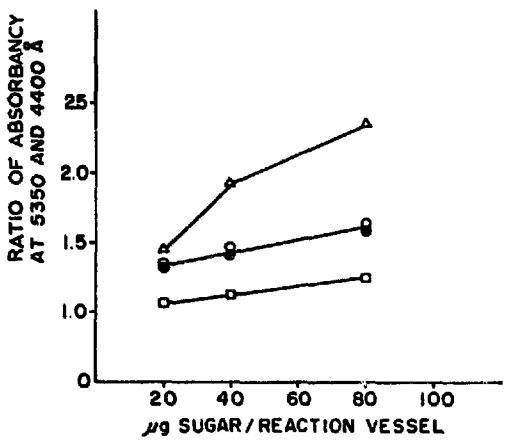

Fig. 1. Performance of the mucopolysaccharide hydrolysate in the carbazol reaction[11] as compared with equivalent concentrations of known sugars. △—△, glucose; ○—○, ●—●, galactose; □—□, mannose.

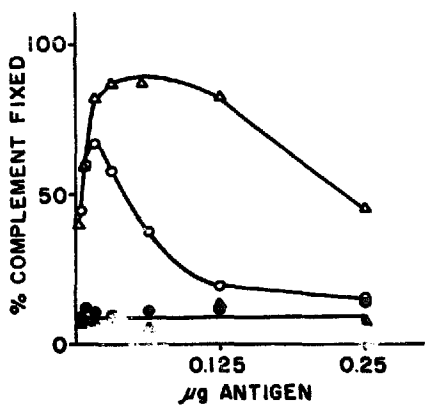

Fig. 2. Complement fixation assay of the purified polysaccharide prepared by Methods 1 (○—○) and 2 (△—△). Peak fixations were reached at 0.015 and 0.05 μg, respectively. The lowest curve was obtained by conducting the assays in the presence of galactose (10 μmoles per reaction tube).

Antigenic assay of the mucopolysaccharide in crude sonicates

A sample of *D. discoideum* incubated for 47 h on washed agar (approx. 18 h after the appearance of mature fruits) and known to contain the mucopolysaccharide, was sonicated and treated with 1 N NaOH for 15 min at 90°. A complement fixation assay was conducted in the presence and absence of galactose (10 μmoles per reaction tube). Fig. 3A shows the results. From the difference in fixation between pairs of tubes containing equivalent concentrations of antigen with and without added galactose, the percentage of the complement fixed that was galactose blockable could be determined. Agar diffusion assays of alkali-treated crude sonicates as well as the purified mucopolysaccharide had revealed the presence of only one major precipitin band that disappeared in the presence of galactose. Thus, the amount of galactose-blockable complement fixation could be taken as a measure of the single antigenic species known to be associated with the mucopolysaccharide. The pretreatment of the sonicates with hot alkali decreased the level of extraneous antigenic activity not blocked by galactose. This was done in order to cut down the background level and permit a more accurate estimate of the difference between fixation in the presence and absence of galactose.

Fig. 3B compares the galactose-blockable complement fixation obtained with an alkali-treated preparation from mature fruits when diluted 1:1 with saline and

when mixed 1:1 with a sonicate of vegetative amoebae. The latter itself had no galactose-blockable antigenic activity and fractionation by the methods described above failed to yield the mucopolysaccharide. The two galactose-blockable fixation curves were identical. Thus extraneous material did not appear to have interfered with the assay, and it could therefore be taken as a valid measure of the antigenic activity associated with the mucopolysaccharide. Furthermore, the results showed that the absence of antigenic activity in the vegetative amoebae was real and not due merely to a masking of activity by an inhibitor of the serological reaction.

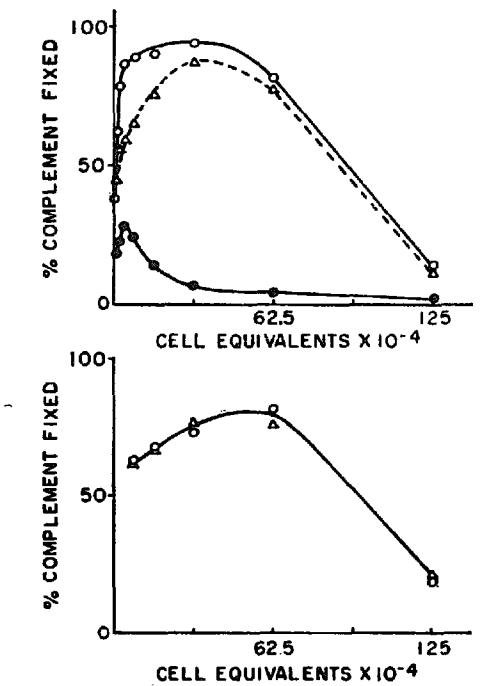

Fig. 3. A (upper). A complement fixation assay of a crude alkali-treated sonicate of mature fruits in the presence (●—●) and absence (○—○) of galactose. The difference in fixation between the two yielded the dotted line, *i.e.* the galactose-blockable fixation (△----△). B (lower). The galactose-blockable fixation of a sonicate of mature fruits diluted 1:1 with saline and 1:1 with an equivalent sonicate of vegetative cells. The preparations were treated with alkali and assayed in the presence and absence of galactose. ○—○, spores; △—△, spores and amoebae.

Developmental kinetics of the mucopolysaccharide

To examine the kinetics of appearance of the mucopolysaccharide antigenically, cells in the stationary phase were harvested from growth plates, washed by centrifugation, and dispensed on washed agar at a density of $2 \cdot 10^8$ cells per plate. At intervals aliquots were harvested in water, sonicated, and heated with 1 N NaOH at 90° for 10–20 min. After neutralization with HCl the preparations were dialyzed overnight against water, made to standard volume, and stored over chloroform in the cold. Galactose-blockable complement fixation was assayed in serial 2-fold dilutions of the samples. In each case, the antigen concentration which yielded half-maximal fixation in the presence of excess antibody could be taken as a measure of the concentration of the antigen[14]. These were then expressed as percentages of the concentration in the mature fruits. Fig. 4 shows the results obtained with two species, *D. discoideum* and *D. mucoroides*. No antigenic activity was detectable in the early stages of the morpho-

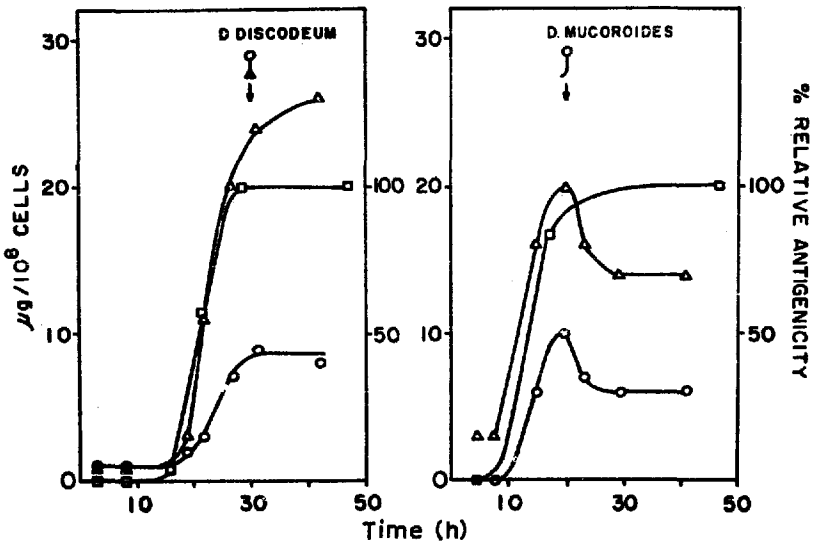

Fig. 4. Developmental kinetics of the mucopolysaccharide in two species. See text for experimental details. □—□, antigenicity; △—△, galactose; ○—○, galactosamine.

genetic sequence. But within a short period of time, the concentration of mucopolysaccharide antigen rose to a peak which, based on the antigenicity of the purified product, constituted 1–1.5 % of the dry weight. This period coincided in both species with the late stages of fruit construction and the appearance of mature spores. The activity was detected earlier in *D. mucoroides* in agreement with its greater overall rate of morphogenesis.

The rise in antigenic activity was accompanied by the accumulation of non-dialyzable, acid-hydrolyzable galactosamine, galactose, and galacturonic acid. Samples of morphogenetically synchronized cells were harvested from washed agar as previously described, sonicated, and treated with $ZnSO_4$ and $Ba(OH)_2$. The supernates were concentrated *in vacuo* and dialyzed overnight. Bound galactosamine was determined in the non-dialyzable fraction after acid hydrolysis by the Elson–Morgan reaction. (No activity was apparent before hydrolysis.) Bound galactose was determined by

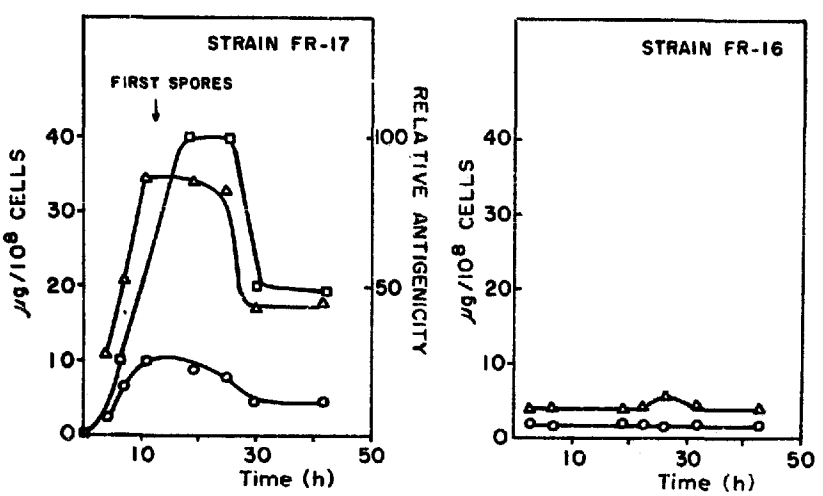

Fig. 5. Developmental kinetics of the mucopolysaccharide in two mutants of *D. discoideum*. □—□, antigenicity; △—△, galactose; ○—○, galactosamine.

the secondary cysteine reaction as the difference between hydrolyzed and unhydrolyzed aliquots. The latter assay was corrected for the residual glucose content as measured by glucostat determinations. Fig. 5 summarizes the results for the two species. The antigenic and biochemical assays of the mucopolysaccharide are seen to be correlated closely both in respect to time of appearance and amount formed.

Similar studies of the species *D. purpureum* have shown that it too forms an acid mucopolysaccharide which cross-reacts with anti-spore sera from *D. discoideum*. A fraction of similar chemical composition has been isolated from fruiting bodies of *P. violaceum*, but no antigenic activity was detectable against either heterologous or homologous anti-spore sera.

The acid mucopolysacchcride in morphogenetically deficient mutants

A total of six mutant strains of *D. discoideum* were examined. Three of these are aggregateless and the remainder fruitless (capable of aggregation but unable to construct mature fruiting bodies). In all of the former and two of the latter no galactose-blockable antigenic activity could be detected. The levels of non-dialyzable galactose, galactosamine, and galacturonic acid were very low, in most cases barely detectable. Fig. 5 summarizes the developmental kinetics of this fraction in one strain, Fr-16, which aggregates normally and forms pseudoplasmodia that migrate but do not transform into fruits.

The remaining mutant, Fr-17, yielded a different pattern. Unlike other fruitless strains, Fr-17 produces both of the terminal cell types (spores and stalk cells) encountered in mature fruiting bodies, as well as many of the biochemical end-products of normal morphogenesis[22]. However, these are contained within a flat amorphous aggregate bearing no geometric resemblance to a normal fruit. In addition, the succession of morphogenetic events is accomplished much faster by the mutant. For example, the first spores appear about 13 h after cell deposition, in less than half the time it takes for wild type to do the same. The data summarized in Fig. 5 indicate that Fr-17 does synthesize an acid mucopolysaccharide containing galactose and galactosamine and with galactose-blockable antigenic activity. This fraction appears at the time of spore formation. In contrast to the wild type, about 50 % is subsequently lost, but since the mucopolysaccharide is water soluble, this may be due to diffusion into the agar substratum.

DISCUSSION

The results reported here have revealed the existence of a set of biochemical events associated with the latter stages of cellular slime-mold morphogenesis, *i.e.* the synthesis of a specific acid mucopolysaccharide containing galactose, galactosamine, and galacturonic acid. This mucopolysaccharide fails to appear in mutants incapable of proceeding through these later morphogenetic stages. It appears much sooner in Strain Fr-17, a mutant whose overall rate of morphogenesis is considerably greater than that of *D. discoideum* wild type. Similarly in the species *D. mucoroides*, the onset of synthesis seems to be coupled to the overall morphogenetic rate.

The sudden accumulation of this material to the extent of 1–2 % of the dry weight, starting from negligible levels, suggests the possibility that one or more new enzymic activities are acquired during the course of morphogenesis: one or perhaps several

mucopolysaccharide synthetases; possibly the enzymes involved in the genesis of galactose, galactosamine and galacturonic acid. (Preliminary experiments have indicated that these compounds become labeled after administration of [^{14}C]glucose.) Given information about the specific enzymes involved, it will be of interest to examine the metabolic controls which regulate their activities and their connection with the overall control of morphogenetic events.

Special mention should be made of the technique by which the antigenic activity of the mucopolysaccharide was measured in crude cell sonicates. Although, to our knowledge, this method has not been previously employed, it may well be of general value for the antigenic analysis of crude extracts, particularly in the case of other polysaccharide antigens. Its validity requires that the antigen under study in the crude mixture be the only one whose reactivity is blocked by the specific haptene employed (in this case, galactose).

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