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Immunochemistry and the Structure of Lung Galactan[1]

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Since the immunologically specific polysaccharide of Type XIV pneumococcus (S XIV) contains galactose, and antibodies to Type XIV pneumococcus are directed against S XIV, the cross reactivity of lung galactan in Type XIV antiserum was tested. Nearly 30% of the antibody was precipitated, indicating a close relationship between the galactose linkages in the two carbohydrates. The galactan also precipitated antiserum to Type II pneumococcus. Since the Type II polysaccharide contains glucuronic acid and no galactose it seemed probable that the uronic acid known to be a minor constituent of lung galactan is actually glucuronic acid. This was verified by paper chromatography. Colorimetric assays of the uronic acid: galactose ratios in the lung galactan and in its cross reaction precipitates showed that the initial ratio of *ca.* 1:50 was not maintained. Thus the lung galactan was not a homogeneous polysaccharide or had been altered in its isolation. The galacto-gluco-xylan of tamarind seed also gave a cross reaction with Type XIV antipneumococcal horse serum but this was not as strong as that exhibited by the lung galactan. These results demonstrate the value of quantitative immunological methods as an aid to the determination of chemical structure.

Quantitative immunochemical methods have been of great service in the clarification of structural chemical relationships, but their application to natural products has been restricted by the small number of reference substances of known constitution and fine structure capable of reacting with antibodies to form precipitates. The type-specific polysaccharides of pneumococcus constitute a large group of such materials of great potential utility, but thus far the constituent sugars and their modes of linkage are known in only a few instances.

An indirect approach to the chemistry of these substances involves the testing of more easily accessible polysaccharides of known constitution against various antipneumococcus sera, since any cross reactivity found would indicate a measure of structural relationship. The recent isolation of a galactan from the residues of beef lungs[4,5] after the separation of heparin and the finding that the galactan[6] gave a precipitate with Type XIV antipneumococcus serum prompted the joint investigation described in the present paper. The unexpected reactivity of the galactan in Type II antipneumococcus serum provided additional instances of the power of immunochemical methods.

Because the specific polysaccharide of Type XIV pneumococcus contains galactose[7] it appeared appropriate to test the reactivity of the lung galactan with Type XIV antiserum in the hope that the structural relationships of the galactan and S XIV might be sufficiently close to permit cross reactivity, *i.e.*, precipitation of at least a portion of the antibody. Qualitative tests showed that precipitation actually occurred. However, there was also marked precipitation of antiserum to Type II pneumococcus. It is known that the specific polysaccharide of this type (S II) contains a repeating unit composed of rhamnose, glucose and glucuronic acid.[8-10] The reaction in Type II serum could therefore be accounted for only if the unknown uronic acid reported to be a minor constituent of the lung galactan[5] turned out to be glucuronic acid. Identification of the uronic acid was thereupon undertaken and confirmation of the serological prediction ensued. Quantitative data on the precipitin reactions in Type XIV and Type II antipneumococcal sera are given in Table I.

TABLE I

PRECIPITATION OF 1.0 ML. ANTIPNEUMOCOCCAL TYPE XIV AND TYPE II HORSE SERA BY LUNG GALACTAN IN THE COLD

Amt. galactan added, mg.	Nitrogen precipitated from Anti-XIV, No. 635, μg.		Anti-II, No. 1054 C-absd.,[a] μg.	Anti-II, No. 930 μg.
0.1	103	92[b]		
.15			42	
.3	179	157		
.45			95[e]	
1	257,[c] 232[d]	207		54[f]
1.5			150	59
2		256		70
3		242	175	
4				75[g]
6		181		

[a] After absorption with pneumococcal C-substance this serum contained 1061 μg. of antibody nitrogen precipitable by S II; of this 20 μg. was precipitated by glycogen and the supernatants were used for the analyses with galactan. All values are calculated to 1 ml. of C-absorbed serum. [b] Data in this subcolumn were obtained with 1.0 ml. of a 1 → 2 dilution of serum with one-half quantities of galactan and were doubled. Supernatants from the precipitations with 2 and 3 mg. gave only 2 μg. of N with tamarind seed polysaccharide. [c] The supernatant gave 664 μg. of antibody N with S XIV. 664 + 257 = 921 μg.; direct analysis of 1.0 ml. of serum with S XIV gave 920 μg. [d] Actually run with 2.5 ml. of antiserum and 2.5 mg. of galactan; calculated to 1 ml. [e] The supernatants, with enough lung galactan to bring them up to the 3 mg. level, each gave 61 μg. of precipitate N. [f] C-Absorbed serum gave 52 μg. of N with 1 mg. of galactan. [g] One-half quantities actually used.

(1) A preliminary account of this work appeared in *Federation Proc.*, **13**, 226, 496 (1954).

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(3) The Ohio State University. Acknowledgment is made to the assistance rendered by Dr. R. A. Gibbons, of this Laboratory, in revising the manuscript for publication.

(4) M. L. Wolfrom, D. I. Weisblat, J. V. Karabinos and O. Keller, *Arch. Biochem.*, **14**, 1 (1947).

(5) M. L. Wolfrom, G. Sutherland and M. Schlamowitz, THIS JOURNAL, **74**, 4883 (1952).

(6) Designated pneumogalactan in reference 5. In this reference the statement is made that the data uniquely established the sequence of D-galactose units as —1→6→1→6—. This is not correct and the

3←1

data allow three other possibilities:

—1→6—1→6—, —1→3→1→3—, —1→3→1→6—.
3←1—6←1 6←1—6←1 6←1

(7) W. F. Goebel, P. B. Beeson and C. L. Hoagland, *J. Biol. Chem.*, **129**, 455 (1939).

(8) B. R. Record and M. Stacey, *J. Chem. Soc.*, 1661 (1948).

(9) S. B. Beiser, E. A. Kabat and J. M. Schor, *J. Immunol.*, **69**, 297 (1952).

(10) M. Stacey, *Endeavour*, **12**, 38 (1953).

The question then arose as to whether the glucuronic acid was an integral part of the lung galactan, or whether it was contained in an impurity, the more so as the electrophoretic diagram of the galactan had indicated an inhomogeneity.[5] It has been possible to provide an immunochemical solution of this problem, also, based on the following reasoning. Precipitation of the galactan in Type XIV antiserum would be mediated through a portion or all of the D-galactose in its repeating units, while precipitation in the Type II serum would be through recurring glucuronic acid residues. If the D-galactose and glucuronic acid were components of the same polysaccharide, the ratios of these sugars in the precipitates formed in the two antisera should be the same. On the other hand, if the D-galactose and glucuronic acid occurred as portions of different polysaccharides, or in different proportions in different polysaccharides, these ratios should differ in the two cases. The latter alternative was found to obtain.

These two successful applications of immunochemical methods do not by any means exhaust the possibilities. From the results of the action of periodate and from hydrolysis of the methylated galactan it was concluded[5] that roughly one-third of the D-galactose was singly bound in glycosidic linkage to one of the other D-galactose units, one-third was doubly bound, and one-third triply. As for the Type XIV pneumococcal polysaccharide, all that is known of its structure is that there are stated to be three galactose residues to one of N-acetylglucosamine.[7] These data are insufficient to permit any decision as to what structures the two polysaccharides have in common. Recently, however, the structure of tamarind seed polysaccharide was described.[11] The principal feature of interest in the present connection is that all of the D-galactose in a methylated portion was recovered as the 2,3,4,6-tetramethyl ether, indicating that there is only one kind of D-galactose linkage in this polysaccharide, namely, that of single units attached to the D-xylose or D-glucose residues which make up the principal chain. A sample of the polysaccharide was obtained through the kindness of Dr. F. E. Brauns, The Institute of Paper Chemistry, Appleton, Wisc., and it was found to precipitate Type XIV antipneumococcus serum. Quantitative data on the reaction are given in Table II.

TABLE II

PRECIPITATION OF 1.0 ML. TYPE XIV ANTIPNEUMOCOCCUS HORSE SERUM 635 BY TAMARIND SEED POLYSACCHARIDE AT 0–2°

Amt. polysaccharide added, mg.	N precipitated, μg.	Amt. polysaccharide added, mg.	N precipitated, μg.
0.1	86	2	58[a]
.2	92	4	31[a]
.4	94, 92[a]	6	2[a]
1	77,[a] 81[a]		

[a] One-half quantities actually used. The supernatants from the analyses with 0.4 mg. gave 148 μg. of additional N with the lung galactan, or 241 μg. of N with both, as against 242 to 257 μg. with galactan directly (Table I).

(11) E. V. White and P. S. Rao, THIS JOURNAL, **75**, 2617 (1953).

Experimental

Materials and Methods.—The lung galactan[6] used was described in reference 5. Tamarind seed polysaccharide (jellose) was prepared as in reference 11. Antisera were generously furnished by the Bureau of Laboratories, New York City Department of Health, and by the Division of Laboratories and Research of the Department of Health, State of New York. Estimations of antibody nitrogen were carried out in duplicate at 0–2° according to Heidelberger and Kendall,[12] with the final determination in the case of the smaller quantities by the Markham method.[13]

Identification of Glucuronic Acid in Lung Galactan by Paper Chromatography.—An amount of 150 mg. of lung galactan[6] of highest purity[5] was suspended in 20 ml. of anhydrous methanol containing 1% hydrogen chloride and allowed to react at room temperature (25–30°) for 1 week. The centrifuged, esterified material (120 mg.) was dissolved in 3 ml. of water and the solution was added under stirring to a solution of 200 mg. of sodium borohydride in 5 ml. of water. After 10 min., the solution was made 0.5 *N* in hydrochloric acid in a total volume of 25 ml. and this was heated in a boiling water-bath (98°) for 20 hr. The cooled solution was then neutralized with sodium hydroxide and concentrated under reduced pressure to 5 ml. An aliquot of this material was subjected to ascending paper chromatography, following the technique of Partridge.[14] The upper layer of 1-butanol:acetic acid:water (4:1:5 parts by vol.) mixture was employed as developer. Authentic samples of D-galactose, D-glucose and D-glucurono-γ-lactone were chromatographed simultaneously on the same paper sheet. The indicator spray reagent was a 3% solution of aniline acid phthalate in 1-butanol and the color was formed by heating the sheet in an oven at 105° for 10–15 min. The hydrolyzate showed three distinct spots corresponding in position to those given by the authentic specimens of D-glucose, D-galactose and D-glucurono-γ-lactone. The D-galactose spot was just above that of D-glucose while that of the D-glucurono-γ-lactone was well above that of D-galactose. The D-glucurono-γ-lactone control showed a second spot corresponding to the free acid.[14] In the hydrolyzate, this spot was obscured by that of D-galactose.

Another portion of the lung galactan (100 mg.) was hydrolyzed directly with 5 ml. of 0.5 *N* hydrochloric acid at 98° for 20 hr. The neutralized and concentrated hydrolyzate was then chromatographed as above. Two spots resulted: a dark one corresponding in position to that of D-galactose and a fainter one corresponding to that of D-glucurono-γ-lactone.

Galactose and Glucuronic Acid Content of Specific Precipitates of Lung Galactan with Type XIV and Type II Antipneumococcal Horse Sera.—Reaction mixtures were set up multiply. One or more portions were analyzed for nitrogen and the others were combined for the sugar analyses. It was found that the antibody could be quantitatively immobilized and the galactan quantitatively extracted from the washed specific precipitates with 5% trichloroacetic acid for 15 min. at room temperature. The residual antibody showed a carbohydrate content normal for serum globulins. Galactose was determined in the extracts by the cysteine-H_2SO_4 reaction of galactose called SCyRI.[15] Triplicate analyses were made on 0.4-ml. aliquots of the trichloroacetic acid extracts (29.6 ml. total vol. for the Type XIV precipitates from six 2.5-ml. portions of Type XIV antiserum and 11.1 ml. total volume for the Type II precipitates from seven 5-ml. portions of Type II antiserum). Internal standards containing an additional 40 μg. of galactan (also in duplicate) were employed as reference standards, all readings being made at 605 mμ against a blank containing all reagents but no galactan. A control containing galactan but no cysteine was also included, but the extinction due to the action of sulfuric acid alone on galactan was small. The total hexose content was also checked by the PCyRI reaction[15] with the result that no significant amount of hexose other than galactose was found.

(12) M. Heidelberger and F. E. Kendall, *J. Exptl. Med.*, **61**, 559 (1935).

(13) R. Markham, *Biochem. J.*, **36**, 790 (1942); see also, E. A. Kabat and M. M. Mayer, "Experimental Immunochemistry," C. C. Thomas, Springfield, Ill., 1948.

(14) S. M. Partridge, *Biochem. J.*, **42**, 238 (1948).

(15) Z. Dische, L. B. Shettles and Martha Osinos, *Arch. Biochem.*, **22**, 169 (1949).

The trichloroacetic acid extracts were diluted to contain 150 μg. of galactose/ml. and the solutions, together with the original galactan (150 μg./ml. as galactan) were assayed for glucuronic acid.[16] Internal standards containing additionally 33 μg./ml. of D-glucuronic acid were used as reference standards and the interference due to the galactose present was corrected for by the inclusion of a galactose standard containing 150 μg. of D-galactose/ml. Analyses were set up in duplicate and tubes containing galactan (or D-galactose) but no carbazole were also included, the correction required for the extinction due to the action of sulfuric acid alone on both galactan and D-galactose in this instance being appreciable. The corrected extinctions due to the glucuronic acid present in the galactan solutions were very small (0.006–0.033), but the corresponding differences in the ratio galactose:glucuronic acid are sufficiently large to be significant (Table III), although the absolute amounts of glucuronic acid found in the precipitates may be subject to quite large error. Another experiment with somewhat smaller amounts of material gave results of the same order. It is therefore apparent that the Type XIV antiserum fractionates the galactan in the direction of more galactose, the Type II antiserum in the direction of more glucuronic acid. This is in accord with immunochemical theory and agrees with the electrophoretic diagram[5] showing an inhomogeneity in the lung galactan.

TABLE III

GALACTOSE AND GLUCURONIC ACID IN SPECIFIC PRECIPITATES FROM 1 MG. OF LUNG GALACTAN PER ML. TYPE XIV ANTIPNEUMOCOCCAL SERUM AND 1.17 MG. OF LUNG GALACTAN PER ML. OF TYPE II ANTISERUM

Substance	N pptd./ml. antiserum (μg.)[a]	Galactan pptd./ml. antiserum (μg.)	Ratio antibody N/galactan (as galactose) in ppt.	Glucuronic acid, μg./150 μg. galactan galactose in ppt.
Type XIV ppt.	246	205	1.2	1.2
Type II ppt.	57	33	1.7	6.0
Original galactan				2.8

[a] Analysis of trichloroacetic acid precipitate. See Table I for direct analyses.

Discussion

The present series of experiments has provided a fourfold demonstration of the power of immunochemical methods in carbohydrate chemistry and has permitted further predictions which remain to be tested.

(1) The precipitation of as much as 28% of the antibody in a Type XIV antipneumococcal horse serum by lung galactan established a structural relationship between the galactan and the specific capsular polysaccharide of Type XIV pneumococcus, previously shown to contain 3 galactose:1 N-acetyl-D-glucosamine[7] in its repeating unit.[17]

(2) The unexpected reactivity of the lung galactan in Type II antipneumococcal serum indicated a relationship between the galactan and the specific polysaccharide of Type II pneumococcus, known to consist of rhamnose, glucose, and glucuronic acid.[8–10] It could therefore be predicted that the small amount of uronic acid found in the galactan would prove to be glucuronic acid, and this was confirmed chemically, as described in the Experimental section.

(3) Since the binding of galactan to Type XIV antibody would be mediated through D-galactose units and its binding to Type II antibody would be through glucuronic acid residues, the determination of the uronic acid:D-galactose ratios in both precipitates sufficed to determine whether or not the uronic acid was an integral portion of the principal galactan component. Table III shows the ratio in the galactan to be 1:45–50, in reasonable agreement with the 1:35–40 previously found.[4] Since the ratio in the polysaccharide portion of the Type XIV precipitate was lower than in the galactan added, and the ratio in the Type II precipitate was higher, it was concluded (a) that the galactan was admixed with a component richer in glucuronic acid; (b) alternatively, that if the glucuronic acid residues were originally distributed unsymmetrically, degradation might have occurred during the preparative manipulations in such a way that one part of the material contained more glucuronic acid than the remainder.

(4) Tamarind seed polysaccharide (jellose) also gave a precipitate with Type XIV antiserum, providing a serological confirmation of an already chemically established structural relationship with the galactan, in this instance the presence in both of D-galactose linked only in position 1. Recently, however, glucose has been found[18] in a repurified sample of E. R. Squibb & Sons' S XIV as well as an early preparation kindly supplied by Dr. W. F. Goebel.[7] It is unlikely, however, that the cross reaction between tamarind polysaccharide and S XIV is due to any appreciable extent to the presence of glucose in both structures since lung galactan (which contains no glucose) removes almost the whole of the antibody in Type XIV antiserum reactive with tamarind polysaccharide (Table I, footnote *b*).

(5) According to the quantitative theory of specific immune precipitation in which both antigen and antibody possess multiple reactive groups,[19] the reactivity of the lung galactan and tamarind seed polysaccharide in Type XIV antiserum is referable to multiple recurrences in these polysaccharides of their common structural feature, D-galactose bound only in position 1. It may therefore be predicted (a) that any polysaccharide with such multiple terminal galactose residues will precipitate Type XIV antiserum,[20] and (b) that one or more of the three galactose residues in the specific polysaccharide of Type XIV pneumococcus will be found to be bound in this fashion. Steps are being taken to test this prediction.

(6) Since only 10% of the antibody in a Type XIV antiserum reacted with the tamarind seed polysaccharide and 28% of the antibody in the same serum reacted with lung galactan and the former portion is part of the latter (Table II, footnote), it is apparent that the galactan is more closely related to the Type XIV substance than is tamarind seed polysaccharide, assuming that the difference is not one of molecular size. It is therefore probable that when the chemistry of the Type XIV substance is

(16) Z. Dische, *J. Biol. Chem.*, **167**, 189 (1947).

(17) For a detailed discussion of serological relationships in terms of the structures of the Types III and VIII pneumococcal polysaccharides, see M. Heidelberger, E. A. Kabat and M. Mayer, *J. Exptl. Med.*, **75**, 35 (1942).

(18) M. Heidelberger, S. A. Barker and M. Stacey, *Science*, **120**, 781 (1954).

(19) M. Heidelberger and F. E. Kendall, *J. Exptl. Med.*, **61**, 563 (1935).

(20) The mucilages of guar, the Carob bean, and Kentucky coffee bean, galactomannans in which all of the galactose is similarly linked, precipitate Type XIV antiserum.

worked out it will be found to have more of the structure of the galactan in common than merely the singly linked terminal galactose residues.

Although the tamarind seed polysaccharide precipitates less of the Type XIV antibody than does the lung galactan, its reaction with the antiserum resembles the homologous type-specific reaction much more closely. The quantities necessary to reach the maximum are small, and an excess of polysaccharide causes inhibition of precipitation. In view of the present lack of knowledge of the exact structure of the galactose units in S XIV, the significance of these observations is difficult to assess.

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A New Procedure for the Isolation of Blood Group A Substance from Human Ovarian Cyst-Fluids[1]

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Material with blood group A activity has been isolated from two pseudomucinous ovarian cyst fluids by the process of electroconvection. The substances so isolated contain more nitrogen, probably as protein, and exhibit greater activity in the test for inhibition of hemagglutination than do substances prepared by a phenol-extraction method. In contrast to the phenol-extraction procedure that frequently yields at least two fractions with blood group A activity, the present method leads to the isolation of a single substance. The substance, in addition, has been found to be antigenic *per se* for the rabbit while other procedures have yielded material non-antigenic for the rabbit unless coupled to a protein.

Although several methods have been described for the isolation of substances having blood group specific A, B and O activity from human and animal sources, the procedure most frequently employed in recent years has been the phenol-extraction method originally devised by Morgan and King.[2,3] One feature that the various methods possess in common is the inclusion of procedures for the removal of protein, the resulting products being largely polysaccharide but containing some amino acids, probably as peptide. While seeking a simpler and somewhat less drastic isolation procedure than those previously described for the isolation of blood group substances from pseudomucinous ovarian cyst fluids, we found that paper electrophoresis could separate the active material from several inactive components. To obtain larger quantities of active substance, pseudomucinous ovarian cyst fluids from Group A secretors were subjected to fractionation by electroconvection; by this means a fraction was obtained that possessed the serological activity expected for blood group A substance. This fraction showed a higher nitrogen content than that of materials isolated by other procedures and gave reactions suggesting that it might be a polysaccharide-protein complex. This fraction had greater activity, as determined by the test for inhibition of hemagglutination, than substances isolated by the phenol-extraction method. In addition this product was antigenic *per se* for the rabbit, in striking contrast to previously studied material with blood group A activity that required coupling to a protein of *Sh. shigae*.[4-6]

Materials and Methods

Two human pseudomucinous ovarian cyst fluids were obtained from patients in the Sara Mayo Hospital of New Orleans through the kind coöperation of Dr. William Harris, Jr. Both cysts showed blood group A activity as determined by the test for inhibition of hemagglutination. Before being used for the experiments herein described, the fluid from cyst 9 had been stored for approximately a year, and that from cyst 13 for a month. During storage both materials were kept at 4–6° with added chloroform as preservative. The clear viscous liquid obtained by centrifugation of the cyst fluids was dialyzed against phosphate buffer *p*H 6.8–6.9 (prepared by dissolving 17.6 g. of KH_2PO_4 and 28.6 g. of Na_2HPO_4 in water to a volume of 5 liters) and was then placed in the electroconvection[7] apparatus, the buffer employed being the same as that against which it was dialyzed. A current of approximately 0.4 amp. and 25 volts was then passed through the apparatus for periods of time varying from about 20 to 100 hours, the temperature being maintained at 4–6°. The top and bottom fractions were removed and analyzed separately. It was possible to ascertain by visual observation that some fractionation had occurred since the bottom portion was brown while the top portion was a clear, colorless, slightly opalescent fluid.

Portions of the original cyst fluid and the top and bottom fractions were then subjected to analysis by means of paper electrophoresis.[8] The solutions were placed in the form of a band, 0.1 ml. of solution per inch of paper, on strips of Whatman No. 3 filter paper that had been previously dipped into buffer and blotted between paper towels to remove excess fluid. After the application of 250 volts for about 8 hours the strips were removed, dried in an oven at 110–120° for 20–30 minutes and treated for 5 minutes with a staining solution prepared by dissolving 40 g. of mercuric chloride and 0.5 g. of brom phenol blue in 400 ml. of 95% alcohol. The strips were then washed in running water until blue bands could be observed against a background essentially free of blue color. To separate sufficient material to permit analysis of the various zones, papers 6 in. wide were employed, the material to be separated and analyzed being pipetted onto a 5-in. section while a smaller separate portion was placed on the edge of the strip. After completion of the separation the edge strip was cut from the large sheet, dried and stained as described above; the main strip of paper was then cut into sections 10–15 mm. wide using the edge strip as a guide. Each section was extracted by being

(1) This investigation was supported by a research grant (RG 2964) from the National Microbiological Institute, National Institutes of Health, Department of Health, Education and Welfare.

(2) W. T. J. Morgan and H. R. King, *Biochem. J.*, **37**, 640 (1943).

(3) E. A. Kabat and A. E. Bezer, *J. Exp. Med.*, **82**, 207 (1945).

(4) W. T. J. Morgan, *Brit. J. Exp. Path.*, **24**, 41 (1943).

(5) W. T. J. Morgan and W. M. Watkins, *ibid.*, **25**, 221 (1944).

(6) S. J. Rainsford and W. T. J. Morgan, *Lancet*, **1**, 154 (1946).

(7) Purchased from the E. C. Apparatus Co., 23 Haven Ave., New York 32, N. Y.

(8) F. V. Flynn and P. de Mayo, *Lancet*, **2**, 235 (1951).