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Fractionation of Rabbit Antiserum (Antiphenylarsonicazo-Bovine Globulins) by Electrophoresis-Convection

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Rabbit antiserum containing antibodies against phenylarsonate and bovine globulin has been fractionated by the method of electrophoresis-convection. A partial separation of the two antibodies in the several gamma globulin fractions of the serum was achieved, thus demonstrating that they differ electrophoretically. The fractionation studies also have revealed the presence of incomplete (inhibiting or non-precipitating) antibodies in both positive and negative sera.

Introduction

Whether one considers that the specific combining sites of antibody molecules arise from a special chemical arrangement of constituent parts or merely a specific spatial arrangement of a common chemical structure, the resulting patterns on the surface of the antibody molecule mgiht be expected to produce a variation in the physical properties of antibody proteins, *i.e.*, differences in specific antibody activity might be accompanied by differences in physical properties. However, there is little or no evidence at present which would substantiate this idea. In fact, Treffers and Heidelberger[2] studying the antigenic specificity of antibodies against different types of Pneumococcus polysaccharides failed to find any antigenic difference although antigenicity is recognized as one of the most sensitive methods of detecting structural differences between protein molecules. There is no doubt that broad groups of different antibodies may show differences in their physical and immunological properties. For example, there are the so-called precipitin and antitoxin types in horse serum, the various types of anti-Rh antibodies in humans, and the possible differences between antibody and normal protein. However, such differences probably have no relationship to the specific combining site of the antibody molecule. In general, such physical differences have not been shown to exist.

In view of the recent developments in the fractionation of serum proteins by electrophoresis–convection methods and the particularly encouraging results obtained by Cann, *et al.*,[3] in the separation of the different types of anti-Rh antibodies the foregoing study was undertaken to determine whether the two antibodies (antiphenylarsonate and antibovine globulin) which are produced in rabbits injected with *p*-azophenylarsonate coupled to bovine globulin, (RBg) could be separated. Tests for possible separation of the two antibodies were based on quantitative precipitin data obtained under as nearly identical conditions as possible so that there would be little question as to the significance of ratios of the two antibodies. During the course of the investigation it became apparent that non-precipitating antibody was present in many sera. These data have also been included.

The electrophoretic separation of the antibodies which has been achieved cannot of course be unambiguously attributed to differences in the properties of the antibody sites themselves. It might also arise from differences in the structure of other parts of the gamma globulin molecules from which the antibodies are formed.

Experimental

Fractionation.—The details of construction and operation of the electrophoresis–convection apparatus have been described previously.[4,5a,5b]

Each of the sera considered here was carried through four or five successive stages of fractionation, the bottom fraction obtained in each stage serving as the starting material for the succeeding stage. The successive stages of fractionation, carried out at *p*H 7.5, 7.0, 6.5, 6.0 and 5.5, are designated as Stage A, B, C, D and E, respectively. Thus, serum diluted with one volume of buffer was fractionated in Stage A at *p*H 7.5. The resulting top fraction is designated as Top A. The bottom fraction, which included the solutions withdrawn from both the lower reservoir and the channel, is designated as Bottom A. Bottom A was refractionated in Stage B at *p*H 7.0, etc. In most instances each stage of fractionation was carried out in duplicate, composites being made of each of the resulting top and bottom fractions.

Fractionations were carried out in phosphate buffer, ionic strength 0.1, for 47–51 hours at field strengths of 1.5–2.5 volts/cm.

About half of the solution withdrawn from the top reservoir in each stage of fractionation was used for serological studies. The remainder of the top fraction was concentrated by pervaporation and analyzed electrophoretically. A portion of the bottom fraction was taken for serological studies. The remainder was diluted to the desired concentration and used in the next stage of fractionation. The bottom fraction of the final stage of fractionation was analyzed electrophoretically.

Electrophoretic Analysis.—The moving boundary technique of Tiselius[6] as modified by Longsworth[7] was used in the electrophoretic analysis. Electrophoretic experiments were carried out on 0.8–1.0% solutions of the top fractions in barbital buffer, *p*H 8.6 and ionic strength 0.1, at a field strength of 8 volts/cm. for two hours. Electrophoretic analyses of sera and bottom fractions were carried out on 2% protein solutions.

Mobilities were calculated in accordance with the suggestions of Longsworth and MacInnes.[8] The apparent concentrations of the electrophoretic components of the sera and the various fractions were determined from the electrophoretic patterns by finding the ratio, in each case, of the component area to the total area, exclusive of the ϵ boundary. The areas were measured on projected tracings of the descending patterns with a planimeter.

Serological Methods and Materials.—Sera were from rabbits which had been immunized for at least three months with bovine globulin–azophenylarsonate which analyzed

(1) Postdoctorate Fellow of the United States Public Health Service, National Institutes of Health.

(2) H. P. Treffers and M. J. Heidelberger, *J. Exp. Med.*, **73**, 293 (1941).

(3) J. R. Cann, R. A. Brown, D. C. Gadjusek, J. G. Kirkwood and P. Sturgeon, *J. Immunol.*, **66**, 137 (1951).

(4) J. R. Cann, J. G. Kirkwood, R. A. Brown and O. J. Plescia, THIS JOURNAL, **71**, 1603 (1949).

(5) (a) J. R. Cann, R. A. Brown and J. G. Kirkwood, *J. Biol. Chem.*, **181**, 161 (1949); (b) J. R. Cann, R. A. Brown, J. G. Kirkwood and J. H. Hink, Jr., *ibid.*, **185**, 663 (1950).

(6) A. Tiselius, *Trans. Faraday Soc.*, **33**, 524 (1937).

(7) L. G. Longsworth, *Chem. Revs.*, **30**, 323 (1942).

(8) L. G. Longsworth and D. A. MacInnes, THIS JOURNAL, **62**, 705 (1940).

40 arsonic acid groups per molecule of globulin. The pools were made from individual sera showing about the same anti-R titers and represented ten or more animals. The first run (Expt. I) was made on a pool of fresh serum and the second (Expt. II) and third runs (Expt. III) were made on pools which had been stored at −20° for about two months.

The serum used in the first experiment analyzed 2.63 mg. of anti-R and 0.64 mg. of anti-Bg per ml. The serum used in the second experiment analyzed 4.84 mg. of anti-R and 0.66 mg. of anti-Bg per ml. The serum used in the third experiment was a pool from immunized rabbits which failed to give any precipitin reactions with either R_3' or Bg antigens.

Precipitin tests were made in the usual manner by adding dilutions of antigen, which varied by a factor of two, to constant amounts of antibody solution. The antibody solutions were first dialyzed to equilibrium against 1.0% NaCl and adjusted, as indicated by preliminary titration, to a concentration which would give not more than 1.0 mg. of antibody per ml. The antigen used to test for antiarsonilate antibodies (anti-R) was 1,3,5-*p*-(*p*-azophenylazo)-phenylarsonic acid resorcinol (R_3') dye. The bovine globulin used throughout was a sample obtained from Armour and Co. Sufficient borate buffer was used in all tests to adjust the *p*H to 8.0. Precipitates were allowed to develop for 60 hours at 4° and then washed and analyzed as previously described.[9] The concentration of antibody was calculated directly on the basis of maximum precipitation with the assumption that all the antigen present had been precipitated.

This method of antibody determination is limited by the preciseness of attaining the true point of maximum precipitation. This point may be missed when twofold differences in antigen dilutions are used but in the present systems the change in the amount of precipitate with varying antigen concentrations was rather gradual near the equivalence point. The error involved was estimated at approximately 15%.

Results

Three different samples of pooled anti-RBg rabbit sera were fractionated but only the fractions of Experiments I and II were characterized electrophoretically. In Experiment I the serum was carried through four stages of fractionation and in Experiment II the serum was carried through five. The results of the electrophoretic characterization of the resulting fractions are presented in Table I. The electrophoretic pattern of the serum used in Experiment II is shown in Fig. 1c; and those of several representative fractions of this serum in Fig. 2. In Experiment III, 60 ml. of serum from animals which apparently had failed to respond to immunization, as judged from serological studies carried out on the unfractionated serum, were carried through four stages of fractionation.

The γ-globulin in the serum of Experiment II resolved into two electrophoretic components. Except for Top A all the fractions obtained in Experiment II showed some evidence of two γ-globulin components. Since the γ-globulin of normal rabbit serum does not resolve into two components, electrophoretic analyses were carried out on a series of pooled antisera in order to determine whether or not the appearance of the slow moving component can be correlated with the anti-R titers of the serum. The electrophoretic patterns of several of these sera are presented in Fig. 1. The mean mobility of the γ-globulin in these sera ranged from -1.50×10^{-5} to -1.71×10^{-5}. No resolution of the γ-globulin was observed in the sera with titers of 0.24, 1.10 and 1.85 mg. anti-R protein per ml. However, the γ-globulin of the serum with titer 3.9 mg. anti-R protein per ml. resolved into two components with mobilities of -1.1×10^{-5} and -1.69×10^{-5}. The slower moving component comprised about 20% of the total γ-globulin. These mobilities are in excellent agreement with those of the components in the serum used in Experiment II. Thus, the appearance of the new component with mobility -1.14×10^{-5} is associated with an increasing anti-R titer.

Sedimentation studies[10] of fraction A, Experiment II which contained 50% precipitable anti-R protein, showed only one component with a sedimentation constant of 6.

Serological.—The serological analysis of the various fractions which are given in Tables II and III indicate at least four significant results of fractionation. First, the amount of anti-R relative to anti-Bg was greatly increased in top fractions, which suggests that the anti-R has a slightly lower mobility than the anti-Bg. Second, the anti-R antibody was greatly concentrated by fractionation (anti-R in Top A, Experiment II was 50% of the total protein). Third, the greatest concentration of anti-R occurred in the third top in Experiment I and in the first top in Experiment II. This change in distribution of anti-R bodies among the fractions of γ-globulin may be correlated with a difference in orginal titer of the sera and the appearance of a new electrophoretic component, -1.14×10^{-5}, in high titer sera. Fourth, there was, in general, an over-all loss of antibody but in many instances individual fractions showed an

TABLE I

ELECTROPHORETIC CHARACTERIZATION OF FRACTIONS

Fraction	Yield, g.	Electrophoretic distribution of components: serum fractions, % γ-Globulin	β-Globulin	α-Globulin	Albumin	Mean mobility,[d] $-10^5 \times \bar{\mu}$ of γ-globulin cm.^{2} sec.$^{-1}$ volt^{-1}
Experiment I						
Top A	0.19	98				1.24
Top B	.20	98				1.33
Top C	.23	95	5			1.73
Top D	.19	96	4			1.79
Bottom D		7	11	11	71	1.95
Experiment II						
Serum		18[a]	10	11	61	1.50
Top A	.27	98				1.18
Top B	.27	98				1.31
Top C	.36	98				1.51
Top D	.27	88[b]	12[c]		Trace	1.77
Top E	.22	67	27[c]		6	1.81
Bottom E		4	8	13	75	2.1

[a] γ-Globulin resolved into two components with mobilities -1.14×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1} and -1.63×10^{-5} at *p*H 8.6. The serum contained 8% of the slow moving component. [b] γ-Globulin resolved into two components with mobilities -1.53×10^{-5} and -1.85×10^{-5} at *p*H 8.6. The fraction contained about 40% of the slow moving component and about 48% of the fast moving component. [c] Includes α-globulins. [d] Mean mobilities were determined in barbital buffer, *p*H 8.6 and ionic strength 0.1.

(9) Frank Lanni and Dan H. Campbell, *Stanford Med. Bull.*, **6**, 97 (1948).

(10) J. R. Cann, R. A. Brown, S. J. Singer, J. Shumaker and J. G. Kirkwood, in preparation.

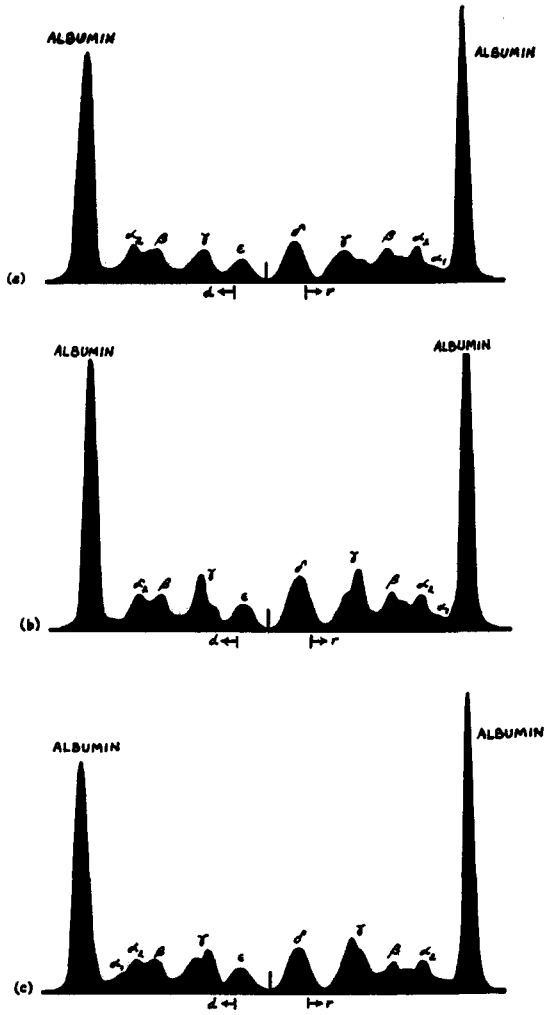

Fig. 1.—Electrophoretic patterns of rabbit RBg antisera containing (a) 1.85 mg. of anti-R protein (b) 3.9 mg. of anti-R protein (c) 4.84 mg. of anti-R protein.

amount of antibody greater than the starting material.

This latter finding occurred so frequently in these and other experiments that the possibility of inhibiting substances being present in the bottom fractions was investigated further. For example, in the D fractions of Experiment I the starting material, which was the remaining bottom C, showed 17 mg. of anti-R and 65 of anti-Bg, but the subsequent top fraction showed a slight increase of anti-R (22 mg.) a small amount of anti-Bg while the bottom showed no anti-R and only 15 mg. of anti-Bg. Tests were then made to determine if recombination of top and bottom would restore the original anti-Bg value. On the basis of anti-R and anti-Bg found in the top and bottom the calculated amount which should have been obtained upon recombination was 0.13 mg./ml. The value actually obtained was 0.33 mg./ml. which was about 2.5 times more than calculated. This value does not give complete recovery of anti-Bg activity but indicates, however, that fractionation separates antibody activity. A similar experiment was run in which the effect of bottom D on whole anti-RBg serum was determined. The R'_3 dye was

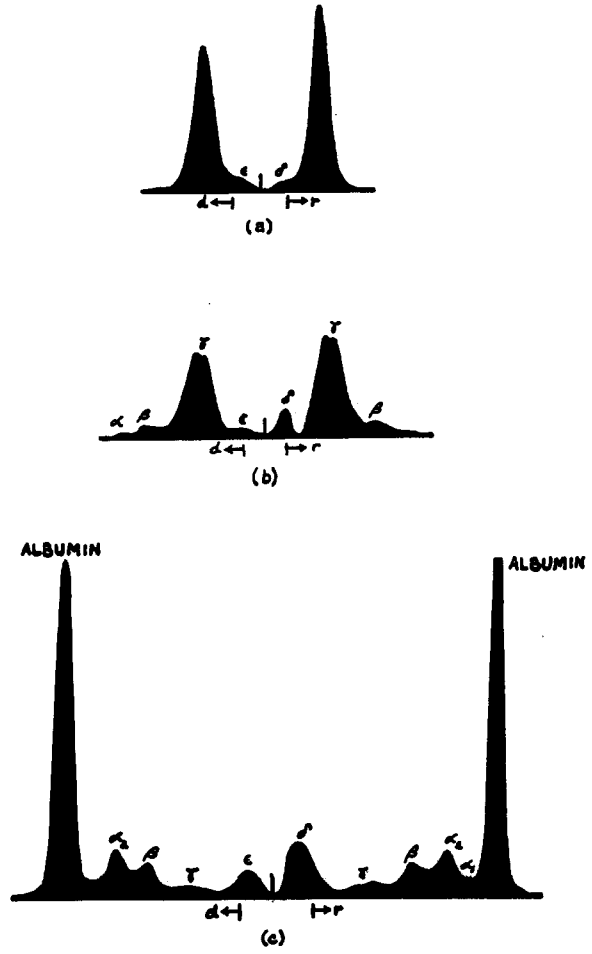

Fig. 2.—Electrophoretic patterns of fractions, Experiment II (a) Top A (b) Top D (c) Bottom E.

used as a test antigen and added to antiserum plus saline, antiserum plus a bottom C fraction from normal serum and to antiserum plus bottom D of Experiment I. The first two tests gave 0.87 and 0.73 mg. of anti-R, respectively, while the bottom D increased the amount of precipitate to 1.37 mg. It was apparent that the bottom D contained some material which would not precipitate R_3' antigen by itself but would attach to a specific precipitate. It was also apparent that under certain conditions this material would inhibit precipitation since release of precipitable antibody was accomplished by fractionation. Thus in Experiment II the top fractions, C, D and E all had more antibody than the original material.

Further studies of the effect of bottom E (Expt. II) on precipitation indicated that this particular fraction consistently produced a slight reduction in the amount of anti-R precipitated and a slight increase in the amount of anti-Bg precipitated under the conditions chosen. For example, a system of anti-RBg serum and R_3' antigen which was adjusted to give about 3.0 mg. of antibody in control tests gave 2.39 when bottom E was added. The same serum gave 0.40 mg. of anti-Bg in control tests and 0.61 mg. when bottom E was added. In all instances bottom E had a strong retarding effect upon the rate of precipitation in all the tests

TABLE II

SEROLOGICAL CHARACTERIZATION OF FRACTIONS

Fraction	Precipitable antibody % of fraction Anti-R protein	Anti-Bg protein	Ratio of Anti-R protein to Anti-Bg protein
	Experiment I		
Unfractionated serum			4.0
Top A	21	1.8	12
Top B	25	1.9	13
Top C	37	2.0	19
Top D	12	0.6	20
Bottom B[a]			4.3
Bottom C[a]			0.3
Bottom D[a]			0.0
	Experiment II		
Unfractionated serum			7.3
Top A	50	1.6	31
Top B	40	0.9	44
Top C	38	1.6	24
Top D	25	0.5	50
Top E	12	0.4	30
Bottom A[a]			5.4
Bottom B[a]			10
Bottom C[a]			2.5
Bottom D[a]			1.1
Bottom E[a]			1.0

[a] No values for bottom because of low antibody content.

TABLE III

TOTAL PRECIPITABLE ANTIBODY CONTENT OF THE FRACTIONS OF ANTI-RBG SERA

Stage of fractionation	Starting material, mg. Anti-R protein	Anti-BG protein	Top fraction, mg. Anti-R protein	Anti-BG protein	Bottom fraction, mg. Anti-R protein	Anti-BG protein	Antibody recoverd,[a] mg. Anti-R protein	Anti-BG protein
			Experiment I					
A[b]	310	76	40	3	212		252	
B	192		50	4	65	15	115	19
C	59	14	88	5	24	94	112	99
D	17	65	22	1	None	15	22	16
			Experiment II					
A	580	80	134	4	190	35	324	39
B	169	31	108	3	113	11	221	14
C	107	11	139	6	38	15	117	21
D	36	14	66	1	12	11	78	12
E	10	9	26	1	3	3	29	4

[a] The over-all recovery was 233 mg. anti-R protein (75%) and 29 mg. of anti-BG proteins (38%) in Experiment I; and 509 mg. anti-R protein (88%) and 25 mg. anti-BG protein (31%) in Experiment II. Total recovery is the sum of the antibody content of the top fractions, the bottom fraction of the last stage of fractionation, and the samples of the bottom fractions used for serological studies prior to the last stage of fractionation. [b] Starting material for A was the original serum.

for the first 12 hours. In fact up to this point analysis of the tests would have demonstrated a marked inhibition effect. The results given above were taken 72 hours after mixing.

In both Experiments I and II considerable antibody activity is lost in the first stage of fractionation and although some of the activity is regained in subsequent fractionation the loss could be the result of an irreversible change in the serum caused by the fractionation procedure. This possibility has been eliminated. A sample of serum was carried through the first stage of fractionation and titers determined on the original serum, top and bottom fraction, and a composite of the two fractions. The sample of the original serum contained 321 mg. of anti-R protein and 7.5 mg. of anti-Bg protein; the top and bottom fractions contained 55 and 127 mg., respectively, of anti-R protein and 0.20 and 5.6 mg., respectively, of anti-Bg protein, the composite contained 285 mg. of anti-R and 7.9 mg. of anti-Bg protein. Although only 57% of anti-R and 76% of the anti-Bg protein were recovered from the fractions, 87 and 105%, respectively, were recovered from the composite. The amount of antibody in the original serum and in the composite were equal within the experimental error.

Although considerable study has been made in the past on sera from rabbits which failed to show any precipitin titer, no demonstrable antibody activity has ever been obtained. However, in view of the results obtained above, a pool of negative serum from rabbits receiving RBg antigen over a period of four to six months was fractionated and tested. The first top was unexpectedly positive for anti-R. About 1.1 mg. of anti-R was obtained in the first top from an original serum sample of 60 ml. The second top was negative, the third top was positive with a yield of 2.7 mg. of anti-R and the fourth (last fraction) was negative. The appearance of antibody in the third top was of considerable interest since the third top of Experiments I and II both contained the maximum amount of antibody. The last bottom fraction of this series was tested for inhibition effects in an R_3' anti-RBg system, a system in which control tests produced 0.87 mg. of anti-R was reduced to 0.78 mg. This inhibition was much less than would be expected of a "negative" serum which yielded appreciable amounts of precipitating antibody upon fractionation.

Discussion

The foregoing results clearly show that antibody molecules which occur in rabbit serum following immunization with bovine globulin–azophenylarsonate can be partially separated by electrophoresis–convection into fractions having different reaction capacities (precipitability) and different specificities (*viz.*, antiarsonillate and antibovine globulin). The separation of inhibiting antibodies from rabbit antiserum confirms an earlier observation made by Heidelberger and Kendall[11] and suggest that so-called precipitin titers, in general, represent the net results of competing antibody molecules of various reaction capacities. The separation of antibody molecules by electrophoresis–convection into fractions having different specificities indicates that at least in this instance, the specific nature of the combining site has some influence on the net charge of the molecule.

Many apparent inconsistencies cannot be explained on the basis of experimental error since in most instances confirmation was easily made and experimental error could be ruled out. For example, in the experiment in which bottom E was added to anti-R and anti-Bg systems a decrease

(11) M. J. Heidelberger and F. E. Kendall, *J. Exp. Med.*, **62**, 467 (1935).

was obtained in the anti-R system, but an increase in the anti-Bg. Since the R_3' antigen had only three combining sites and bovine globulin would probably have many more, one might expect the R_3' system to be inhibited more easily by incomplete antibodies while the large number of combining sites on the globulin molecule could accomodate both incomplete and precipitating antibodies and result in an actual increase in precipitated protein. Interpretation must, however, await further investigation since it is obvious too little is known at present regarding the significance of the relative concentration of the various antibodies and mechanisms involving competition for antigenic combining sites.

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Investigations on Lignin and Lignification. VI. The Comparative Evaluation of Native Lignins[1]

BY S. F. KUDZIN, ROBERT M. DEBAUN AND F. F. NORD

The native lignins of white Scots pine, oak, birch and maple and the native lignin of white Scots pine obtained after decay by *Lentinus lepideus* were compared by chemical and physical methods. The lignins were methylated with diazomethane, oxidized with nitrobenzene and alkali, subjected to paper chromatography and tested colorimetrically with the phosphotungstic–phosphomolybdic reagent for phenols. Infrared spectra of the softwood lignins and of the methylated softwood and hardwood lignins are presented. The lignins from sound and decayed white Scots pine contained the same number of methylatable groups, while the other lignins differed from the white Scots pine and from each other in this respect. None of the lignins yielded syringaldehyde upon oxidation, excepting that derived from maple wood. All the lignins responded similarly to the phenol reagent, except lignin from oak wood. The lignins from sound and from decayed white Scots pine were shown to be similar in every respect investigated.

Chemically prepared lignins from softwoods and hardwoods are known to be materially different, especially as regards methoxyl content. Although aspen native lignin[2] has been shown to differ from softwood native lignins, the native lignins of oak, birch and maple[3] possess many properties similar to those of softwood lignins.[4] Furthermore, the native lignins from sound and decayed softwoods also possess similar properties.[4c,d,e]

We have extended the comparison of the native lignins from sound white Scots pine and the native lignin from that wood after decay by *Lentinus lepideus* and of the native lignins of the hardwoods oak, birch and maple by means of methylation with diazomethane, infrared spectra of the methylated lignins, quantitative determination of the vanillin and syringaldehyde obtained on oxidation with nitrobenzene and alkali, paper chromatography, and colorimetric response to the phenol reagent.[5] The previously reported[4] spectra of the lignins from white Scots pine are interpreted with respect to the functional groups present.

Methylation with diazomethane is generally considered to be specific for phenolic, enolic and acidic hydroxyl groups. In addition to the purely comparative value, the oxidation of the hardwood lignins was undertaken to corroborate our previous suggestion[3] that oak and birch native lignins were devoid of syringyl groups, whereas the maple native lignin appeared to contain such structures. The lignins were subjected to paper chromatography in order to ascertain the homogeneity of the samples. Quantitative response to the phenol reagent was estimated as a relative measure of the groups reacting with this substance. A similar reagent[6] was used in the comparison of native lignin preparations obtained from cork.[7] Although the reagent is affected by phenolic hydroxyl groups, the chemistry of the reaction is not altogether clear.[8,9] The reagent is a phosphotungstic–phosphomolybdic acid of the 1:18 series according to the earlier nomenclature.[10]

Experimental

The native lignins studied were those previously reported.[3,4c]

Methylation.—A dioxane solution of diazomethane for the methylation of one gram of native lignin was prepared by adding one gram of nitrosomethylurea to a cooled mixture of dioxane (14 ml.) and 45% potassium hydroxide (2 ml.). The dioxane solution was then decanted and allowed to stand over pellets of potassium hydroxide for several hours. This solution was then added to a 10% solution of native lignin in dioxane, and the mixture was allowed to stand overnight, centrifuged and precipitated into ether. This procedure was repeated until a constant methoxyl value was obtained.

Oxidation of Native Lignin and Quantitative Determination of Vanillin and Syringaldehyde.—A chromatographic separation[11] was employed for these studies.

Paper Chromatography of the Native Lignins.—Dioxane solutions of the native lignins were placed in a front along the bottom of a piece of Schleicher and Schuell paper No. 696. The dioxane solutions were allowed to dry, and the paper rolled into a cylinder, held fast with paper clips and

(1) Presented at the *Lignin Round Table* held during the XIIth Internat. Congress of Chemistry, New York, N. Y., 1951. These data are taken from parts of the dissertations of S.F.K. and R.M.D. submitted to the Graduate School of Fordham University in partial fulfillment of the requirements of their degrees of Doctor of Philosophy, 1951. For paper No. V of this series see THIS JOURNAL, **73**, 1358 (1951).

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