duced catalase."[7] The temperature coefficient of the "over-all" reaction between catalase and hydrogen peroxide has been reported[8] as Q_{10} = 1.4 (0–20°). I have been able to confirm this value in experiments conducted by the titration method.

If one wishes, therefore, to express results in terms of *Kat. f.*, the measurement with the dropping mercury electrode should be made at a temperature in the neighborhood of 20°, and the value of K corrected to 0° by the use of this known value of Q_{10}. The *Kat. f.* of a preparation of crystalline catalase from beef liver[9] was 34,800 by the electrical method, corrected to 0°, and 36,000 by the titration method at 0°. The *Kat. f.* of crystalline catalase is now known to be variable in different preparations.[10] The value obtained is within the limits established by Sumner, Dounce and Frampton.

(7) Keilin and Hartree, *Proc. Roy. Soc.* (London), **B124**, 397 (1938).
(8) Stern, *J. Biol. Chem.*, **114**, 473 (1936).
(9) Sumner and Dounce, *ibid.*, **127**, 439 (1939).
(10) Sumner, Dounce and Frampton, *ibid.*, **136**, 343 (1940).

Other units of catalase potency involving a measurement of the reaction velocity constant[11] may be determined directly or by the use of a temperature correction according to the definition of the unit.

This work was done with the technical assistance of Miss Mary McManus.

Summary

A method is described for the measurement of the velocity of the decomposition of hydrogen peroxide by catalase, utilizing the dropping mercury electrode, without a polarograph or other automatic recording device. The method is applicable at ordinary room temperatures, giving good agreement with titration measurements. At temperatures around 0° it yields consistently lower velocity measurements than those obtained by titration, suggesting the presence at lower temperatures of a measurable concentration of an intermediate enzyme-substrate compound.

(11) Williams, *J. Gen. Physiol.*, **11**, 309 (1927).

BOSTON, MASS. RECEIVED APRIL 19, 1941

[CONTRIBUTION FROM THE CHEMICAL LABORATORY, UNIVERSITY OF TORONTO]

The Action of Diazomethane on Lactones and on Lignins

BY E. Y. SPENCER AND GEORGE F. WRIGHT

The reaction of diazomethane with acidic hydroxyl groups has been utilized by some workers as a means of evaluating the amount of such hydroxyl groups in lignins.[1] Others have arbitrarily assumed that isolated lignin was a homogeneous substance having one acidic hydroxyl group per recurring unit in the "molecule." On the basis of diazomethane reaction they have assigned a unit molecular weight to the substance.[2] Such an assumption would seem to be unwarranted from several points of view. First, evidence has been accumulating to indicate that lignin in wood is bound through its phenolic hydroxyl groups. These linkages appear in the extracted materials as phenolic esters of the organic acid (acetic or formic) used as extractant as well as phenol glycosides or phenol glycuronides and their acetates. Such linkages become apparent when the acetic or formic acid-extracted lignin is subjected to either alkaline or acid hydrolysis.[3] These hydrolyses liberate carbohydrate (part of which has been identified as xylose), the organic acid used in the extraction process, and an isolated lignin having a much higher phenolic hydroxyl content, estimated by reaction with diazomethane, than was found in the original extracted lignin. This phenolic hydroxyl content is likewise greater than that found in isolated lignins obtained by more drastic extraction methods involving use of mineral acid or alkali at elevated temperatures. It would indeed appear from the analytical data[3] that these phenolic linkages include most of the hydroxylic groups in the non-saccharide portion of the lignin complex.[4] In

(1) Fuchs and Horn, *Ber.*, **62**, 1691 (1929); Freudenberg and Sohns, *ibid.*, **66**, 262 (1933).
(2) Brauns and Hibbert, THIS JOURNAL, **55**, 4720 (1933); Brauns, *ibid.*, **61**, 2120 (1939).
(3) (a) Bell, Wright and Hibbert, unpublished results completed at McGill University in August, 1937. (b) Lieff, Wright and Hibbert, THIS JOURNAL, **61**, 1477 (1939).
(4) In this Laboratory lignin is regarded, according to the botanical definition, as the non-cellulosic incrusting material in wood xylem. The relationship of this lignin to the materials isolated by various methods and designated by prefix such as Klason lignin, methanol lignin, etc., depends entirely on the method of extraction. Attempts to extract lignin free from detectable carbohydrate in no way demonstrate that the incrusting substance in wood is not partly saccharide in nature. The fact that acetic acid birch lignin contains combined carbohydrate when it is isolated is considered here as evidence that lignin, the cementing material, contains carbohydrate in the wood

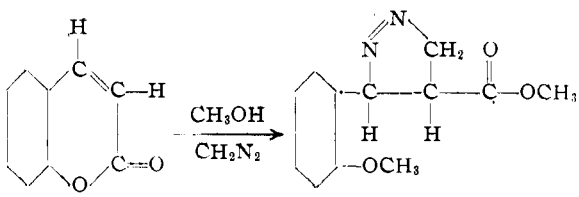
view of these results it would seem to be somewhat dangerous to designate as "phenolic hydroxyl content" that portion of the total which may only be freed in different degree using different extraction processes, more especially when this "phenolic hydroxyl content" serves as an estimation of "unit molecular weight."

Secondly, diazomethane may act upon functional groups other than phenolic hydroxyls by a solvolysis reaction. Azlactones have been found to solvolyze under these conditions to form carbomethoxy derivatives when diazomethane is present.[5] Saccharomonolactone, on the other hand, seems not to be methoxylated with diazomethane at the lactone linkage, but suffers loss of water between C_4 and C_5 with subsequent methoxylation of the resultant enol by diazomethane. Additional methoxylation occurs, not only at the carbonyl group,[6] but also at the hydroxyl group adjacent to it. These examples suffice to show that diazomethane methoxylation is an unsatisfactory method for evaluation of phenolic hydroxyl groups.

It occurred to us that methoxylation by diazomethane of the isolated lignins[2] might be owing to a simple lactone linkage since hydrolysis or methanolysis of the small amount of this linkage present might readily occur under the conditions of diazomethane methoxylation. We chose valerolactone and coumarin as typical lactones with which to test this hypothesis.

The first of these compounds, valerolactone, when treated with diazomethane in dry methanol solution, was found to consume the reagent slowly over a period of days. When the yellow color of the diazomethane persisted after eight days, the solvent was removed and the residue distilled to yield a product containing 8.8% methoxyl. This was fractionated to yield 57% of unchanged valerolactone and a higher boiling fraction which was demonstrated to contain methyl 4-hydroxypentanoate by conversion into the more stable acetyl derivative. The higher boiling fraction therefore contained 43% of this ester which has a methoxyl content of 23.3%.

A different reaction occurred with coumarin in absolute methanol. The diazomethane was consumed over a ten-day period. A crystalline compound was isolated from the reaction product which conformed by analysis to the composition of 3-carbomethoxy-4-(*o*-methoxyphenyl)-pyrazoline. In this case diazomethane has reacted with both phenolic and carboxylic hydroxyl as well as with the ethylenic linkage. The latter reaction is not unprecedented.[7]



While these reactions with typical lactones are slower than the replacement of acidic hydrogen by a methyl group, the contribution that they may make to a reaction with lignin intended to evaluate such hydroxyl renders the diazomethane reaction of little value as an analytical tool in lignin chemistry.

If the lactone linkage in lignin is similar to the coumarin type, which seems likely in view of previous work,[8] then all of the products obtained by treating isolated lignin with diazomethane ought to contain nitrogen. We tested a series of such samples of isolated lignins obtained by different extraction methods and diazomethane-methoxylated by procedures involving presence of and apparent absence of solvolyzing media such as methanol and water. In every case slightly less than 1% of nitrogen was present, although a control analysis on several of the isolated lignins before methoxylation showed absence of nitrogen. The nitrogen was therefore not present before diazomethane treatment, nor was its presence owing to the infusible by-product that is frequently found during diazomethane reactions. This by-product was found to be nitrogen-free. The nitrogen thus seems to be an integral part of isolated lignins extracted by alkali, mineral acid and organic acids, after they have been treated with diazomethane.

The relationship of this nitrogen content to the lignin linkage which might be characteristic of it was studied by qualitatively analyzing a number of modified lignin samples for nitrogen. Because of the low nitrogen content the ordinary elemental analysis was altered to give satisfactory tests with nitrogen-free samples to which known amounts of urea were added. The amount of Prussian blue coloration was found to be a rough measure of the nitrogen content.

(5) Fischer and Hoffman, *Z. physiol. Chem.*, **245**, 139 (1937).

(6) Schmidt and Zeiser, *Ber.*, **67**, 2120 (1934); Schmidt, Zeiser and Dippold, *ibid.*, **70**, 2402 (1937).

(7) (a) Pechmann and Burkard, *ibid.*, **33**, 3594 (1900); (b) Hansen. *ibid.*, **64B**, 943 (1931).

(8) Spencer and Wright, THIS JOURNAL, **63**, 1281 (1941).

Application of this test to diazomethane-methoxylated birch acetic acid lignins showed that the methanol soluble fraction thus treated did not lose all of its nitrogen when boiled for seven hours with 28% sulfuric acid. The linkage is therefore more stable than one would expect for the nucleus which would result as an intermediate in the reaction of diazomethane with an aldehyde or ketone linkage.[9] It would seem, then, that presence of one of the latter linkages is less probable than the coumarin-type of linkage since the pyrazoline would be much more likely to survive this severe treatment.

Since reliable purification procedures have not been available to lignin chemists, the homogeneity of the substance at hand is always questionable. There might therefore exist the possibility that a coumarin-type lactone linkage, although present in the wood, was not intrinsically a part of the lignin but was associated with it by the process of extraction. This might possibly be the case if the resins had been incompletely extracted from the wood before the lignin extraction was carried out. Reactions were therefore carried out which might be expected to eliminate such a contaminant if it were present. Thus alkali–dimethyl sulfate treatment should produce a methoxylated product from which more soluble contaminants could be eliminated by solvent fractionation. Comparison of the nitrogen content in a diazomethane-methoxylated acetic acid-birch lignin before and after reaction with dimethyl sulfate shows that this is probably not the case; retention of the nitrogen indicates that a coumarin-type linkage in the lignin structure is more probable.

Another experiment intended to demonstrate the same point is also significant concerning the general question of phenolic hydroxyl groups in lignin. It has been shown that the ether-soluble fraction of yellow birch acetic acid lignin can be methoxylated by diazomethane from an initial 19.7% methoxyl to about 21.9%, but if this isolated lignin be first hydrolyzed by action of 10% alkali, then diazomethane increases the methoxyl value from 23.3 to 34%.[3a] Parenthetically it may be noted that the difference in methoxyl content between the saponified and unsaponified samples allows an estimation of the weight of fragment which has been separated from the lignin complex by alkaline hydrolysis. We have now shown that this increase in methoxyl content can be effected from a value of *ca.* 24 to *ca.* 32% by allowing the isolated lignin (OCH_3 = 19.7%) to stand for several weeks in a methanol–hydrochloric acid solution and subsequently diazomethane-methoxylating the resulting product. The carbohydrate fraction obtained from this hydrolysis is still being investigated, but at least a part has been re-identified as xylosazone[3] under conditions which suggest that this pentose is a fragment of a uronic acid. This finding supports the former hypothesis that carbohydrate is bound to lignin by a phenolic glucoside type of linkage. It is pertinent to the discussion in this paper that the acid-hydrolyzed, diazomethane-methoxylated lignin contained about the same amount of nitrogen as an identical acetic birch lignin which had not been acid-hydrolyzed before methoxylation with diazomethane. The functional group which is responsible for this combination of nitrogen is therefore not in that portion of the lignin complex which is assumed to be carbohydrate.

It is obviously unsafe to draw any quantitative conclusion from the results of diazomethane action on isolated lignins. However, one might speculate upon the fact that the methoxyl increase which has been attributed to phenolic hydroxyl[2] may be explained equally satisfactorily as due to an incomplete reaction involving methoxylation and pyrazoline formation owing to a coumarin type of lactone linkage.

Experimental

Methyl 4-Acetoxypentanoate.—When 37.5 g. (0.375 mole) of valerolactone in 375 cc. of absolute methanol was treated at 1° with an ether solution of the diazomethane from 113 g. of nitrosomethylurethan, the yellow color disappeared in four days. The diazomethane from 17 g. of nitrosomethylurethan was then added; the color persisted for eight days. Diazomethane polymer was filtered off and the filtrate distilled *in toto* at 67–78° (8 mm.). The methoxyl content of this distillate was 8.8%. Upon redistillation two fractions were collected: b. p. 65–70° (1 mm.), $n^{24}D$ 1.4303, weight 14 g.; b. p 68–75.5° (1 mm.), $n^{24}D$ 1.4311, weight 20.1 g., 10.1% methoxyl. The latter fraction was treated with acetic anhydride and pyridine[3] and the acetylated product wastefully fractionated from valerolactone at 12 mm. to yield 6.4 g., b. p. 92–93°, $n^{22}D$ 1.4260; saponification equivalent, calcd., 87; found, 89. An intermediate fraction contained 17.1% OCH_3; calcd. for the pure ester 17.8% OCH_3.

3-Carbomethoxy-4-(*o*-methoxyphenyl)-pyrazoline.—When 1.46 g. (0.01 mole) of coumarin (purified by decomposition of the bisulfite addition product at 200°) was dissolved in 30 cc. of absolute methanol and treated at 0° with the diazomethane from 6.2 g. of nitrosomethylurethan,

(9) Arndt and Eistert, *Ber.*, **61B**, 1118 (1928); Arndt, Eistert and Ender, *ibid.*, **62B**, 44 (1929).

the yellow color disappeared in one week. One-tenth of the original amount of diazomethane was then added, which persisted after three days. After filtration from diazomethane polymer the solution was evaporated under 8 mm. to leave an oil. When this was dissolved in ether, 0.7 g. of yellow crystals precipitated (30% of theoretical). No further yield could be obtained from the residual oil. After two crystallizations from ether the compound melted at 94.5°; saponification equivalent calcd., 234; found, 233.

Anal. Calcd. for $C_{12}H_{14}O_3N_2$: C, 61.5; H, 5.98; N, 11.97; OCH_3, 26.5. Found: C, 61.8; H, 5.93; N, 12.23; OCH_3, 25.8.

Acid Hydrolysis of Ether-soluble Acetic Acid Extracted Yellow Birch Lignin.—One gram of this ether-soluble fraction prepared by the directions of Bell, Wright and Hibbert (OCH_3, 19.7%) was dissolved in 50 cc. of methanol and treated with 4 cc. of concentrated hydrochloric acid. The solution became warm, a slight gas evolution being noted. After eighteen days the solvent was removed under 6 mm. pressure. The residue was now insoluble in benzene, partly soluble in chloroform and completely soluble in dioxane and in aqueous alkali. It was dissolved in dioxane and precipitated into ether to yield 0.46 g. of a first fraction; OCH_3, 23.2%. The precipitating liquors, evaporated to dryness, dissolved in chloroform and precipitated into 28–38° petroleum ether yielded a second fraction, 0.32 g.; OCH_3, 24.3%.

In a second experiment the residue resulting from vacuum evaporation of the methanol–hydrochloric acid solution was taken up in water and centrifuged. The supernatant liquor after continuous ether extraction to remove 0.32 g. of distillable material gave a weak Molisch test which was markedly increased after addition of hydrochloric acid to a concentration of 8% and subsequent two hour reflux. From this solution 0.03 g. of xylosazone, m. p. 159°, was obtained, which was identical with that obtained by formic acid reflux of acetic acid birch lignin[3a] and with an authentic sample.

Diazomethane Methoxylation of Acid Hydrolyzed Lignins.—Each of the fractions obtained above was dissolved in 50 cc. of sodium benzophenone-dried dioxane and was treated with an excess of diazomethane prepared by procedure A, subsequently described, over a nine-day period. Diazomethane was added from time to time to ensure presence of an excess. At the end of this time the solvent was removed from each under 8 mm. and each was precipitated from benzene into 28–38° petroleum ether. Fraction 1 yielded 0.268 g.; OCH_3, 32.5%. Fraction 2 yielded 0.05 g.; OCH_3, 31.9%.

Nitrogen Content of Methoxylated Lignins.—The lignin samples were methoxylated with diazomethane according to two procedures. Procedure A involves the addition of an ether solution of diazomethane which was prepared from nitrosomethylurethan and methyl alcoholic potassium hydroxide to an acetone or dioxane solution of the lignin; it therefore may contain some methanol. Procedure B employs diazomethane evolved from an ethylene glycol solution and therefore is free from methanol.

The qualitative analysis of these lignins involved a micro adaptation[10] of the ordinary macro procedure, since either micro or macro procedures alone failed to give satisfactory tests on samples known to contain 1% of nitrogen. A sample of 0.1 g. of lignin was added over a half-minute period to a 75-mm. test-tube containing about 0.15 g. of molten sodium. After continued heating for one minute the tube was dropped into 5 cc. of water, which was then boiled, filtered and evaporated to about 0.3 cc. One-half of the concentrate was stirred with one drop of 4% ferrous ammonium sulfate solution on a spot plate. After one minute the spot was acidified with concentrated hydrochloric acid to give the tests recorded below. The tests were evaluated by amount of Prussian blue color. Diazomethane polymer gave a negative test by this procedure.

The quantitative analyses for nitrogen were carried out on 20–40 mg. samples.

Spruce alkali-lignin[11] was treated with diazomethane by procedure B to give a derivative containing 22.7% methoxyl; this sample gave a strong qualitative test for nitrogen which was quantitatively evaluated as 0.94%. Substantially the same results were obtained with spruce methanol-lignin[12] which, when diazomethane methoxylated by procedure B, contained 24.1% OCH_3, 0.70% N and gave a positive qualitative test for nitrogen.

In order approximately to evaluate the qualitative nitrogen test a sample of acetone-soluble spruce formic acid-lignin,[13] which contained 12.8% OCH_3 and gave a negative nitrogen test, was mixed with 1.5 weight per cent. of urea The qualitative nitrogen test was comparable with those obtained from the diazomethane-methoxylated samples.

Birch acetic acid lignin[3a] gave a negative test for nitrogen. Fractions 1 and 2 of the acid-hydrolyzed ether-soluble portion of this lignin both gave strong nitrogen tests after methoxylation by procedure A and gave analyses for 0.80 and 0.71% N, respectively. The diazomethanemethoxylated (procedure A) methanol-soluble portion of birch acetic acid-lignin (OCH_3 24.2%) gave a somewhat weaker nitrogen test which was slightly less positive either after subsequent dimethyl sulfate methoxylation (36.6% OCH_3) or after several hours of boiling with 28% sulfuric acid. In every case tests on the reagents used were negative.

Summary

1. Valerolactone reacts with diazomethane in methanol to give methyl 4-hydroxypentanoate.

2. Coumarin with diazomethane in methanol forms 3-carbomethoxy-4-(*o*-methoxyphenyl)-pyrazoline.

3. Qualitative and quantitative elemental analyses show that diazomethane-methoxylated lignins contain a small amount of nitrogen. This is interpreted as evidence that lignins contain a coumarin type of lactone linkage.

4. The presence of bound phenolic hydroxyl groups in the lignin complex has been demonstrated by acid hydrolysis of the isolated acetic acid birch lignin.

TORONTO, CANADA RECEIVED DECEMBER 30, 1940

(10) Foulke and Schneider, *Ind. Eng. Chem., Anal. Ed.*, **10**, 104 (1938).

(11) Marshall, Brauns and Hibbert, *Can. J. Research*, **13**, 103 (1935).

(12) Brauns and Hibbert, *ibid.*, **13**, 28 (1935).

(13) Wright and Hibbert, THIS JOURNAL, **59**, 125 (1937).