TABLE I

ENZYMATIC INCORPORATION OF O_2^{18} INTO *cis–cis*-MUCONIC ACID

Experiment I. Catechol (0.6 mmole), 4.8 mg. of purified pyrocatechase (specific activity 112 units/mg. protein),[a] 0.6 millimole of glutathione and 4.5 millimoles of potassium phosphate buffer (*p*H 7.5) were incubated in a 500-ml. Erlenmeyer flask in a total volume of 60 ml. H_2O^{18}, 0.701 atom % excess. Experiment II. A 500-ml. Büchner suction flask was modified with a high vacuum stopcock at the top; the hose connection was sealed with a rubber vaccine cap. The same reaction components as in Expt. I were employed except that H_2O was used instead of H_2O^{18} and to the degassed container was added a O_2^{18}–N_2 mixture with an approximate ratio of O_2^{18}/N_2 of 18.2%.[b] In Expt. II the O^{18} content of the flask was determined before the addition of the enzyme and after the completion of the reaction. The atom % excess was 1.354 and 1.331 respectively. The reactions were run at 25° with gentle mechanical shaking and the reaction rate was followed by the increased absorption at 260 mμ. Aliquots were removed by syringe through the rubber vaccine cap in Expt. II. After two hours of incubation 2 ml. of 2 *N* H_2SO_4 was added to the reaction mixture. *cis–cis*-Muconic acid was isolated and was recrystallized from absolute ethanol. The recrystallized material was pyrolyzed by the method of W. E. Doering and E. Dorfman (THIS JOURNAL, **75**, 5595 (1953)[c]. The O^{18} content was determined by a Consolidated Nier Model 21-201 Mass Spectrometer, measuring $CO^{16}O^{18}/CO_2$ (46/44) ratio.

Expt.	Medium	Atom % O^{18} in Catechol	Atom % O^{18} in *cis–cis*-Muconic acid[d]	Atom % excess Experiment	Atom % excess Theory[e]
I	$O_2^{16} + H_2O^{18}$	0.207	0.207	0.000	0.701
			0.207	0.000	
II	$O_2^{18} + H_2O^{16}$	0.207	1.421	1.217	1.343
			1.433	1.229	

[a] M. Katagiri and O. Hayaishi, unpublished procedure. [b] O_2^{18} was prepared by electrolysis of H_2O^{18} obtained from the Stuart Oxygen Co. [c] We are indebted to Mr. S. Ishihara of the National Bureau of Standards for use of the pyrolysis equipment. [d] Calculated for the oxygen atoms incorporated. [e] Theoretical atom % excess when two oxygen atoms are derived from O^{18}.

oxygen transferase rather than a dehydrogenase and no hydration reaction is involved in the over-all process. *cis–cis*-Muconic acid semialdehyde is therefore excluded as an intermediate since any known mechanism of enzymatic aldehyde oxidation involves hydration. A compound such as (III) appears to be a more likely intermediate in the pyrocatechase reaction. Orthobenzoquinone appears unlikely as an intermediate since H_2O_2 was previously shown not to participate in the reaction.[1,4] This compound, however, cannot be completely ruled out as an intermediate because of the possibility of a tightly bound enzyme–H_2O_2 complex acting as a peroxidase.

The similarity of pyrocatechase to other enzymes which catalyze oxidative rupture of aromatic rings of certain phenolic compounds was recently reviewed by Crandall.[5] In addition to pyrocatechase, homogentisicase,[6] 3-hydroxyanthranilic acid oxidase[7,8] and protocatechuic acid oxidase[4] appear to belong to this new class of metallo-protein enzymes which introduce two oxygen atoms directly across the aromatic bond adjacent to the phenolic group with simultaneous rupture of the aromatic structure.

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DECOMPOSITION OF PRIMARY HYDROPEROXIDES

Sir:

We should like to report that *n*-butyl hydroperoxide when decomposed by heating the neat liquid at 85° in absence of added catalyst gives, as the primary gaseous product, hydrogen. Furthermore this behavior is also general for the higher primary hydroperoxides; isobutyl, *n*-amyl, isoamyl, *n*-heptyl, *n*-octyl and *n*-decyl hydroperoxides all give hydrogen as the major gaseous component on heating to 100°. Oxygen was not produced in significant amounts in any case.

Milas[1] states that "at relatively low temperatures primary hydroperoxides decompose to give aldehydes and water, while secondary and tertiary hydroperoxides give the corresponding alcohols and oxygen." That hydrogen was the gas evolved in the decomposition of *n*-butyl hydroperoxide was therefore totally unexpected.

A preliminary study on *n*-butyl hydroperoxide reveals two major reactions: approximately 50% goes to hydrogen and butyric acid and 40% goes to *n*-butyl *n*-butyrate and water. The remaining minor products are carbon dioxide, carbon monoxide, propane, propyl butyrate, butyl propionate, propionic acid and an unknown compound, probably an hydroxy acid.

Redistilled *n*-butyl hydroperoxide,[2] 0.605 g. (0.0067 mole), which was shown by gas-liquid partition chromatography[3] to contain no appreciable amounts of impurities, was heated in the vapor of boiling trichloroethylene (b.p. 86°) for 47 hours until gas evolution had ceased and the peroxide titer was zero. The gas, 83.9 ml. at standard conditions, was collected over mercury; it showed the following analysis: hydrogen, 80.0% (0.0030 mole); carbon dioxide, 4.4% (0.00016 mole); carbon monoxide, 0.1%; propane, 6.8% (0.00025 mole); oxygen, 0.5%; residue 8.2%. The identity of the propane, as well as carbon monoxide and carbon dioxide, was confirmed by its infrared spectrum. The liquid products, 0.532 g., were separated by gas–liquid partition chromatography[3] on a silicone oil–Celite column and identified by infrared spectra. The composition of the liquid mixture was determined, by direct comparison of the partitionograms of the unknown with a synthetic mixture,[4] to be as follows: water, 9.9% (0.0029

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(3) (a) A. T. James and A. J. P. Martin, *Analyst*, **77**, 915 (1952); (b) A. J. P. Martin and A. T. James, *Biochem. J.*, **50**, 679 (1952).

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mole); *n*-butyric acid, 55.9% (0.0034 mole); *n*-butyl *n*-butyrate, 27.5% (0.0010 mole); *n*-butanol, 3.7%; *n*-butyraldehyde, 0.8%; propionic acid, 0.9%; *n*-butyl propionate and *n*-propyl *n*-butyrate, traces; and an unknown hydroxy (?) acid, 1.3%. The analysis of the major component was confirmed by conventional chemical separation and identification.

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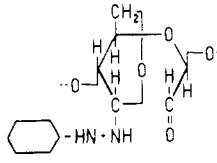
THE FORMAZAN REACTION IN PROVING THE STRUCTURE OF PERIODATE OXIDIZED POLYSACCHARIDES

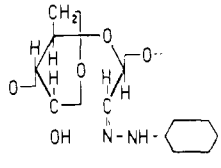
Sir:

We have reported[1] that the *aldehydo*-phenylhydrazones of sugars couple with diazo compounds to build *formazans*, but that the phenylhydrazones of ring structures derived from the hemiacetal form do not. We now propose to make use of this observation in a study of the precise structures of polysaccharides oxidized with periodic acid and of the phenylhydrazones obtainable from them.[2,3]

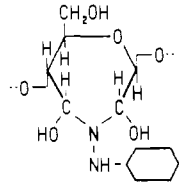
As with polysaccharides oxidized with periodic acid, each individual oxidized monosaccharide reacts with only *one* mole of aromatic amine, *e.g.*, of phenylhydrazine.[4,5] In the literature the following three formulas are suggested for the monosaccharides,[6,7] and their phenylhydrazones,[8,9] respectively.



I



II

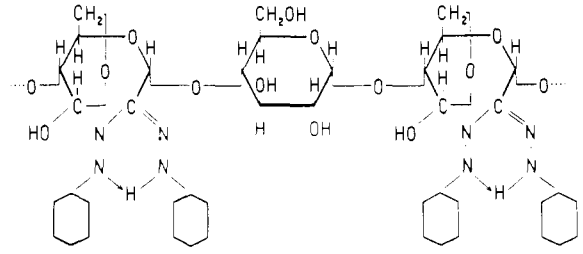


III

(I) is an aldehyde-hemiacetalphenylhydrazone, II, a hemiacetal-aldehydo-phenylhydrazone, and III, a "hemialdal" structure. Of these, only II contains an aldehydo-phenylhydrazone structure suitable for yielding a formazan.

On coupling with diazotized aniline in ice-cold pyridine solution, the phenylhydrazones of several polysaccharides (cellulose, starch, inulin, xylan and dextrin) oxidized wirh periodic acid, we obtained bright-red *diphenyl-formazans*, and thereby obtained unambiguous proof of the presence of structure II in these compounds.

On this basis, we suggest this formula for the structure of formazans obtained from polysaccharides, *e.g.*, from starch, oxidized with periodic acid



Procedure.—The polysaccharide samples were oxidized at room temperature with 3 to 5% sodium metaperiodate for 24 and 48, and in the case of insoluble polysaccharides (*e.g.*, cellulose or xylan) for 120 hours, respectively. The corresponding phenylhydrazones were prepared as described by Barry and Mitchell.[4] The bright yellow phenylhydrazones were then dissolved or suspended in pyridine or a 1:1 mixture of pyridine and ethanol. Dropwise addition with ice-cooling, of a diazotized aniline solution led to coupling to give the formazan. The vividly red solution or suspension was poured into ice water, and the polysaccharide formazans separated. With concentrated sulfuric acid they yielded the dark-blue color-reaction characteristic of the formazans.

By regulating the rate of oxidation it is possible to determine how many formazan groups form per monosaccharide group. For instance, if a *cellulose* wad is oxidized for 120 hours as described by Jayme and Sätre,[3] it will contain only one formazan group to three monosaccharides, and retain its original fibrous structure.

Anal. Calcd. for the phenylhydrazone $(C_{24}H_{34}O_{14}N_2)_x$: N, 4.88. Found: N, 5.04. Calcd. for the formazan $(C_{30}H_{38}O_{14}N_4)_x$: N, 8.26. Found: N, 7.42.

If *potato starch* is oxidized with 5% sodium metaperiodate for 24 hours, or *inulin* with 4% sodium metaperiodate for 48 hours, the resulting product will contain two formazan groups to three monosaccharides.

Anal. Calcd. for the phenylhydrazone $(C_{30}H_{38}N_4O_{13})_x$: N, 8.46. Found: N for starch, 8.31; for inulin 8.40. Calcd. for the formazan $(C_{42}H_{46}N_8O_{13})_x$: N, 12.87. Found: N for starch, 11.80; for inulin, 12.50.

A *xylan* sample oxidized with 3% periodate solution for 120 hours and a *dextrin* sample oxidized for 48 hours applying the above-described procedure likewise yielded the corresponding polysaccharide formazans. (Found: N for xylan 11.19; N for dextrin, 12.05).

This new reaction, yielding sparingly soluble formazans of vivid red color and high nitrogen content, will facilitate establishing as yet undecided structures of polysaccharides.

Experiments to form new active groups (tetrazoliums, thioaldonic-acid-phenylhyrazides, metallic complexes) in the molecule of polysaccharides by

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