acetyl introduced which prevents gel formation represents only one acetyl group for about eight uronide groups. Second, this quantity seems practically independent of the methoxyl content between 7 and 10%. At the higher percentage of methoxyl one of about three free carboxyl groups could be hindered in hydrogen bond formation if the acetyl group should enter positions indicated by arrows in Fig. 2. At the lower percentage only one free carboxyl group out of about four could be hindered. The fact that this increase in available carboxyl groups is not reflected in the percentage of acetyl required to prevent gelation appears to deëmphasize the role of carboxyl to carboxyl bridges in formation of high solids pectin gels. Further discussion will require more data on pectin gels than presently available.

Acknowledgment.—The authors thank A. D. Shepherd and A. L. Smith for extraction of the sugar beet pectin and E. F. Jansen for the sample of acetylesterase.

Summary

A series of pectin acetates were prepared. When 2.6 or more per cent. acetyl is present in the molecule, the ability of the pectin to form high-solids gels is markedly reduced if not eliminated. Jellying ability was restored by partial acid hydrolysis of the pectin acetate.

Acid hydrolysis of sugar beet pectin produced a jellying pectin.

RECEIVED MAY 31, 1949

[CONTRIBUTION FROM THE GEORGE M. MOFFETT RESEARCH LABORATORIES, CORN PRODUCTS REFINING COMPANY]

The Action of Nitrogen Dioxide on Corn Starch and its Fractions

BY RALPH W. KERR

Since the early reports of Unruh and Kenyon[1] and of Yackel and Kenyon[2] showing the specific action of nitrogen dioxide on cellulose, some thought has been given to the possible use of the reaction in a study of structure in a related carbohydrate, starch. It is now generally believed that the common starches, such as corn, consist of a mixture of polymer types, one of which is linear, the α-glucose units being joined only by 1–4 glucoside linkages, and the other is a branched polymer, the branches being formed through 1–6 glucoside linkages. Although chemical data to support this viewpoint are convincing within the experimental limitations of the general methods which have been employed, such as exhaustive methylation and periodate oxidation, these methods applied to starch involve certain considerations which would make confirmation by an independent approach very desirable.

The work of Unruh and Kenyon would tend to show that when nitrogen dioxide acts on a polyglucoside such as cellulose, the action is primarily directed to the oxidation of the primary alcohol groups to carboxyls. These carboxyl groups may of course be readily determined by appropriate methods and distinguished from carbonyl groups formed in the primary phase of possible side reactions between the nitrogen dioxide and any hydroxyl group other than the primary alcohol group on carbons number six in the polyglucoside chain. Moreover, the work of Unruh and Kenyon would tend to show that the action of nitrogen dioxide on cellulose in forming uronic acid carboxyl groups could be extended, virtually to completion. Structurewise, amylose is thought to be quite similar to cellulose and should, accordingly, give a theoretical yield also of glucuronic acid groups. On the other hand, since amylopectin is thought to be branched through hydroxyl groups on carbons number 6, then for every branch in the structure there should be one less than the theoretical number of uronic acid carboxyl groups when oxidation with nitrogen dioxide is complete. Consequently, oxidation with nitrogen dioxide should provide a method to determine quantitatively the difference in structure between the two major fractions in starch.

In our early preliminary studies of the action of nitrogen dioxide on unfractionated, whole corn starch, using procedures essentially as described for obtaining a theoretical yield of polyglucuronide from cellulose, it was readily apparent that the results fell somewhat short of the theoretical yield. Mench and Degering[3] have already reported that when starch is treated in chloroform solution of nitrogen dioxide, the carboxyl content was somewhat less than that of an anhydropolyglucuronic acid chain. These results may be expected on the basis of analytical determinations which have shown[4] that the starch contains very nearly 70% of the branched type of polymer and only 30% of linear, or cellulose-like polymer. Therefore, qualitatively, at least, the method appeared to show promise and investigations were begun on the action of nitrogen dioxide on starch fractions. The following is a preliminary report of these studies.

(1) C. C. Unruh and W. O. Kenyon, THIS JOURNAL, **64**, 127 (1942).

(2) E. C. Yackel and W. O. Kenyon, *ibid.*, **64**, 121 (1942).

(3) J. W. Mench and Ed. F. Degering, *Proc. Indiana Acad. Science*, **55**, 69 (1945).

(4) R. W. Kerr and O. R. Trubell, *Paper Trade J.*, **117** (no. 15) 25 (1943).

Experimental

Oxidation with Nitrogen Dioxide.—In preliminary experiments the solid starch samples were treated with nitrogen dioxide in the vapor state by the cyclic method essentially as described by Yackel and Kenyon for cellulose.[2] The starch samples were dried *in vacuo* over phosphorus pentoxide or sulfuric acid to equilibrium moisture content of about 2.5%.

In later experiments, where indicated, an effort was made to remove the water formed during the oxidation at an earlier period in the cycle and thus to maintain the entire reaction system in as nearly an anhydrous state as possible. This was accomplished by adding phosphorus pentoxide to the reaction chamber as well as by maintaining a supply in the reagent reservoir. The starch sample was spread out into very thin layers on trays placed horizontally. Between every two trays of starch a large petri dish was placed containing phosphorus pentoxide.

All reactions were allowed to proceed at room temperature and the temperature of the reactor at no time exceeded 30°.

Samples were removed periodically and washed in closed centrifuge bottles by shaking with 70% alcohol followed by centrifuging and decanting until the wash liquor was free from acid. The product was then washed twice with absolute ethanol and dried *in vacuo* over calcium chloride.

Preparation of Nitrogen Dioxide.—Nitrogen dioxide was purified by distillation over phosphorus pentoxide (b. p. 21°).

Determination of Uronic Acid Carboxyl Groups.—Uronic acid carboxyl groups were determined in all cases by modification of a published[5] carbon dioxide evolution method. A 15% hydrochloric acid solution was used as lower concentrations gave less reproducible results. A refluxing time of six hours was chosen from the data of Figs. 1 and 2. The carbon dioxide evolution from oxidized starch is relatively rapid during the first two or three hours and is greatly reduced but appreciable in the four to six-hour interval. In the following two-hour interval evolution is equivalent to about 0.1% of carboxyl. Galacturonic acid monohydrate behaves similarly and the theoretical amount of gas was obtained in six hours. In contrast original starch evolved the gas at a constant rate per hour equivalent to 0.06% carboxyl.

Preparation of Starch Samples.—Corn starch was prepared by gelatinizing 30 g. of defatted corn starch in 400 ml. of water held at the boiling point for fifteen minutes followed by atomizing the paste into 4 liters of acetone. The fine powder was collected, washed five times with 200 ml. of acetone, filtered and dried *in vacuo*.

A fraction of the linear polymer in corn starch was prepared in pure crystalline form according to the method of Kerr and Severson.[6] A portion of this amylose sample was prepared in retrograded form by making a hot solution of 15 g. of the dried crystalline material in 2000 ml. of boiling water and allowing the hot solution to cool and stand for several days at 3°. The bulky precipitate was centrifuged and dehydrated by 5 washes in 500 ml. of acetone following which it was dried *in vacuo*.

The amylopectin, or B-fraction, of corn starch was prepared by fractionating autoclaved, defatted corn starch with butanol, according to the method of Schoch.[7]

Oxidation with Ferricyanide.—The oxidation of starch samples was carried out and the results expressed essentially as described by Cleveland and Kerr,[8] the method being adapted from procedures used by Gore and Steele:[9] 0.250 g. of starch was gelatinized in 25 ml. of water in a loosely stoppered Erlenmeyer flask by immersing in a boiling water-bath for fifteen minutes. Then 25 ml. of an alkaline solution containing 16.5 g. of potassium ferricyanide and 22 g. of sodium carbonate per liter, was added and the mixture heated an additional fifteen minutes in the boiling water-bath. The flask was cooled immediately and acidified with 60 ml. of a solution which contained 200 ml. of acetic acid, 70 g. of potassium chloride and 20 g. of zinc sulfate heptahydrate per liter. Finally, 20 ml. of 20% potassium iodide was added and the liberated iodine titrated with 0.05 *N* thiosulfate solution. For samples of higher reducing value a proportionately lesser amount of the carbohydrate was used. Blanks were run on 25 ml. of the ferricyanide reagent. The ferricyanide number was calculated as the ml. of tenth normal thiosulfate per gram of the starch.

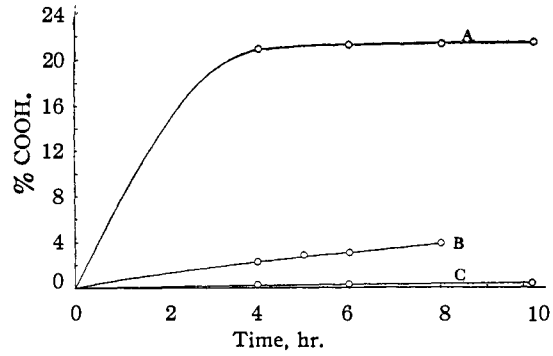

Fig. 1.—Liberation of carbon dioxide by boiling hydrochloric acid at various times from: A, galacturonic acid monohydrate; B, saccharic acid; C, defatted corn starch.

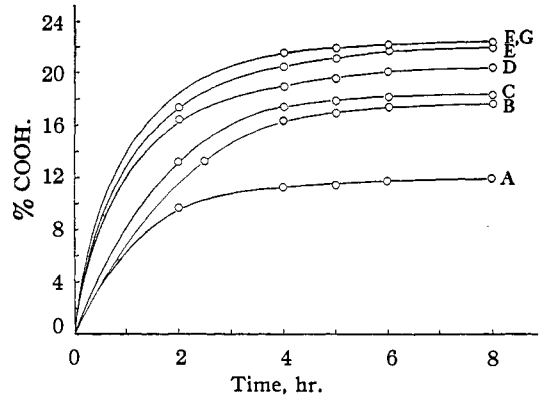

Fig. 2.—Liberation of carbon dioxide by boiling hydrochloric acid at various times from corn starch oxidized by nitrogen dioxide for: A, 24; B, 48; C, 72; D, 96; E, 116, and F, G, 164 and 212 hr.

Oxidation with Periodic Acid.—A solution was prepared containing 7.04 g. of periodic acid and 136 g. of sodium acetate trihydrate per liter, adjusted to *p*H 4.3 with acetic acid. This solution was standardized by adding 1 ml. of 20% potassium iodide to 25 ml. of the reagent in presence of sufficient sodium bicarbonate to give a slightly alkaline reaction and back titrating with 0.1 *N* sodium arsenite. One gram of starch sample was stirred with 1000 ml. of the periodic acid solution at 25°. At regular intervals, 25-ml. aliquots were removed and the unconsumed periodic acid determined by titration with sodium arsenite. The moles of periodic acid consumed per molar unit of dry starch are shown in Table I for three starches containing (A) zero uronic acid groups (B) 11.73% and (C) 21.68% uronic acid groups.

(5) R. L. Whistler, A. R. Martin and M. Harris, *J. Research Natl. Bur. Standards*, **24**, 13 (1940).

(6) R. W. Kerr and G. M. Severson, THIS JOURNAL, **65**, 193 (1943).

(7) T. J. Schoch, *ibid.*, **64**, 2954 (1942).

(8) F. C. Cleveland and R. W. Kerr, *Cereal Chem.*, **25**, 133 (1948).

(9) H. C. Gore and H. H. Steele, *Ind. Eng. Chem., Anal. Ed.*, **7**, 324 (1935).

TABLE I

OXIDATION OF STARCH SAMPLES WITH PERIODATE

Time, hr.	Moles of periodic acid consumed A	B	C
1	0.56	0.65	0.70
2	.80	.80	.86
3	.88	.89	.95
4	.96	.97	1.00
5	.97	1.00	1.06
6	1.02	1.04	1.12
24	1.14	1.16	1.28

Discussion

The oxidation of defatted and dehydrated corn starch with nitrogen dioxide in a gas–solid phase reaction proved to be a slow process and influenced by variables in addition to those already reported for cellulose. As shown in Fig. 3, only 13.20% carboxyl groups were produced in one hundred and sixteen hours. However, the same starch, pre-

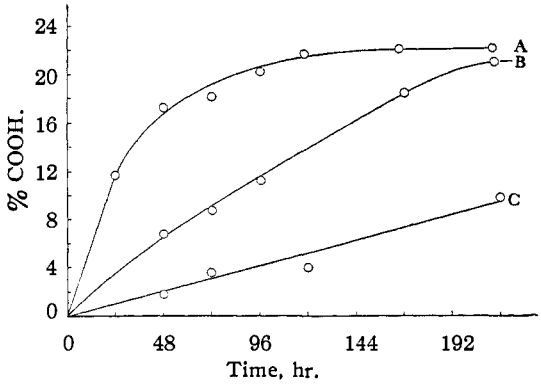

Fig. 3.—Formation of glucuronic acid carboxyls by oxidation with nitrogen dioxide: A, dehydrated, gelatinized corn starch; B, repetition of A, under more anhydrous conditions; C, granular corn starch.

gelatinized and dehydrated in acetone, was oxidized at a much greater rate and to a higher level of uronic carboxyl content. The results, also summarized in Fig. 3, show that the starch attained a carboxyl content of 20.12% in 96 hours and leveled off at 22.09% in 164 hours. This analytical value was unchanged at 22.09% at 212 hours. The theoretical carboxyl content for a polyanhydroglucuronide is 25.57%. The reaction with nitrogen dioxide was applied to fractions of corn starch and here again, with the linear fraction, at least, it was found that physical form played an important part in determining the rate of reaction. As shown in Fig. 4, the crystalline amylose was found to contain only 12.07% carboxyl in 116 hours, whereas the same amylose in retrograded form had 21.35%. The B-fraction of corn starch, which is very much less prone to orient in the crystalline and retrograded forms, was found to contain 21.57% carboxyl in 48 hours and this value fell off slightly to 21.36 per cent. at 116 hours. Thus about 21.5% carboxyl appears to be the limit of oxidation of the B-fraction with nitrogen dioxide.

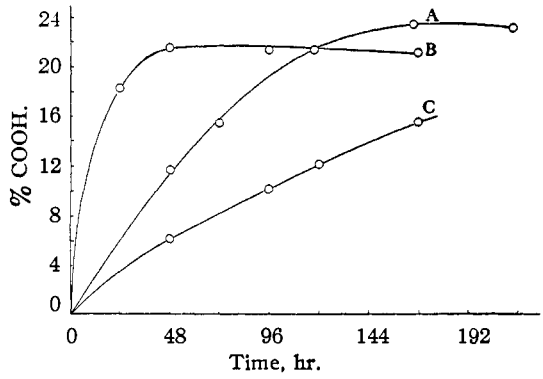

Fig. 4.—Formation of glucuronic acid carboxyls by oxidation of corn starch fractions with nitrogen dioxide: A, corn amylose in retrograded form; B, corn amylopectin, or B-fraction; C, corn amylose in crystalline form.

Continued oxidation of the linear corn starch molecules gave a maximum carboxyl content of 23.31% carboxyl after 164-hr. of oxidation. The value fell off slightly to 22.99% at 212 hr. Therefore, qualitatively at least, oxidation with nitrogen dioxide shows that there are less free primary alcohol groups (C-6, hydroxyls) on B-fraction molecules than on the linear ones, and as might be expected from the fact that whole starch is a mixture of both types, the limit value for starch is intermediate to those of the two fractions in the starch.

However, none of the starch products tested, not even the supposedly linear fraction attained the theoretical carboxyl content for a polyanhydroglucuronide, and inasmuch as physical form of the carbohydrate was found to affect the rate of oxidation, if not the final limit, it would seem possible that in all cases the rate was sufficiently reduced to permit interfering side reactions to become appreciable in extent. The present procedure cannot, accordingly, be given a quantitative interpretation.

A further study was made of the procedure in an effort to determine possible side reactions and and their effect on the carboxylation studied. It seemed reasonable to suppose that nitrogen dioxide might also oxidize, to some extent at least, terminal aldehyde groups on number one carbons, where they exist, to carboxyls and also secondary alcohols on carbons 2 and 3 to carbonyl, aldehydes (with a splitting of C–C valences between carbons 2 and 3) and finally to acids.

As was found also by Kenyon and co-workers[10] we found that the presence of a second carboxyl group on the glucose unit very materially stabilized the uronic acid carboxyl group on carbon 6 to decarboxylation when treated with boiling 15% hydrochloric acid. Thus, for example, saccharic acid showed very great stability to decarboxyla-

(10) E. W. Taylor, W. F. Fowler, Jr., P. A. McGee and W. O. Kenyon, THIS JOURNAL, 69, 342 (1947).

tion in the carboxyl group determination used by us and described in the Experimental section. In our procedure saccharic acid liberated carbon dioxide to the equivalent of 2.21% carboxyl at four hours, 3.15% at six hours and 3.89% at eight hours as shown in Fig. 1. Therefore, it would appear that if nitrogen dioxide oxidized carbons other than number 6 to carboxyl, and particularly terminal carbons number one, our analytical procedure for uronic acid carboxyls would give a result which was too low.

A large sample of starch was oxidized for 116 hours and was thoroughly purified for analysis by extensive washing in water and alcohol mixtures. The acidity of this sample was then determined by the calcium acetate procedure outlined by Unruh and Kenyon[1] and was found to be equivalent to 22.60% carboxyl. Only 21.68% uronic acid carboxyl was found by decarboxylation. Therefore it appears that nitrogen dioxide does oxidize the starch molecule at places other than at carbon 6 and that the production of these additional carboxyls causes the uronic acid carboxyl determination to give a fictitiously low result.

Additional evidence was found for secondary reactions in that the starch became strongly reducing in character during the course of oxidation. The purified nitrogen dioxide starch described above, was oxidized by alkaline ferricyanide according to procedures outlined in the Experimental section. It was found that whereas untreated corn starch gave a ferricyanide number of 1.0, the oxidized starch gave a value of 100. The very large increase in ferricyanide number may indicate either the formation of carbonyl groups on carbons 2 and 3 or aldehyde groups on carbons 1, by hydrolysis, which in turn become oxidized to carboxyl groups.

Little evidence could be found to support the view that these carbonyl groups were formed to any large extent on carbons 2 and 3, forming either ketone groups or a di-aldehyde starch according to the well-known action of periodate on polyglucosides.[11] A sample of the starch used in the ferricyanide oxidation, a sample of untreated corn starch and another sample of starch oxidized to a level intermediate between the two were oxidized with periodic acid. The fractional moles of periodic acid consumed per molar unit of dry starch were compared at different times as shown in Table I. Substantially no difference was observed in the behavior of the several starches tested during consumption of the first mole of the periodate. It would be expected that if nitrogen dioxide had caused extensive oxidation on carbons 2 and 3, then the oxidized starch would have consumed periodate less readily. If anything, the untreated starch (A) reacted a little less readily than those oxidized with nitrogen dioxide, (B) and (C). In later studies, Kenyon and co-workers[12] have come to the conclusion also that nitrogen dioxide oxidation of carbons 2 and 3 in the glucose units of cellulose, to carbonyl groups, occurs to a very small extent, some 1.7% carbonyl groups being formed after an extensive reaction period.

On the other hand, several considerations favor the view that nitrogen dioxide, being acidic in nature in the presence of water causes considerable hydrolytic cleavage in the starch molecule, creating a new terminal aldehyde (which then may be subsequently oxidized to a saccharic acid group) for each glucoside linkage hydrolyzed. First, the viscosity of the starch is very greatly reduced during the nitrogen dioxide treatment. Whereas untreated corn starch showed a Scott viscosity test[13] of 90, the purified, nitrogen dioxide-treated starch described above gave a Scott viscosity test of only 26.[14] Furthermore, in treating starch with nitrogen dioxide there is a regular, progressive decrease in specific rotation. Untreated corn starch has a specific rotation of $[\alpha]^{25}D$ 203° whereas an aqueous 1% solution of the analytical sample which had 21.68% carboxyls, gave a value $[\alpha]^{25}D$ 147.5°.[15]

An attempt was made to perform the nitrogen dioxide oxidation of starch under more anhydrous conditions so as to minimize the possible formation of saccharic acid groups. Corn starch was oxidized by a modified procedure as described in the Experimental section. The same gelatinized starch was used as in a previous run and the results for both experiments are plotted in Fig. 3. It will be seen that, unfortunately, anhydrous conditions surrounding the starch drastically reduce the oxidation rate. For example, whereas in the first experiment 20.12% carboxyls were produced in 96 hr., only 13.5% were formed when the starch was surrounded by phosphorus pentoxide. Furthermore, even though the starch in the first experiment leveled off at about 22.1% carboxyls after 164 hours, starch under more anhydrous conditions leveled off at a value of only 21.2% carboxyls between 200 and 300 hours. It may be concluded that moisture, which is to be avoided if hydrolysis is to be prevented, promotes the desired oxidation reaction.

Accordingly, it may be concluded that in order for the nitrogen dioxide reaction to be of quantitative value in a determination of the differences in structure between the supposedly linear, and B-fraction molecules in starch, conditions must be developed so that the oxidant will produce from

(11) For a résumé of the action of periodic acid on starch see Kerr, "Chemistry and Industry of Starch," Academic Press, New York, N. Y., 1944, p. 227.

(12) P. A. McGee, W. F. Fowler, Jr., C. C. Unruh and W. O. Kenyon, THIS JOURNAL, **70**, 2700 (1948).

(13) R. W. Kerr, "Chemistry and Industry of Starch," Academic Press, New York, N. Y., 1944, p. 85.

(14) Formation of di-aldehyde starch, with rupture of carbon to carbon valences does not cause a reduction in size of the starch molecule (see reference (11)), whereas, obviously, hydrolysis does.

(15) The principal polymeric bond in starch is an alpha 1–4, glucoside linkage, which is considerably less stable to aqueous acid than the beta 1–4, glucoside linkage in cellulose and accordingly, starch could very well be hydrolyzed under conditions where cellulose is not.

amylose a chain composed entirely of polyanhydroglucuronide groups which are substantially as long as the original starch molecules, if indeed there are perfectly linear molecules in starch. It follows from our work that catalysts or adjuncts other than water or moisture, are required which will promote the reaction and that water should be eliminated as soon as it is formed in the oxidation reaction. Otherwise correction factors are required in the determination of uronic acid carboxyls which appear to be relatively large in respect to the final value which is of importance in starch structure studies and one of the serious disadvantages inherent in other chemical approaches, such as methylation, would not have been overcome.

The assistance given by Mr. O. R. Trubell in performing several of the analyses reported in this communication is gratefully acknowledged.

Summary

A study was made of the reaction between nitrogen dioxide in the gaseous state and corn starch and its fractions in the solid state. The percentage of uronic acid carboxyl groups found in amylose after oxidation was greater than in oxidized amylopectin; whole starch gave a value intermediate to the two fractions.

These results qualitatively support the view that branching through glucoside linkages to carbons number 6 is more extensive in amylopectin than in amylose molecules.

It was found also that the physical form of the carbohydrates, as well as moisture associated with, or formed in the starch, influenced the oxidation reaction rate.

Additional data were given to show that moisture also facilitates hydrolysis of glucoside linkages which makes possible the formation of saccharic acid groups at the points of scission. Carbon-6 carboxyl groups in saccharic acid were found to be relatively more stable in a decarboxylation reaction with hydrochloric acid than uronic acid carboxyls.

ARGO, ILLINOIS RECEIVED MAY 20, 1949

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY]

On the Reaction Inactivation of Tyrosinase during the Aerobic Oxidation of Catechol[1]

BY ISAAC ASIMOV AND CHARLES R. DAWSON

Tyrosinase is a copper-bearing phenolase widely spread throughout the plant kingdom, notably in the common mushroom, *Psalliota campestris*. This enzyme possesses the property of catalyzing the aerobic oxidation of both monohydric and *o*-dihydric phenols.[2] One of the most striking characteristics of the aerobic oxidation of catechol as catalyzed by the enzyme tyrosinase, is the marked inactivation of the enzyme that occurs during the early course of the reaction. The enzyme inactivation appears to be an integral part of the mechanism whereby tyrosinase catalyzes the oxidation, since the loss of activity, which is markedly in evidence even during the first minute of the reaction,[4] is not brought about by any known product of the enzyme reaction, nor by catechol in an anaerobic system.[5,6] Furthermore, the inactivation during the early course of the reaction cannot be attributed to an instability of the enzyme in dilute solution, or to salt, heat or *p*H effects, since it has been found by the authors that control systems with only catechol lacking showed no loss in enzyme activity over comparable periods in the absence of agitation.[7]

By means of chronometric measurements, Miller, *et al.*,[8] showed experimentally that when the amount of quinone (Q) formed during the tyrosinase-catalyzed oxidation of catechol, was plotted reciprocally against the time of reaction (t), (*i. e.*, $1/Q$ *vs.* $1/t$), a linear relationship was obtained

$$\frac{1}{Q} = \left[\frac{b}{a}\right]\left[\frac{1}{t}\right] + \frac{1}{a} \qquad (1)$$

This relationship may also be expressed

$$Q = at/(b + t) \qquad (2)$$

in which form it has been termed in these laboratories, the "chronometric equation." (The terms, a and b, in these equations are constants, the dimensions of which are equivalent to those used for Q and t, respectively, and which vary in value with

(1) From a dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science of Columbia University by Isaac Asimov, present address: Department of Biochemistry, Boston University School of Medicine.

(2) Since *p*-cresol and catechol are commonly used as experimental substrates these catalytic activities are termed cresolase and catecholase activities, respectively.[3]

(3) J. M. Nelson and C. R. Dawson, *Advances in Enzymology*, **4**, 99 (1944).

(4) W. H. Miller and C. R. Dawson, THIS JOURNAL, **63**, 3375 (1941).

(5) C. R. Dawson and B. J. Ludwig, *ibid.*, **60**, 1617 (1938).

(6) B. J. Ludwig and J. M. Nelson, *ibid.*, **61**, 2601 (1939).

(7) When such control systems (lacking in catechol) are subjected to agitation, a loss in enzyme activity does result, presumably because of protein denaturation at the air–liquid interface. However, such surface denaturation does not appear to contribute to the inactivation of the enzyme observed during the first three minutes of the aerobic oxidation of catechol in agitated systems ("reaction inactivation") since the same rate and extent of inactivation is observed in unagitated systems.

(8) W. H. Miller, M. F. Mallette, L. J. Roth and C. R. Dawson. THIS JOURNAL, **66**, 514 (1944).