615

REACTION OF BORATE WITH SUBSTANCES OF BIOLOGICAL INTEREST

By CHARLES A. ZITTLE, *Philadelphia, Pennsylvania*

CONTENTS

I. Introduction 493
II. Reaction of Borate with Simple Polyhydroxy and Related Compounds 494
A. Types of Compounds Giving Reaction 494
B. Types of Complexes Formed 496
C. Change in Properties of Polyhydroxy Compounds 499
D. Equilibria Involved. Reversibility 501
III. Reaction of Borate with Polysaccharides 502
A. Vegetable Gums and Mucilages 502
1. Vegetable Gums and Mucilages That React with Borax 502
2. Factors That Influence Complex Formation 505
3. Complex Formation in Nonaqueous Solvents 506
4. Complex Formation with Tannin and Polyvinyl Alcohol 506
5. Nature of the Borate-Polysaccharide Complex 507
B. Blood Group Substances 509
1. Isolation of Blood Group Substance with Borate 509
2. Change in Properties in the Presence of Borate 511
3. Component Monosaccharides Probably Responsible for Reaction 513
C. Bacterial Polysaccharides 513
IV. Reaction of Borate with Vitamins and Other Substances That Are Components of Coenzymes 514
A. Adenosine 5-Phosphate (Muscle Adenylic Acid) 514
B. Pyridoxine (Vitamin B_6) 515
C. Riboflavin (Vitamin B_2) 515
D. Dehydroascorbic Acid 516
V. Effects of Borate on the Activity of Certain Enzymes 516
A. Polyhydroxy Compound as Coenzyme or Substrate 516
B. Inhibitions of Unknown Mechanism 518
C. Inhibition Probably Ionic (Alkaline Phosphatase) 518
VI. Reaction of Borate with Viruses 520
VII. Physiological Activity of Borate 521
A. Plants 521
B. Microorganisms 521
C. Animals 522
References 524

I. Introduction

The reaction of boric acid with polyhydroxy and related compounds has been known and used for a long time. For example, the ability of mannitol to render boric acid more acidic forms the basis of an

analytical method by which boric acid is determined by titration. The reaction is intensified by raising the pH, and, in borax, mannitol can be estimated by its increase in optical rotation. The principal requirement for reaction with borate—that the hydroxyl groups must be adjacent and *cis*—has made the reaction useful in elucidating the structure of polyhydroxy compounds. Since many compounds of biological interest (vitamins, coenzymes, enzyme substrates, polysaccharides) contain hydroxyl groups in a favorable position to react with borate, the reaction should be of use in elucidating the mechanism of action and the properties of these substances. An outstanding favorable characteristic of the reaction is the ease with which it can be reversed, thus permitting recovery of the components in their original form. References, in most cases brief, to the reaction of borate with substances of the kind mentioned, as well as reports of effects on viruses and enzymes, have appeared in the literature. The reaction has been of use in the purification of blood group polysaccharide, for one of the consequences of the reaction with borate in this case is a reduction of the solubility of the polysaccharide in salt solutions. Borate may be of use also in exploring some of the biological reactions of the blood group and other polysaccharides. Further, borate might be useful in investigating the reaction of viruses with blood cell or tissue receptor sites of polysaccharide nature. In view of the many coenzymes reacting with borate, a large field should be open for exploration of the reaction of coenzymes with both holoenzyme and substrate. Other applications for the borate reaction will appear in the course of the review. It will be seen also that, in addition to the specific reactivity of borate with hydroxyl groups, in some enzyme inhibitions borate is probably acting strictly as an anion.

II. Reaction of Borate with Simple Polyhydroxy and Related Compounds

A. TYPES OF COMPOUNDS GIVING REACTION

Böeseken (9) has recently reviewed the use of boric acid for determining the configuration of carbohydrates. Increase in conductivity of 0.5 *M* boric acid at 25° C. on addition of the polyhydroxy compound is used as a measure of the interaction. Most pertinent to the present review are the types of compounds giving the reaction. The aliphatic glycols react with boric acid when the hydroxyl groups are adjacent and *cis;* however, the simple 1,2-glycol reacts feebly, presumably because an unfavorable position results from mutual repulsion of the hydroxyl groups. Compounds with more than two hydroxyl groups react more strongly, and the intensity of the reaction increases with increase in the number of the adjacent hydroxyl groups (glycerol < erythritol < adonitol < arabitol < mannitol). Although the unreactivity of glycol (1,2-ethanediol) and 1,2-propanediol has been confirmed (58a), in high concentrations these compounds do react sufficiently for boric acid to be titrated in their presence (90).

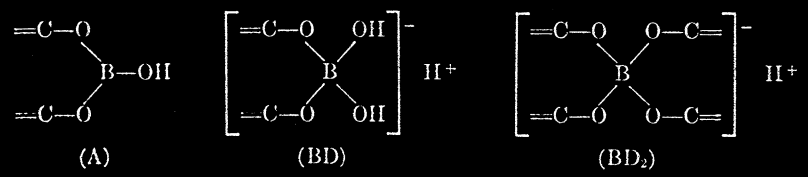
Aromatic ortho-dihydroxy compounds (catechol, pyrogallol, etc.) react strongly with boric acid (9). In the case of the *saturated* six-membered ring (inositol), the reaction with boric acid is negligible, presumably because the atoms are distorted from a single plane. Krantz and associates (4,58a) have made similar observations. Adjacent hydroxyl groups in five-membered rings are reactive with boric acid (4,9). Böeseken (9) has extended these observations to a consideration of the sugars, and it appears that adjacent, *cis*-hydroxyl groups in the pyranose structure are less reactive than in the furanose ring. For some reason, ketoses (D-fructose, D-sorbose) are much more reactive with boric acid than aldoses (D-mannose, D-galactose) (9). The hydroxyl group arising from lactol formation can participate in reactions with boric acid (α-D-glucose) (9).

α-Hydroxy (lactic, etc.) and aromatic *o*-hydroxy (salicylic, etc.) acids are also strongly reactive with boric acid, the necessary OH groups presumably being supplied by hydration of COOH to $C(OH)_3$ (9). Tannins are reactive with boric acid and borax on account of the tri(3,4,5)-hydroxybenzoic acid (22). 1,2-Diketo compounds like benzil and diacetyl, certain keto acids (85), and diketo sugars (77) also react with borate, as does the triketo compound alloxan (64). The hydroxyl groups in these cases probably arise from hydration of the ketone group. Two molecules of borate react with the resulting tetrahydroxy compound (85). Hydroxyl groups in 1,3 positions are in some cases reactive (pentaerythritol (58a); pyridoxine (94); 1,3-butylene glycol (90); 1,2-isopyropylidene-D-glucofuranose (101a)).

The ability of borate to form complexes with diols has been discussed from the standpoint of the physical dimensions of the borate molecule, a factor that is equally important for the specific oxidation of diols by certain oxidants such as periodate and lead tetraacetate (37a).

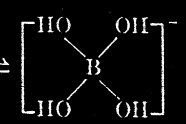
B. TYPES OF COMPLEXES FORMED

Isbell *et al.* (46), in a study of certain sugars and sugar alcohols in the presence of tetraborate, have made a lucid review of prior work. Together with other workers they believe the compounds formed are of the following type:



(A) (BD) (BD₂)

The reactions involved are:

(1) $H_3BO_3 + H_2O \rightleftharpoons$  $+ H^+$

(B)

(Sodium tetraborate in solution is equivalent to equimolecular amounts of sodium borate ($NaBO_2$) and boric acid.)

(2) $B + D\ (diol) \rightleftharpoons BD + 2\ H_2O$

(3) $BD + D \rightleftharpoons BD_2 + 2\ H_2O$

(4) $BD + H^+ \rightleftharpoons A + H_2O$

From mass law equations, the relative amounts of the several compounds obtained with different concentrations of the reactants were calculated. Figure 1 shows the changes in components with increase in the tetraborate:diol ratio. The systems studied were unbuffered and with low tetraborate:diol ratios were quite acid due to the complex formation. The changes exerted by the increase in alkalinity which accompanies the increase in the tetraborate ratio are shown by the dotted lines.

Isbell *et al.* (46) state that compounds of the BD_2 type should be formed preferentially in concentrated solutions of the carbohydrate containing small amounts of borate. Consequently, the change in optical rotation caused by the addition of small quantities of tetraborate to a carbohydrate solution can be ascribed principally to the formation of a compound of the BD_2 type. Compounds of the BD type should predominate in dilute solutions containing large quantities of tetraborate and little carbohydrate. The proportion of type A compounds is higher in concentrated solutions than in dilute, and the change in optical rotation with change in concentration at a constant tetraborate:carbohydrate ratio provides a means of judging the direction and magnitude of the optical rotation of the compounds of type A. The relative amounts of compounds of each type also

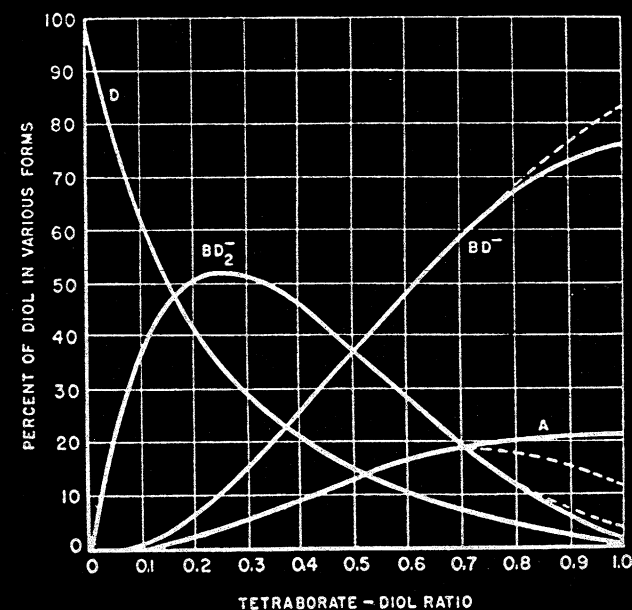


Fig. 1. Hypothetical equilibrium diagram for the tetraborate-diol system: (D) percentage of uncombined diol; (BD_2) percentage of diol present as BD_2 compound; (BD) percentage of diol present as BD compound; (A) percentage of diol present as type A compound (46).

vary with pH. In general, with increase in pH, compounds of type BD and BD_2 increase at the expense of type A.

Isbell *et al.* (46) measured the optical rotation and pH of mixtures of sodium or potassium tetraborate and glucose, fructose, sorbose, sucrose, mannitol, and sorbitol. The amounts of the complexes formed from boric acid and a carbohydrate by the Böeseken procedure (9) are sufficient to cause measurable changes in acidity but too small to detect by optical rotation measurements. In the presence

of the alkali tetraborates, however, large quantities of complex borates are formed, and it is possible to study compound formation by optical methods. Isbell *et al.* (46) plotted the optical rotation for concentrations of the diol of 2 to 10 g. per 100 ml. *versus* various concentrations of tetraborate. Figure 2 shows the results obtained with fructose.

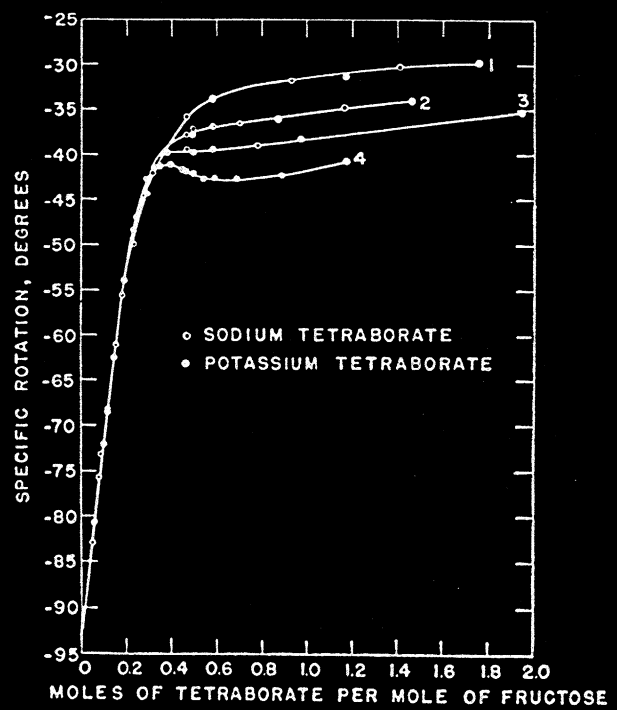
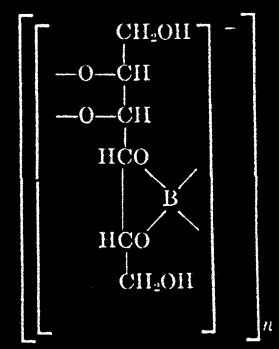


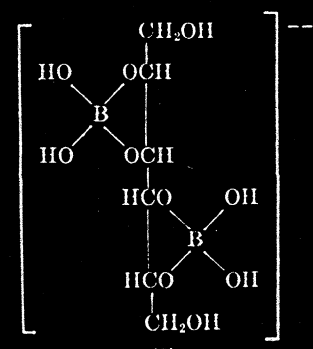
Fig. 2. Specific rotation of fructose in the presence of alkali tetraborates: (1) 2 g. fructose per 100 ml.; (2) 4 g. fructose per 100 ml.; (3) 6 g. fructose per 100 ml.; (4) 10 g. fructose per 100 ml. (46).

From the specific rotation of the mixtures and from the earlier considerations of the equilibria involved, the type of complex formed, as well as its specific optical rotation, were determined. In most cases the change in optical rotation was linear at low tetraborate:diol ratios, reflecting a quantitative formation of the type BD_2 compound.

In contrast with the results with fructose and most of the other compounds studied, the specific rotation of the mannitol-borax complex was independent of the mannitol concentration, and the rotation was linear up to a tetraborate:mannitol ratio of 0.5. The authors conclude that type A compound is probably not formed and the type BD_2 formed contains only 1 molecule of mannitol for each borate group due to formation of the compound shown in formula (1), where the mannitol molecule is a didiol. The BD compound is represented by formula (2). This example is of interest because the polysaccharides with their multiplicity of groupings would be expected to form compounds of the same types.



(1)



(2)

The change in the specific rotation of sucrose in the presence of tetraborate is slight (a maximum of about 6°) in conformity with the absence of *cis*-hydroxyl groups. The change, however, is real, and the authors believe it indicates a compound of type A analogous to the compounds formed by 1,3-diols (90). However, *trans*-1,2-diol groups should be considered also, for the recent studies of Reeves (88a) with cuprammonium-glycoside complexes have shown that certain ring configurations make it possible for hydroxyls of *trans*-1,2-diol groups to be in a favorable position for complex formation. The angle between the planes in which the adjacent hydroxyl groups lie relative to the carbon-carbon axis is the determining factor. The reaction of borate with sucrose is of interest, since the many polyglucose compounds of natural origin would be expected to react also in this fashion.

C. CHANGE IN PROPERTIES OF POLYHYDROXY COMPOUNDS

The enhanced acidity of boric acid in the complex with polyhydroxy compounds has been mentioned in a previous section. This has been

measured either by conductivity (9), or by titration (58a,74). That the complex has a negative charge is shown from studies in an electrical field; glucose and maltose in the presence of borax migrate to the anode (17). As noted in the previous section, changes in optical rotation occur, and are particularly striking with the polyalcohols, which have low initial rotations. The solubility of compounds undergoing complex formation is altered. Riboflavin is considerably more soluble in the presence of borate (31), as is a methyl ether of the flavone quercetin (62). The extraction of the flavones rutin and quercetin from natural materials is increased by the presence of borate (57); the compounds can be recovered by crystallization after the solutions are acidified. The solubility of calcium gluconate is increased 30-fold by boric acid (69,82). As one might expect, the solubility of the boric acid is increased also. On the other hand the solubility of polysaccharides in salt solutions is decreased by borate (110,113). Some of the glycol-boric acid compounds are volatile (80,90). The volatility of the monomethyl ether of glycerol with boric acid, as compared with the nonvolatility of the glycerol complex, suggested that in the latter cross linkage involves the third hydroxyl group (90). Certain flavones react with boric acid in acetone, giving a highly specific yellow color (105a–b).

The chemical reactivity of compounds in complexes with borate is greatly altered, indicating some of the obvious effects expected for compounds serving as substrates for enzymes. Oxidation of 5-keto-D-gluconic acid, dehydro-L-ascorbic acid, and 2,3-diketo-L-gulonic acid by cupric ion and methylene blue is prevented by borate (77). Although these are examples of complex formation involving keto groups, oxidation of the enediol group in ascorbic acid is not inhibited (77). Oxidation of a number of mono- and disaccharides by alkaline copper solutions is decreased by the presence of borate (66); oxidation by an acid copper reagent (Barfoed) is unaffected. Oxidation by alkaline iodine solution, which reacts only with the aldehyde group, is less rapid with borate present, but the final values are unchanged (66). Benzil could not be reduced polarigraphically in the presence of borate (85). The green flame given by boric acid in ethanol, arising from the combustion of the volatile ethyl borate, is not given by borate in combination with calcium gluconate. A positive test is obtained when the compound is decomposed by adding H_2SO_4 (69). Michaelis (75,76) reported that borate interfered with the measurement of oxidation-reduction potentials of certain dyestuffs containing paired hydroxyl groups.

In a few instances, light absorption studies have been performed. In the case of riboflavin, where the light absorption is due to the alloxazine ring, complex formation between the ribityl portion and borax caused no change in the absorption spectrum (31). In the case of benzil, where the —C(=O)—C(=O)— group contributed to the light absorption, a marked reduction in absorption occurred in the presence of borate (85). The absorption of ultraviolet light by pyridoxine is strikingly altered by the reaction with borate (94). In this instance, the light-absorbing phenolic hydroxyl group is involved in the complex formation.

Borate alters the chromatographic adsorption of carbohydrates. By the use of borate, ribose-5-phosphate and arabinose-5-phosphate have been separated (16). Certain physical properties, such as freezing point (67) and viscosity (22,68) of the borate complexes, have also been investigated. Changes in viscosity are greatest with the multireactive, high-molecular polysaccharides (22,60). Changes in sedimentation in the ultracentrifuge have also been observed with polysaccharides (60).

D. EQUILIBRIA INVOLVED. REVERSIBILITY

The studies of Isbell *et al.* (46) showed that complex formation proceeded linearly with addition of borate to diols, indicating a firm union between the components. The concentration range over which this occurs is determined by the nature of the diol. A number of equilibrium studies have been performed with the boric acid–mannitol system with a range of concentrations and pH values. In the pH range 2.62 to 6.55 and a wide range of concentration, Deutsch and Osoling (23) obtained from pH measurements the following constants for the equilibria indicated:

(1) $$B^- + M \rightleftharpoons BM^- \qquad K_1 = 3.0 \times 10^2$$

(2) $$B^- + 2\,M \rightleftharpoons BM_2^- \qquad K_2 = 5.1 \times 10^4$$

It will be recalled from the studies of Isbell *et al.* (46) that owing to the didiol nature of this polyalcohol M is probably one-half the mannitol molecule.

Ross and Catotti (91) studied the same system in the pH range

3.88 to 4.81; the simplifying assumption was made that concentrations of B^- and BM^- were negligible. For the formulation:

$$HB + 2\,M \rightleftharpoons BM_2^- + H^+$$

from which the inverse relationship to the concentration of H^+ is apparent, a value of 1.00×10^{-4} was obtained for the mass action constant. This value can be divided by the dissociation constant of boric acid (6.4×10^{-10}) to give a K_2 value of 15.6×10^4 for equation (2) above. For the reaction of the diketone benzil with borate in 0.25 *M* NaOH and 48% ethanol, in which 2 moles of borate combine with 1 mole of benzil, Pasternak (85) has reported a *K* value of about 10^3.

In spite of the strong association between polyhydroxy compound and borate the reaction is easily and rapidly reversed by dialysis (22,113), change in pH (22,85), and heat (77), and in the case of gel formation by the addition of an excess of a low-molecular polyhydroxy compound (22). Gels (BD_2 type) once formed are not readily liquefied (transformed to BD type) by excess borate which conforms to the above values for K_1 and K_2 for the different products. Pasternak (85) has reported the rate of formation of the benzil-borate complex in 0.25 *M* NaOH; dissociation rates could not be determined in acidified solutions, for the rate was much faster than indicated by the rate of formation of the complex in alkali and the equilibrium constant, and too fast to be measured polarigraphically. It was concluded that decomposition in acid solutions must proceed by a different mechanism than in alkaline solutions. A recent discussion (92a) of the hydrolysis of aliphatic borate esters in acidic solutions may apply to the hydrolysis of certain types of the diol complexes.

III. Reaction of Borate with Polysaccharides

A. VEGETABLE GUMS AND MUCILAGES

1. Vegetable Gums and Mucilages That React with Borax

The ability of borax to react with certain vegetable gums characterizes several of this class of substances (71). Gel formation with borax has been proposed as a means for distinguishing locust bean gum from several other gums (47). Because of the sensitivity of the reaction, it was suggested as a means for testing for and estimating boric acid and borates (35). Deuel, Neukom, and Weber (21) and Moe, Miller, and Iwen (78), however, were the first to point out the probable nature of the reaction by analogy with the reaction of borax with simpler polyhydroxy compounds. Subsequently, in a study of numerous polysaccharides, Deuel and Neukom (22) demonstrated the expected correlation between the intensity of the gel reaction and the presence of a reactive carbohydrate. Since the polysaccharides contain a multiplicity of hydroxyl groups, compound formation of the BD_2 type would lead to cross linkages between the polysaccharide molecules, with gel formation or an increase in viscosity, and changes in other properties related to molecular size. Deuel and Neukom pictured the network formed as shown in Figure 3. Their tests were carried out by adding portions of a saturated solution of borax to aqueous

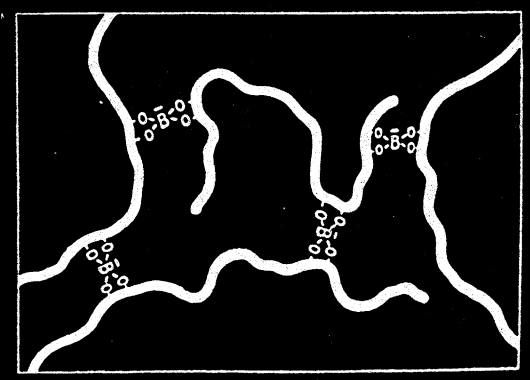


Fig. 3. Representation of the coupling of linear polysaccharides in the borate-didiol complex (22).

solutions of the gums. The sensitivity of the reaction ranged from a positive gel reaction with only a 0.2% solution of some polysaccharides to others that required 10% solutions and the addition of solid borax to demonstrate the effect. Some difficultly soluble polysaccharides brought into solution with dilute cuprammonium reagent gave a positive reaction when borax was added. Table I gives a summary of the results obtained by Deuel and Neukom. Carubin, one of the most reactive polysaccharides, with boric acid neither became viscous nor formed a gel. Unlike monosaccharides none of the polysaccharides studied increased the acidity of a boric acid solution.

TABLE I

REACTION OF BORAX WITH AQUEOUS SOLUTIONS OF POLYSACCHARIDES (22)

Name and source of polysaccharide	Chemical structure of polysaccharide*
First Group: Gel formation with solutions 1% or less in concentration	
Salep mannan, mucilage from tubers of *Orchis morio*	Mannan (polymannose). Some acetyl groups. Linear molecule.
Carob bean gum (carubin), from the seeds of *Ceratonia siliqua*	Galactomannan. Linear molecule.
Mucilage from seeds of fenugreek (*Trigonella foenum-graecum*)	Galactomannan.
Mucilage from seeds of the honey locust (*Gleditsia triacanthos*)	Galactomannan.
Mucilage from seeds of alfalfa (*Medicago sativa*)	Galactomannan.
Second Group: Gel formation with solutions 5% or greater in concentration	
Mannan from bakers' yeast	Polymannose. Branched structure.
Alginic acid and its glycol ester	Polymannuronic acid.
Glycerol ester of pectic acid	Glycerol ester of polygalacturonic acid.
Mucilage from bark of slippery elm (*Ulmus fulva*)	L-Rhamnose, D-galactose, D-galacturonic acid.
Mucilage from flaxseed (*Linum usitatissimum*)	L-Rhamnose, L-galactose, D-xylose, D-galacturonic acid.
Gum arabic (*Acacia* species)	L-Rhamnose, D-galactose, L-arabinose, D-glucuronic acid. Branched structure.
Cherry gum (*Prunus avium*)	D-Mannose, D-galactose, L-arabinose, D-glucuronic acid.
Third Group: Gel formation in solution in very dilute cuprammonium	
Psyllium mucilage (*Plantago psyllium*)	D-Galactose, D-xylose, D-galacturonic acid, L-arabinose.
Tragacanth (*Astragalus* species)	D-Galactose, L-fucose, L-arabinose, D-xylose, D-galacturonic acid.
Fourth Group: Weak gel formation, but solutions become viscous and ropy	
Mucilage from tamarind seeds (*Tamarindus indica*)	Xylose, glucose, and galactose.
Galactan from the seeds of white lupine (*Lupinus albus*)	Polygalactose.

* References are cited by Deuel and Neukom (22) for the information on structure. Literature pertaining to the structures of these polysaccharides has been thoroughly reviewed recently (48), and many of the polysaccharides are discussed in recent books (73, 87).

Additional reports have added to the number of polysaccharides giving this reaction. Gel formation with borax has been reported for the galactomannans of carob and honey locust beans, and guar seed (*Cyamopsis tetragonalaba* (*psoralioides*)) (78). The effect of borax on the mucilage from tamarind seeds has been observed by others (92). The gum from the cashew tree (*Anacardium occidentale*) containing arabinose and galactose, reacts with borax (99). A polysaccharide of mannose and glucose from the roots of *Amorphophallus oncophyllus* also gives the borax gel test (108).

Of the compounds tested by Deuel and Neukom, alginic acid was unusual in that the sodium salt showed no reaction with borax whereas the ammonia salt and salts of organic bases like cyclohexylamine showed gel formation, as did the glycol ester of alginic acid. Pectin (partial methyl ester of polygalacturonic acid) gave a negative borax test, as expected, but the monoglycerol ester (20) gave a gel presumably owing to the reactivity of the glycerol hydroxyl groups. Under the conditions employed by Deuel and Neukom, the following polysaccharides gave no reaction with borax: starch, glycogen, methylcellulose, inulin, pectin, agar, carrageen, and quince seed mucilage. The solidity of a lichenin gel was increased by adding borax. The structure of these compounds suggests that they would have little or no reactivity with borax. Under certain conditions, however, they are reactive, presumably *trans*-1,2- or 1,3-hydroxyl groups being involved. For example, the increase in viscosity of starch and dextran solutions with addition of borax is of commercial importance (83,88). The solidity of an agar gel is increased by adding borax (100).

2. *Factors That Influence Complex Formation*

The effects of a number of factors on the polysaccharide-borax complex formation was studied by Deuel and Neukom (22). In Figure 4 are shown the effects of concentrations of borax and carubin and the pH on the viscosity of such solutions. Deuel and Neukom attribute the decline in viscosity at high concentrations of borax to the high pH, but it seems that much of the decrease must be due to the formation of the BD type of complex. Williams (105) has reported that carob bean gum gels could be liquefied by excess of borax. Once formed, such gels are difficult to liquefy (35), but solutions can be obtained by adding the gum solution to the borax (114), in which case the BD type of complex is formed initially.

Gel formation of the polysaccharides with borax can be reversed with low-molecular compounds that react with borax; fructose, glucose, mannitol, glycerol, glycol, and glyoxal are active; sucrose is inactive. Data are presented by Deuel and Neukom showing the effects of fructose, glycerol, and sucrose on the viscosity of carubin-

borax solutions. These polysaccharide gels are quickly reversed by dialysis.

Treating a carubin solution with potassium periodate, which oxidatively splits 1,2-glycols (87), had little effect on the viscosity of the solution without borax, but it prevented gel formation with borax.

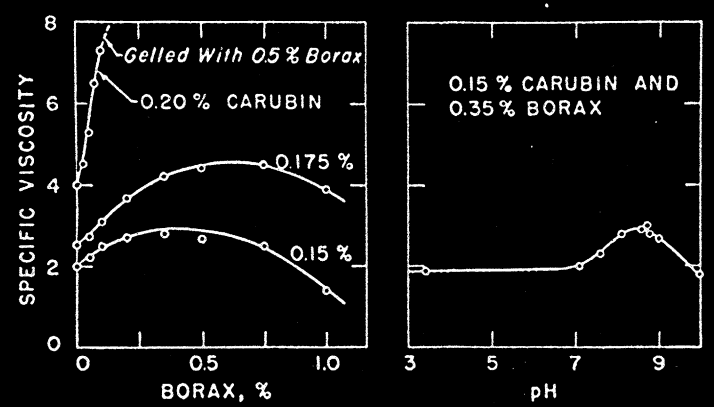


Fig. 4. The influence of concentration and *p*H on the viscosity of carubin-borax solutions at 20°C. (22).

3. *Complex Formation in Nonaqueous Solvents*

In view of Irany's (44,45) observation that boric acid and borax react with polyhydroxy compounds in nonaqueous media, Deuel and Neukom studied the effects of borax and boric acid when added to the polysaccharides in formamide. Both borax and boric acid produced gels, but only the boric acid gels became liquid when a little water was added. The boric acid gel showed a strong syneresis and gradually became flocculent, indicating that the didiol complex was stable. Dilute solutions of polysaccharides are more reactive in this system, as compared with the aqueous system, but here too the reaction appeared to be specific for neighboring *cis*-hydroxy groups.

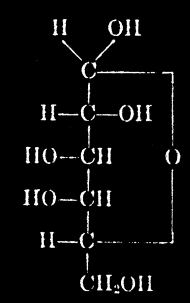
4. *Complex Formation with Tannin and Polyvinyl Alcohol*

On account of the trihydroxy benzoic acid component, tannins are reactive with borax. Since the molecular weight is low (about 1500), gel formation was obtained only when a 40% solution was used to which powdered borax was added. Boric acid, although its acidity is enhanced by tannin, did not form a gel, suggesting that the complex is type A. In formamide, 10% solutions of tannin gelled with boric acid (22).

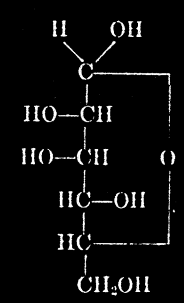
The reaction of borax with polyvinyl alcohol in aqueous solution is of interest, as it may be an example of a reaction with a 1,3-diol. Deuel and Neukom (22) confirmed the observations of Marvel and Denoon (71a) that polyvinyl alcohol was precipitated from aqueous solutions with boric acid and that gels were obtained with borax. Addition of periodate reversed the reaction, but at the same time reduced the macromolecular structure so the extent of the involvement of 1,2- and 1,3-hydroxyl groups could not be decided (22). The boric acid reaction could not be reversed by the addition of fructose. A type A complex was indicated. The gel obtained with borax could be reversed by adding fructose. In this case, the complex appears to be the BD_2 type.

5. *Nature of the Borate-Polysaccharide Complex*

Deuel and Neukom discuss their results in terms of the reactive component carbohydrates of the polysaccharides. D-Galactose (formula 3), its corresponding methylpentose—fucose, and galacturonic acid; D-mannose (formula 4), its corresponding methylpentose—rhamnose, and mannuronic acid are of this class. Glucose, glucuronic acid, xylose, and arabinose, the other carbohydrates most frequently encountered in the plant polysaccharides, do not have the required paired hydroxyl groups. The hydroxyl group on carbon atom 1 paired with carbon 2 is also reactive, however; hence glucose and xylose would be reactive in their α form. Since this group takes part in bond formation with other carbohydrates, only the hydroxyl



(3)



(4)

groups on carbon atoms 1 and 2 of the terminal carbohydrate in a chain would be free to react with borate.

With many of the polysaccharides, the presence of the required hydroxyl groups is evident. In the galactomannans, the hydroxyl groups on carbon atoms 2 and 3 of the mannose are available, since mannose is bound to galactose by a 1,4-glycosidic linkage (78). Terminal galactose units would also be reactive (carbon atoms 3 and 4). Apparently galactose in compounds like the galactan of alfalfa is bound in such a manner that only the terminal galactose is reactive.

The implications of the mechanical properties of the gels given by the different polysaccharides are discussed by Deuel and Neukom (22). The high viscosity and ropiness (threads about 1 meter long can be drawn out) shown by concentrated solutions (more than 10%) of the galactan of white lupine are attributed to complex formation with the galactose end groups of this polysaccharide. Since more dilute solutions of the tamarind seed mucilage gave the borax test this polysaccharide may contain more of the terminal galactose units. Other polysaccharides, like salep mannan and carubin, give a three-dimensional network with borax, manifested by absence of syneresis, and stickiness of the gel. Also gels that are broken up by stirring quickly return to their former state. Even boiling does not make the gel completely fluid. Deuel and Neukom state that gel formation is understandably more likely with a linear molecule like that of salep mannan than with a branched structure like the yeast mannan. In the latter instance (36), for example, only one of the six mannose residues in the repeating unit contains paired hydroxyl groups in the *cis* position. The higher the molecular weight, the greater is gel formation. Gel formation by carubin decreased progressively as it was hydrolyzed enzymically (22).

The authors discuss the contribution that a study of the borax reaction can make to elucidating the structure of the polysaccharides. Further studies of the comparative reactivity of the hydroxyl groups in five- and six-membered saturated rings are required, however, for a fuller understanding of the reaction as applied to polysaccharides. Studies with borate of the type performed by Reeves (88a) with the cuprammonium reagent should provide much needed information. Reeves' measurements of complex formation with various substituted hexoses and pentoses revealed the paired hydroxyl groups involved. Consideration of the possible ring configurations of the molecules showed that certain configurations permitted *trans*-, as well as *cis*-, hydroxyl groups to be in a favorable position for complex formation (88a). The unreactivity with borate of paired *cis*-hydroxyl groups in six-membered saturated ring molecules and the reactivity of polysaccharides suggest that when such rings are in chains the hydroxyl groups are in more favorable positions. The chemical data (48,78) appear to exclude the furanose five-membered ring in the polysaccharides as the explanation for the reactivity of the hydroxyl groups with borate.

B. BLOOD GROUP SUBSTANCES

1. Isolation of Blood Group Substance with Borate

Large amounts of polysaccharide are present in alkaline phosphatase preparations obtained from calf intestinal mucosa by tryptic digestion (93,110). In an effort to fractionate this material further, borate was added to solutions that were fractionated with ammonium sulfate with the hope that it would influence the solubility of the polysaccharide portion (110). Borate decreased the solubility of the polysaccharide (110), and by this means with little difficulty a relatively pure substance was obtained (112,113). This material had no phosphatase activity and was found to be blood group polysaccharide (112,113). This borate-ammonium sulfate fractionation was equally applicable to commercial hog gastric mucin (112,113), a frequently used starting material for preparing blood group substance. The following procedure (113) was used for purifying the blood group polysaccharide from this material:

2.0 g. of mucin (Wilson Labs. Type 1701-W) was suspended in 173 ml. of water and 27 ml. of 0.5 *M* $NaHCO_3$. As preservative, 1.0 ml. of chloroform was added, and the mixture was left for 18 hours at room temperature and stirred occasionally. The mucin was almost completely dissolved, and only a small amount of sediment was removed from the milky solution by centrifuging. 125 g. of ammonium sulfate, the amount necessary to precipitate the proteins, was added to this solution; the large amount of precipitate which formed was removed by filtration. Seven volumes of the clear solution were mixed with one volume of 0.2 *M* sodium tetraborate. The mixture was kept at 7° for 2–3 hours and stirred vigorously to convert to a flocculent precipitate the gel which first formed. The precipitate formed slowly, and quantitative precipitation was usually shown by a clear supernatant fluid. The precipitate was collected by centrifuging, dissolved in 30 ml. of water, and dialyzed to eliminate ammonium sulfate and borate. After dialysis, the polysaccharide was precipitated by addition of 2 volumes of acetone and 1.0 ml. or more of 2 *M* sodium acetate per 20 ml. of aqueous solution.

The precipitate was readily dried by triturating in acetone with a spatula. The yield was 11.5% of a white, granular product, with the usual serological properties of blood group A substance. This material was free of protein without further treatment, as shown by the chloroform-amyl alcohol test (113). In electrophoresis, there was one main component; reasons were given (113) for believing that the second, faster component, present to the extent of about 35%, was an "acid" polysaccharide previously reported to be in mucin.

In this procedure, the concentration of ammonium sulfate was 3.58 *M* (78% saturated). In a brief earlier report (112), a procedure was described in which the solution was 57% saturated with ammonium sulfate, a concentration, however, which does not give a water-clear filtrate. Landy and Batson (65), however, used this concentration of ammonium sulfate and subsequent addition of borate. Their product required deproteinization with chloroform-amyl alcohol, but after this treatment it was 96% homogeneous electrophoretically. Ikawa and Niemann (43a) also used the borate procedure for preparing blood group A substance, and have given some physical and chemical data for the product.

The quantitative influence of the borate on the solubility of the blood group polysaccharides has not been determined. In the material from calf intestinal mucosa the polysaccharide was precipitated from a solution that was 57% saturated with ammonium sulfate. The bulk of the proteins remained in solution and were precipitated with additional ammonium sulfate after the polysaccharide fraction was removed. From these examples, it is evident that the method can be adapted to the solubilities of the particular components involved.

The reaction of borate with a preparation of blood group substance was observed earlier by Morgan and King (81), but the probable parallelism with the reactions of borate with simple carbohydrates was not noted. Interesting effects were described when "undegraded" blood group substance was used: "the addition of an equal volume of 0.05 *M* borate buffer (pH 8.5) to a 1 percent solution of the A substance gives rise to gel formation; the system, however, still retains pronounced elastic properties. The elastic quality may readily be shown by rotating a thin gelatinous solution in a suitable vessel and observing the return motion that follows immediately the original rotational motion has stopped. The sol-gel change is reversible. Dialysis of the gel at 0° against distilled water leads to the formation of the original viscous solution. These reversible sol-gel changes do not occur with degraded preparations of A." A recent report (1) from the same laboratory, without reference to their previous observations (81), states that a purified A substance did not give an elastic gel with borate. Ikawa and Niemann (43a) made the same test with their preparations of blood group A substance, and none gave a gel. Apparently some component of gastric mucin, present in Morgan's first preparation of blood group substance (81), is even more reactive with borate with respect to gel formation than is the blood group substance. The ability of borax to lake red blood cells (52a) may be due to a reaction with the blood group substance of the cell surface.

2. *Change in Properties in the Presence of Borate*

The reaction of borate with blood group substance is strikingly shown by the changes in electrophoretic and sedimentation properties. The studies to be described were performed with the blood group substance from calf intestinal mucosa. This substance appears to differ principally from that obtained from hog gastric mucin in that it is more highly charged, probably because of the presence of esters of sulfuric acid (113).

The electrophoretic mobility of the polysaccharide was determined in borate-free buffers (phosphate) and in solutions in which the phosphate was replaced, either wholly or partially, by borate–boric acid mixtures. Experimental details are given in the original paper (59). The mobility of the polysaccharide as a function of pH is shown by the solid lines in Figure 5. The effect of the borate at several pH values is shown by the dotted lines. The increase in mobility with addition of borate was paralleled by a sharpening of the electrophoresis boundaries. The failure of borate to change the mobility of the polysaccharide below pH 5.5 may be ascribed to inadequate concentrations of the ionized form of the complexes, a result of the combined effects of limited combination and low degree of ionization. The increased combination of borate with diol induced by the mounting concentration of borate ion, and the greater ionic dissociation of the complexes resulting from rise in pH were together responsible for the rapid increase in mobility above pH 5.5.

To separate the effects of borate ion concentration (which determined the nature and extent of diol-borate combination) from that of pH (which determined the degree of ionic dissociation of the diol-borate complexes), preparation II of the polysaccharide was studied in solutions of different borate concentration and constant pH. Figure 6 shows the change in the mobility with increase in borate concentration. The observed results have been interpreted in terms of changes in the relative amounts of the BD_2, BD types of complex (59).

The viscosity and sedimentation in an ultracentrifuge of the blood group substance in borate have also been investigated (60). The viscosity was studied for concentrations of blood group substance from 2.5 to 15 mg./ml., and for borate concentrations up to 0.05 *M*. The viscosity increased at high diol-borate ratios but decreased at low diol-borate ratios, results reminiscent of the carubin data 22).

Sodium borate, at pH 8.6, in concentrations up to 0.05 *M*, progressively increased the sedimentation constant of dilute solutions of

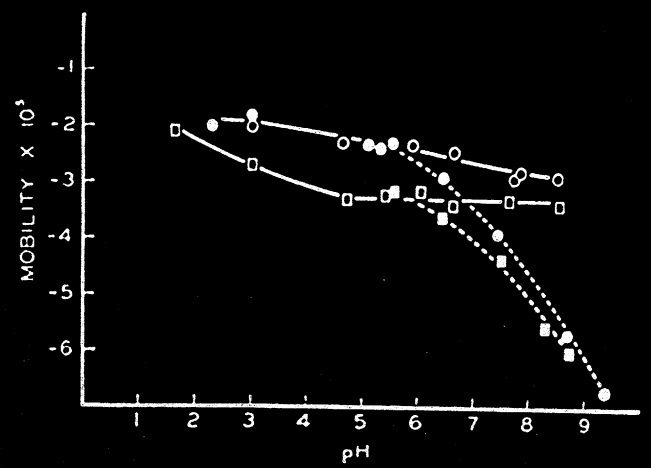


Fig. 5. Mobility of the blood group polysaccharide (10.0 mg./ml.) as a function of *p*H: (□) preparation I in borate-free solution; (○) preparation II in borate-free solution; (■) preparation I in borate solution; (●) preparation II in borate solution (59). Preparation I was homogeneous in electrophoresis; preparation II contained 10-15% of slowly migrating constituents (113).

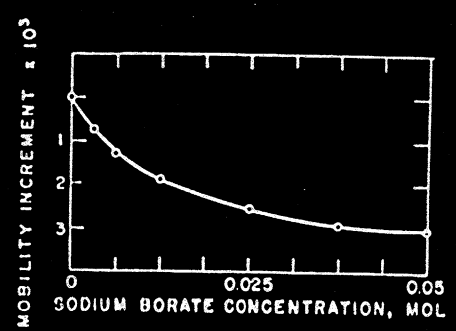


Fig. 6. Mobility increments of blood group polysaccharide (10.0 mg./ml.) (preparation II) at *p*H 8.6 as a function of sodium borate concentration (59).

the blood group polysaccharide (5.0 mg./ml.), corrected for density and viscosity, from 7.1 to 8.0 *S*. In concentrated solutions (15 mg./ml.), borate up to 0.025 *M* increased the sedimentation constant still further (up to 11.0 *S*); in 0.05 *M* borate the sedimentation constant was independent of polysaccharide concentration throughout the same range as in borate-free solutions. Both the viscosity and sedimentation data were interpreted in terms of the BD_2, BD types of complex, the former being mostly responsible for the changes observed. Regardless of the borate content of the solvent, the sedimentation diagram of the polysaccharide consisted of a single boundary, a manifestation of the rapid establishment of equilibrium between the sedimenting units (BD_2, BD, and D).

3. *Component Monosaccharides Probably Responsible for Reaction*

Blood group substance contains D-galactose and L-fucose (5,10) both of which have neighboring hydroxyl groups in the *cis* position for reacting with borate. However, if these carbohydrates are bound together by 1,4 linkages as is frequently found, only the terminal molecules have paired hydroxyl groups. The requirement for specific groupings is emphasized by the fact that hyaluronic acid, which is composed of *N*-acetyl-D-glucosamine and D-glucuronic acid, did not react with borate (113).

The blood group substances are composed of protein and polysaccharide and have distinctive serological properties. Blood group activity and cross reactivity with *Pneumococcus* type 14 antibody appear to reside in different parts of the polysaccharide molecule (1,49). Studies performed in borate may throw additional light on the groupings involved in each instance. Other animal polysaccharides that should react strongly with borate are gonadotropic hormone, which seems to be closely related to blood group substances (29,103), and the mucoprotein from egg white, which contains 10% of mannose, together with glucosamine (28). Borate bound to this mucoprotein might be the nondiffusible borate compound postulated by Hove *et al.* (43) to explain the distribution of borate between the white and yolk of the egg (8–10:1).

C. BACTERIAL POLYSACCHARIDES

In view of the great variety of polysaccharides obtained from microorganisms (27), it is not surprising that there have been observations suggestive of reactions of polysaccharides with borate. No detailed investigations, however, have been made in this field.

A neutral polysaccharide from the group A hemolytic streptococci

was negatively charged in electrophoresis in borate, whereas it had zero net charge in phosphate buffer (109). The composition of this polysaccharide is not known. This observation led to the use of borate to separate the polysaccharide from calf intestinal mucosa (110). It has also been observed that a polysaccharide from the tubercle bacillus had its electrophoretic mobility increased in the presence of borate (102). This polysaccharide contains mannose as one of its component carbohydrates (37,89).

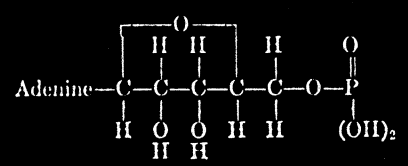
Lopatkin has reported that cultures of the Shiga-Kruse dysentery bacillus are agglutinated by boric acid or borax. Deuel and Neukom have pointed out (22) that this phenomenon might be due to the presence in this organism of a polysaccharide containing galactose and rhamnose (27). Inhibition of the "swarming" of *Proteus vulgaris* by boric acid was considered to be due to complex formation with a component polysaccharide (98). Gel formation by a mannan from yeast with borax (22) was mentioned in a previous section.

Boric acid and borax are inhibitory to many microorganisms, but this is more likely to represent a reaction with enzymes rather than with polysaccharides. It will be referred to briefly in a later section.

IV. Reaction of Borate with Vitamins and Other Substances That Are Components of Coenzymes

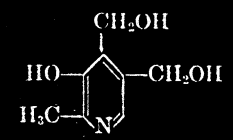
A number of reactions of borate with vitamins and coenzymes have been reported in the literature. Other compounds in this category can be expected to react because of their components. Riboflavin phosphate, coenzymes I and II, and vitamin B_{12}, reported to contain ribose (11), fall into this group. Pantothenic acid might also be expected to react with borate. In spite of adjacent hydroxyl groups in the *cis* position, inositol reacts little or not at all with borate (9,58a).

A. ADENOSINE 5-PHOSPHATE (MUSCLE ADENYLIC ACID)



Klimek and Parnas (53) found that adenosine 5-phosphate reacted with boric acid; 40 mg. of the neutralized phosphate together with 1.0 ml. of 0.2% neutralized boric acid became acid enough to require 1.25 ml. of 0.01 *N* NaOH to restore the original pH. Yeast adenylic acid, on the other hand, did not react with boric acid; this is to be expected, since here the phosphate is bound to carbon atom 3, thus eliminating the *cis*-hydroxy pair on carbon atoms 2 and 3.

B. PYRIDOXINE (VITAMIN B_6)



Pyridoxine reacts strongly with borate (94); this is evident from the subsequent nonreactivity of pyridoxine with 2,6-dichloroquinone chlorimide, change in the absorption spectrum, increase in acid strength, and in thermostability. The inability of pyridoxine compounds substituted at the 4-hydroxymethyl group to react with borate and the titration data indicate that the 3-phenol and 4-hydroxymethyl groups of pyridoxine react with borate, giving the BD_2 type of complex. The complex is fully active as a vitamin, indicating that it is completely dissociated in the animal organism to give the unchanged vitamin.

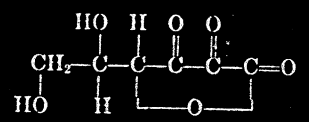
The reaction of borate with pyridoxine is interesting since it exemplifies the reaction of borate with another type of 1,3-diol. However, it may not be possible to study the coenzyme function of pyridoxine or pyridoxal with borate since combination with phosphate, which gives the coenzyme, is apparently at the phenolic group (carbon 3) (51). This substitution would prevent the borate reaction.

C. RIBOFLAVIN (VITAMIN B_2)

Kuhn and Rudy first showed that the optical rotation of riboflavin was reversed by borax (61). Frost (31) investigated the riboflavin-borate complex further and found that the solubility of riboflavin could be increased 25-fold by adding borate and heating. The effect of heat was not clear, but riboflavin tended to crystallize out of insufficiently heated solutions. The absorption spectrum was not changed by the borate. Addition of acid to the complex generally caused precipitation of the riboflavin in a short time. A clear-cut stoichiometric relationship between the components of the complex

was not apparent; in the pH range of 6.4 to 6.6, 0.5% boric acid held in solution about 0.3% riboflavin, a molecular ratio of about 1:100. Evidence that the ribityl group is the site of the reaction is the fact that the solubility of 6,7,9-trimethylisoalloxazine and tetraacetylriboflavin is not affected by borate. The chemical reactivity of the isoalloxazine group (reduction with sodium hydrosulfite, reoxidation by air, photolysis to lumiflavin) is not altered by borate. Also, although none of the hydroxyl groups of riboflavin can be benzoylated in the presence of borate, without borate a tetrabenzoate can readily be obtained. In microbiological and animal experiments the riboflavin-borate complex showed full biological activity.

D. DEHYDROASCORBIC ACID



The oxidation of dehydroascorbic acid, the reversible oxidation product of vitamin C with antiscorbutic activity, by cupric ion or methylene blue is prevented by borate (77), suggesting that a complex is formed by interaction at the diketo group. In spite of the presence of an enediol group —C(OH)=C(OH)— at carbon atoms 2 and 3, ascorbic acid apparently does not react with borate, for its oxidation by the same reagents is not inhibited by borate (77).

V. Effects of Borate on the Activity of Certain Enzymes.

The effects of borate on enzymes are divided into three groups: (*a*) those in which polyhydroxy compounds (substrate or coenzyme) seem to be involved, (*b*) those in which the mechanism of the inhibition is unknown, and (*c*) an example of inhibition by borate which appears to be ionic, namely, its effect on alkaline phosphatase.

A. POLYHYDROXY COMPOUND AS COENZYME OR SUBSTRATE

In most cases, references to inhibition by borate are brief. Enzymes with riboflavin-containing prosthetic groups are apparently not sensitive to borate. Potato aldehyde oxidase was not affected by 0.05 *M* borate at pH 7.2 (107), but the oxidation of mescaline (3,4,5-trimethoxyphenylethylamine) catalyzed by a rabbit liver preparation at pH 7.8 was strongly inhibited by 0.008 *M* sodium borate. The oxidation of tyramine, on the other hand, was not affected by the same concentration of borate (7). Xanthine oxidase of milk was not affected by 0.01 *M* borate at pH 8.5 (115). Cytochrome c reductase, with coenzyme II as the reducing agent, was apparently unaffected by 0.04 *M* borate at pH 9.0 (40). The resistance of these enzyme systems to borate is puzzling, for when the coenzymes are hydrolyzed by enzymes borate is inhibitory. Further, the ribityl side chain of riboflavin is specific for its vitamin activity; only the D-arabityl group can replace it (26). So it would appear that any combination with this group would interfere with its physiological activity. It may be that once bound to protein the hydroxyls of the ribityl group are masked.

A recent paper reports (91a) that borate is inhibitory to xanthine oxidase from milk. Oxidation of the xanthine was measured spectrometrically (ΔE_{290}) in pyrophosphate buffer at pH 8.5; variation of the concentration of xanthine from 8.5 to 68.5 $\times 10^{-6}$ *M* without and with 0.016 *M* borate showed that the inhibition was of the competitive type. A report that borate was not inhibitory to xanthine oxidase (115) was based on manometric experiments with 0.005 *M* xanthine, a concentration too high to permit borate to show inhibition. The demonstration (91a) that borate is specifically inhibitory to the riboflavin-containing xanthine oxidase, presumably by interaction with the ribityl group, is of broad implications in view of the many enzyme systems that contain the ribityl or ribose group. The competitive nature of the inhibition is of additional interest because it indicates that the ribityl group participates in the formation of the enzyme-substrate complex.

Examples of the inhibition by borate of the action of enzymes on substrates that are coenzymes in other enzyme systems are the following: A phosphatase highly specific for adenosine 5-phosphate (the 3-substituted compound is not hydrolyzed) is stated to be inhibited by borate; phosphate was less inhibitory (38). A nucleotide pyrophosphatase from the potato, which hydrolyzes the pyrophosphate bond in coenzyme I between adenosine 5-phosphate and nicotinamide mononucleotide, is strongly inhibited by borate at pH 8.5 (58). Both these inhibitions probably result from the combination of borate with the ribose portion of the molecules.

There are other examples which appear to reflect a combination of

borate with substrate, with concomitant inhibition of the specific enzyme. Q-enzyme from the potato, which catalyzes the transformation of amylose to amylopectin, was inhibited by borate (33). The synthesis at pH 6.0 of starch from glucose 1-phosphate was inhibited 21% by 0.1 *M* borate, whereas the reverse process of starch degradation at pH 6.8 was accelerated 17% (107).

Oxidation of dihydroxylphenyl-L-alanine by various plant oxidases was inhibited by as little as 0.01 *M* boric acid (70). Oxidation of glycolic and lactic acids was affected less by borate, and ascorbic acid not at all. The effect of borate on the enzymic oxidation of these four compounds was roughly parallel to their complex formation with borate. Surprisingly, a plant enzyme that oxidizes catechol was not consistently affected by 0.1 *M* borate (32). The action of the enzyme glucosulfatase was also inhibited by borate (96), a 0.001 *M* concentration having a perceptible effect. However, phosphate is even more inhibitory and sulfate is inhibitory, so the effect may be ionic.

B. INHIBITIONS OF UNKNOWN MECHANISM

The following enzymes are reported to be inhibited by borate: soybean (19), jack bean (97), and bacterial (84), ureases, arginase (79), cholinesterase (30), pepsin (8), and phosphodiesterase (111). Since urease is inhibited by phosphate in a competitive manner (34), the borate inhibition may be the same type, and it seems likely that both exert their influence as anions and that the borate does not react with a diol group. Borate is much more inhibitory to Mn-activated arginase than is phosphate, whereas the opposite is true of the Mg-activated alkaline phosphatases of animal tissues (115). Borate, however, is more inhibitory than phosphate to alkaline phosphatase from top yeast (39a). In borate inhibition of pepsin, Bersin and Berger (8) believed the borate exerted its influence by combining with the protein substrate as an anion. The phosphodiesterase acting on ribonucleic acid appeared to be inhibited noncompetitively by borate and competitively by phosphate (111). More detailed studies are needed to elucidate the mechanism of this inhibition.

C. INHIBITION PROBABLY IONIC (ALKALINE PHOSPHATASE)

Borate has frequently been reported to be inhibitory to the enzyme alkaline phosphatase (18, cited in ref. 115). Recently a detailed investigation has been made of the inhibitory effect of borate on the alkaline phosphatase from cow's milk and from calf intestinal mucosa (115). Phenyl phosphate was the substrate. Like other anions, borate affected both the position and sharpness of the pH optimum.

By varying the concentration of borate and plotting against the reciprocal of the phosphatase activity, straight lines were obtained from which concentrations giving 50% inhibition were read. For the milk phosphatase, this was 0.050 *M* tetraborate; for the mucosa enzyme, 0.019 *M*. Experiments were also performed with several concentrations of borate and various concentrations of the substrate.

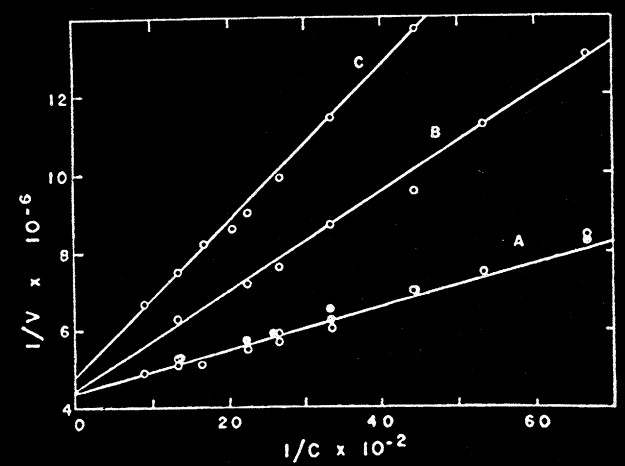


Fig. 7. Effect of borate on calf intestinal mucosa phosphatase in relation to concentration of substrate (115). Velocity (V) is expressed in moles per 5 minutes. Concentration of substrate (C) is in moles per liter. (A) no borate; (○) 0.035 *M* ethanolamine; (●) 0.071 *M* ethanolamine; (B) 0.005 *M* tetraborate; (C) 0.01 *M* tetraborate.

The results obtained for the mucosa phosphatase are shown in Figure 7, where the reciprocals of the substrate concentration and the enzyme velocity are plotted. The ethanolamine buffer in which the assays were performed was not inhibitory. Data for the enzyme-substrate dissociation constants and the inhibition constants were calculated. Since neither the results given for the mucosa phosphatase nor the results with the milk phosphatase showed a shift of the $1/V$ intercepts, it was concluded that the inhibition was competitive, as contrasted with the noncompetitive, in which an intercept shift does occur.

This means that the borate was bound at a site which also bound the substrate. Since phosphate, and even carbonate are inhibitory by a similar mechanism, it was concluded that borate, like phosphate

and carbonate, acts as an anion, probably on a heavy metal site in the enzyme.

The type of inhibition shown by borate acting on an enzyme containing a coenzyme of the kinds discussed earlier might be either competitive or noncompetitive. The outcome would be determined by whether the borate-reactive diol grouping of the coenzyme contacted the substrate directly. To decide whether a diol group was involved, it would be necessary to study other anions to determine whether the borate was a specific inhibitor.

VI. Reaction of Borate with Viruses

Several instances have been reported recently of an inhibitory effect of borate on viruses. Boric acid was moderately inhibitory to vaccinia virus (24). Borate (0.1 *M*) was much more detrimental than was phosphate to the agent of chicken tumor I when incubated at 37° for 3 hours (14). The greatest destruction occurred at pH values above neutrality. This effect cannot represent simply a reaction with diol groups, for it was apparently irreversible; the concentration of borate in the solutions used for assay was only 0.005 to 0.0005 *M*. Borate also appears to exert an inactivating influence on the T_7 bacteriophage of *Escherichia coli* (52). A small increase (3%) in the sedimentation rate of this phage in borate, as compared with other buffers, may indicate an actual union between borate and phage, although physical factors alone may account for this small difference.

It appeared (112) that the borate reaction might be of use in studying the reaction between virus and blood cell or tissue receptor sites. Evidence for the polysaccharide nature of these sites has been reviewed recently by Anderson (2). Destruction of these sites by periodate (39), indicating paired hydroxy groups, and the ability of blood group substance apparently to unite with virus since it hindered the virus-receptor union (15,41), suggested (112) that borate might react with the receptor site. There was a possibility that hemagglutination might be inhibited by borate or even that the infectious process itself might be affected. Borate might be expected to interfere also in the phage-bacterium interaction where polysaccharides are involved as well (3). However, in studies in which borate was the suspending medium for influenza virus hemagglutination studies (12,13), the results were not favorable to such a hypothesis. With one strain (13) agglutination was decreased by borate, but with another strain (12) agglutination was increased, and in both cases other compounds (arsenite, arsenate) exerted comparable effects.

VII. Physiological Activity of Borate

A. PLANTS

That plants require small amounts of borate is well known. The extensive literature on this subject has been reviewed recently (6). In a number of instances (70), borate has been shown to have an effect on enzymes from plant sources, but in no case did the results appear to throw light on the borate requirement of plants. Winfield (106) has discussed the possible role of borate in plant metabolism from the standpoint of its ability to form complexes with polyhydroxy compounds, particularly pyridoxine and riboflavin. The reaction of flavones with boric acid, and a high correlation between the borate and flavone content in flowers and fruits suggested that the two substances are present in plants in combination (98a). It should be noted that the plant's requirement for borate can readily be exceeded, and then it becomes toxic to the plant (25).

B. MICROORGANISMS

Few investigations have been made of the borate requirements of the lower forms of life. Such studies would be of interest in view of the borate requirement of plants. Studies performed with fungi (*Aspergillus niger*, *Penicillium glaucum*) indicate that they do not require borate (107).

Boric acid and borax are deleterious to both bacteria (63) and fungi, but the former are affected by concentrations (0.1 to 0.3%) which do not appear to harm fungi. This fact has been utilized in making plate counts of fungi (101) and in reducing contamination in penicillin production (54). Even in bacteria-free culture, borax enhanced penicillin production (55). The yellow-green fluorescent compound described by Kuhn (63) apparently was not formed. Winfield (107) did not observe this compound either. There is considerable strain difference in the response of fungi (*Aspergillus* (82), *Penicillium* (54,55)) to borate. Several aspects of the metabolism of *Penicillium* in the presence of borate have been reported (55,56). Borate, as well as *m*-inositol, reversed the inhibitory effect of malonate on fermentation by *Clostridium saccharobutyricum* (90a). At 0.17% con-

centration borax markedly increased the action of penicillin against *Salmonella typhosa*, but 1% was antagonistic to the action of penicillin (8a). Borate is inhibitory to all stages of the life cycle of the malaria parasite (95). The large amounts of polysaccharide in protozoa (42) may be relevant. Novak (82a) has reviewed the literature on the germicidal action of boric acid and borates.

Kuhn (62) has observed that boric acid can be a sex-determining factor for the bisexual green algae *Chlamydomonas*. The bisexual alga secretes both male and female sex-determining substances, the latter a methyl ether of quercetin. By combining with the quercetin derivative, boric acid permits the excess of the male factor to determine the sex. Kuhn reported (63) that the growth of tomato pollen was affected by boric acid; a concentration of 0.001% gave 90% germination in 15% sucrose. Apparently, in this instance, the complex of boric acid and a monomethyl ether of quercetin functioned as a growth factor. In this paper (63) Kuhn reviews biological research with boric acid and discusses the biological significance of boric acid.

C. ANIMALS

There is no evidence that borate in other than minimal amounts is required by animals. This has been discussed in a paper by Hove, Elvehjem, and Hart (43) and briefly reviewed recently (72).

The physiological activity of borate in the animal body has been investigated recently by Frost and Richards (32) and Pfeiffer, Hallman, and Gersh (86). The literature on the topic has been comprehensively reviewed by Pfeiffer *et al.* (86,86a). The oft-quoted report that boric acid appears in the urine within a minute after immersion of the feet in saturated boric acid (50) could not be confirmed by Pfeiffer *et al.* (86). Boric acid does not readily pass through the intact skin, but it does pass through injured areas. The authors state that ointments or powdered boric acid should never be used on such areas, nor should solutions be used for irrigation of body cavities. From cases described in the literature, the authors conclude that the fatal dose is 15–20 g. for an adult and 5–6 g. for an infant. In experiments lasting up to 6 months, Wiley found much smaller doses (0.5 g./day) to be disturbing (104). Pfeiffer *et al.* determined the fatal dose of boric acid for mice, rats, guinea pigs, and dogs. Boric acid made neutral was slightly more toxic to mice (subcutaneously) than was the unneutralized. Sorbitol, mannitol, or 0.9% NaCl given orally did not protect mice against the boric acid. A mixture of Ringer solution and plasma (1:1) did offer protection. The borate in poisoned animals was found principally in the brain and liver, with smaller amounts in the body fat.

In chronic toxicity experiments with dogs, the boric acid was excreted rapidly (86), as all other investigators have found. Apparently, the boric acid was excreted in the free form, as was also observed by Wiley (104). After the initial peak of boric acid excretion (1–2 hours), the phosphate excretion began to rise, and at 6 hours it exceeded by 5 times the control value. Wiley also found disturbed phosphate metabolism in human volunteers (104). Pfeiffer *et al.* (86) found that in chronic toxicity experiments the most pronounced pathological changes were in the kidney and the central nervous system. The fact that the plateau of urinary excretion was not reached until 14 to 18 days suggested that there was a cumulative effect.

Fractionation of the brain lipides showed that there was boron in the acetone-soluble fraction, which was ether insoluble and water soluble. There was some evidence that phosphate had been displaced from this fraction. It was tentatively concluded that a boro-glycerate complex might be involved (86).

Boric acid reduces the ability of alloxan to produce diabetes in rats (64). Since boric acid stabilizes the lactim form of alloxan an enhanced diabetogenic action was expected.

In view of the ability of borate to bind many substances of physiological importance, one might expect it to be more toxic. The animal body is probably spared greater toxic manifestations from moderate doses of borate by the ease with which compound formation is reversed and the fact that borate is rapidly excreted through the kidney at a very low threshold.

The literature on borate and its ability to form complexes with polyhydroxy compounds has been summarized principally with the thought that the reaction would provide another tool for studying biological mechanisms. Studies of this type would be expected also to throw light on the need of plants for small amounts of borate, and probably to explain the toxic effects of higher doses on both plants and animals.

References

1. Aminoff, D., Morgan, W. T. J., and Watkins, W. M., *Biochem. J.*, 46, 426 (1950).
2. Anderson, S. G., *Federation Proc.*, 8, 631 (1949).
3. Ashenburg, N. J., Sandholzer, L. A., Scherp, H. W., and Berry, G. P., *J. Bact.*, 59, 681 (1950).
4. Bell, F. K., Carr, C. J., Evans, W. E., and Krantz, J. C., Jr., *J. Phys. Chem.*, 42, 507 (1938).
5. Bendich, A., Kabat, E. A., and Bezer, A. E., *J. Am. Chem. Soc.*, 69, 2163 (1947).
6. Berger, K. C., in *Advances in Agronomy*, Vol. I. Academic Press, New York, 1949, p. 321.
7. Bernheim, F., and Bernheim, M. L. C., *J. Biol. Chem.*, 123, 317 (1938).
8. Bersin, T., and Berger, S., *Z. physiol. Chem.*, 283, 74 (1948).
8a. Bigger, J. W., *Rept. 4th Intern. Congr. Microbiol.* 1947, 95 (1949); *Chem. Abstracts*, 43, 4339 (1949).
9. Böeseken, J., in *Advances in Carbohydrate Chemistry*, Vol. IV. Academic Press, New York, 1949, p. 189.
10. Bray, H. G., and Stacey, M., *ibid.*, p. 37.
11. Brink, N. G., Holly, F. W., Shunk, C. H., Peel, E. W., Cahill, J. J., and Folkers, K., *J. Am. Chem. Soc.*, 72, 1866 (1950).
12. Briody, B. A., *J. Infect. Diseases*, 83, 283 (1948).
13. Briody, B. A., *ibid.*, 83, 293 (1948).
14. Bryan, W. R., Maver, M. E., Moloney, J. B., Thomas, M. A., and Sells, M. T., *J. Natl. Cancer Inst.*, 10, 647 (1949).
15. Burnet, F. M., McCrea, J. F., and Anderson, S. G., *Nature*, 160, 404 (1947).
16. Cohen, S. S., and Scott, D. B. McN., *Science*, 111, 543 (1950).
17. Coleman, G. H., and Miller, A., *Proc. Iowa Acad. Sci.*, 49, 257 (1942).
18. Cram, D. M., and Rossiter, R. J., *Can. J. Research*, E27, 290 (1949).
19. Dei, K., *Tohoku J. Exptl. Med.*, 42, 93 (1942); *Chem. Abstracts*, 42, 5068 (1948).
20. Deuel, H., *Helv. Chim. Acta*, 30, 1523 (1947).
21. Deuel, H., Neukom, H., and Weber, F., *Nature*, 161, 96 (1948).
22. Deuel, H., and Neukom, H., *Die makromolekulare Chemie*, 3, 13 (1949).
23. Deutsch, A., and Osoling, S., *J. Am. Chem. Soc.*, 71, 1637 (1949).
24. Dunham, W. B., and MacNeal, W. J., *J. Bact.*, 44, 413 (1942).
25. Eaton, F. M., *J. Agr. Research*, 69, 237 (1944).
26. von Euler, H., Karrer, P., and Malinberg, M., *Helv. Chim. Acta*, 18, 1336 (1935).
27. Evans, T. H., and Hibbert, H., in *Advances in Carbohydrate Chemistry*, Vol. II. Academic Press, New York, 1946, p. 203.
28. Fredericq, E., and Deutsch, H. F., *J. Biol. Chem.*, 181, 499 (1949).
29. Friedmann, R., *Nature*, 164, 626 (1949).
30. Frommel, E., Herschberg, A. D., and Piquet, J., *Helv. Physiol. Pharmacol. Acta*, 2, 169 (1944).
31. Frost, D. V., *J. Biol. Chem.*, 145, 693 (1942).
32. Frost, D. V., and Richards, R. K., *J. Lab. Clin. Med.*, 30, 138 (1945).
33. Gilbert, G. A., and Swallow, A. J., *J. Chem. Soc.*, 1949, 2849.
34. Harmon, K. M., and Niemann, C., *J. Biol. Chem.*, 177, 601 (1949).
35. Hart, R., *Ind. Eng. Chem., Anal. Ed.*, 2, 329 (1930).
36. Haworth, W. N., Heath, R. L., and Peat, S., *J. Chem. Soc.*, 1941, 833.
37. Heidelberger, M., and Menzel, A. E. O., *J. Biol. Chem.*, 118, 79 (1937).
37a. Heidt, L. J., Gladding, E. K., and Purves, C. B., *Paper Trade J.*, 121, 81 (1945).
38. Heppel, L. A., and Hilmoe, R. J., *Federation Proc.*, 9, 184 (1950).
39. Hirst, G. K., *J. Exptl. Med.*, 87, 301 (1948).
39a. Hoffman-Ostenhof, O., and Moser, H., *1st Internatl. Congr. Biochem. Abstracts*, 1949, 345.
40. Horecker, B. L., *J. Biol. Chem.*, 183, 593 (1950).
41. Horsfall, F. L., Jr., and McCarty, M., *J. Exptl. Med.*, 85, 623 (1947).
42. Hotchkiss, R. D., *Arch. Biochem.*, 16, 131 (1948).
43. Hove, E., Elvehjem, C. A., and Hart, E. B., *Am. J. Physiol.*, 127, 689 (1939).
43a. Ikawa, M., and Niemann, C., *Arch. Biochem.*, 27, 441 (1950).
44. Irany, E. P., *Ind. Eng. Chem.*, 35, 1290 (1943).
45. Irany, E. P., in Alexander, J., *Colloid Chemistry*, Vol. VI. Reinhold, New York, 1946, p. 1089.
46. Isbell, H. S., Brewster, J. F., Holt, N. B., and Frush, H. L., *J. Research Natl. Bur. Stds.*, 40, 129 (1948).
47. Jacobs, M. B., and Jaffe, L., *Ind. Eng. Chem., Anal. Ed.*, 3, 210 (1931).
48. Jones, J. K. N., and Smith, F., in *Advances in Carbohydrate Chemistry*, Vol. IV. Academic Press, New York, 1949, p. 243.
49. Kabat, E. A., Baer, H., Bezer, A. E., and Knaub, V., *J. Exptl. Med.*, 88, 43 (1948).
50. Kahlenberg, L., and Barwasser, N., *J. Biol. Chem.*, 79, 405 (1928).
51. Karrer, P., and Viscontini, M., *Helv. Chim. Acta*, 30, 52 (1947).
52. Kerby, G. P., Gowdy, R. A., Dillon, E. S., Dillon, M. L., Csaky, T. Z., Sharp, D. G., and Beard, J. W., *J. Immunol.*, 63, 93 (1949).
52a. Kibrick, A. C., and Harris, D., *J. Biol. Chem.*, 185, 265 (1950).
53. Klimek, R., and Parnas, J. K., *Biochem. Z.*, 252, 392 (1932).
54. Knight, S. G., and Frazier, W. C., *J. Bact.*, 50, 505 (1945).
55. Koffler, H., Knight, S. G., Emerson, R. L., and Burris, R. H., *ibid.*, 50, 549 (1945).
56. Koffler, H., Knight, S. G., Frazier, W. C., and Burris, R. H., *ibid.*, 51, 385 (1946).
57. Koones, H. F., U. S. Pat. 2,450,555 (Oct. 5, 1948).
58. Kornberg, A., and Pricer, W. E., Jr., *J. Biol. Chem.*, 182, 763 (1950).
58a. Krantz, J. C., Jr., Carr, C. J., and Beck, F. F., *J. Phys. Chem.*, 40, 927 (1936).
59. Krejci, L. E., Sweeny, L., and Zittle, C. A., *Arch. Biochem.*, 22, 253 (1949).
60. Krejci, L. E., Sweeny, L., and Hambleton, J., *ibid.*, 22, 262 (1949).
61. Kuhn, R., and Rudy, H., *Ber.*, 68, 169 (1935).
62. Kuhn, R., Löw, I., and Moewus, F., *Naturwissenschaften*, 30, 407 (1942).
63. Kuhn, R., *Wien. Chem.-Ztg.*, 46, No. 1–2, 1 (1943).
64. Kuhn, R., and Quadbeck, G., *Naturwissenschaften*, 35, 318 (1948).
65. Landy, M., and Batson, H. C., *J. Immunol.*, 62, 477 (1949).
66. Levy, M., and Doisy, E. A., *J. Biol. Chem.*, 77, 733 (1928).
67. Levy, M., *ibid.*, 84, 763 (1929).
68. Macpherson, H. T., and Percival, E. G. V., *J. Chem. Soc.*, 1937, 1920.
69. Macpherson, H. T., and Stewart, J., *Biochem. J.*, 32, 76 (1938).
70. MacVicar, R., and Burris, R. H., *Arch. Biochem.*, 17, 31 (1948).
71. Mantell, C. L., *The Water-Soluble Gums*. Reinhold, New York, 1947.
71a. Marvel, C. S., and Denoon, C. E., Jr., *J. Am. Chem. Soc.*, 60, 1045 (1938).

72. McClure, F. J., *Ann. Rev. Biochem.*, **18**, 341 (1949).
73. McIlroy, R. J., *The Chemistry of the Polysaccharides.* Arnold, London, 1948.
74. Mellon, M. G., and Norris, V. N., *Ind. Eng. Chem.*, **16**, 123 (1924).
75. Michaelis, L., *J. Biol. Chem.*, **87**, 33 (1930).
76. Michaelis, L., and Eagle, H., *ibid.*, **87**, 713 (1930).
77. Militzer, W. E., *J. Biol. Chem.*, **158**, 247 (1945).
78. Moe, O. A., Miller, S. E., and Iwen, M. H., *J. Am. Chem. Soc.*, **69**, 2621 (1947).
79. Mohamed, M. S., and Greenberg, D. M., *Arch. Biochem.*, **8**, 349 (1945).
80. Morell, S. A., and Lathrop, E. C., *J. Am. Chem. Soc.*, **67**, 879 (1945).
81. Morgan, W. T. J., and King, H. K., *Biochem. J.*, **37**, 640 (1943).
82. Moyer, A. J., Umberger, E. J., and Stubbs, J. J., *Ind. Eng. Chem.*, **32**, 1379 (1940).
82a. Novak, M., *Bull. Natl. Formulary Comm.*, **18**, 94 (1950).
83. O'Donoghue, J. R., *Sci. Proc. Roy. Dublin Soc.*, **25**, No. 2, 9 (1949); *Chem. Abstracts*, **43**, 9500 (1949).
84. Oshima, K., *Japan. J. Med. Sci., II, Biochem.*, **5**, 149 (1944); *Chem. Abstracts*, **44**, 3557 (1950).
85. Pasternak, R., *Helv. Chim. Acta*, **30**, 1984 (1947).
86. Pfeiffer, C. C., Hallman, L. F., and Gersh, I., *J. Am. Med. Assoc.*, **128**, 266 (1945).
86a. Pfeiffer, C. C. and Jenney, E. H., *Bull. Natl. Formulary Comm.*, **18**, 57 (1950).
87. Pigman, W. W., and Goepp, R. M., Jr., *Chemistry of the Carbohydrates.* Academic Press, New York, 1948.
88. Radley, J. A., *Starch and Its Derivatives.* 2nd Ed., Chapman & Hall, London, 1943.
88a. Reeves, R. E., *J. Am. Chem. Soc.*, **71**, 212, 215, 1737, 2116 (1949).
89. Renfrew, A. G., *J. Biol. Chem.*, **89**, 619 (1930).
90. Rippere, R. E., and LaMer, V. K., *J. Phys. Chem.*, **47**, 204 (1943).
90a. Rosenberg, A. J., *Compt. rend.*, **222**, 1310 (1946).
91. Ross, S. D., and Catotti, A. J., *J. Am. Chem. Soc.*, **71**, 3563 (1949).
91a. Roush, A., and Norris, E. R., *Arch. Biochem.*, **29**, 344 (1950).
92. Savur, G. R., and Sreenivasan, A., *J. Biol. Chem.*, **172**, 501 (1948).
92a. Scattergood, A., Miller, W. H., and Grammon, J., Jr., *J. Am. Chem. Soc.*, **67**, 2150 (1945).
93. Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.*, **149**, 369 (1943).
94. Scudi, J. V., Bastedo, W. A., and Webb, T. J., *ibid.*, **136**, 399 (1940).
95. Seeler, A. O., and Malanga, C., *Proc. Exptl. Biol. Med.*, **63**, 194 (1946).
96. Soda, T., *J. Faculty Sci. Imp. Univ. Tokyo, Sect. I*, **3** (Part 3), 149 (1936).
97. Sumner, J. B., and Somers, G. F., *Chemistry and Methods of Enzymes.* Academic Press, New York, 1947, p. 158.
98. Sykes, J. A., and Reed, R., *J. Gen. Microbiol.*, **3**, 117 (1949).
98a. Taubóck, K., *Naturwissenschaften*, **30**, 439 (1942).
99. Tiomno, F. R., *Rev. quim. ind. (Rio de Janeiro)*, **15**, No. 168, 23; No. 172, 19 (1946); *Chem. Abstracts*, **41**, 2921 (1947).
100. Tseng, C. K., in Alexander, J., *Colloid Chemistry*, Vol. VI. Reinhold, New York, 1946, p. 715.
101. Tyner, L. E., *Soil Sci.*, **57**, 271 (1944).
101a. Vargha, L. v., *Ber.*, **66**, 704 (1933).
102. Watson, D. W., Thesis, Univ. Wisconsin, 1941.
103. Whitten, W. K., *Nature*, **163**, 534 (1949).
104. Wiley, H. W., U. S. Dept. Agr., Bur. Chem., *Circ. 15*, **1904**, 27.
105. Williams, A. L., *Analyst*, **53**, 411 (1928).
105a. Wilson, C. W., *J. Am. Chem. Soc.*, **61**, 2303 (1939).
105b. Wilson, C. W., Weatherby, L. S., and Bock, W. Z., *Ind. Eng. Chem., Anal. Ed.*, **14**, 425 (1942).
106. Winfield, M. E., *Australian J. Exptl. Biol. Med. Sci.*, **23**, 111 (1945).
107. Winfield, M. E., *ibid.*, **23**, 267 (1945).
108. Wise, L. E., *Arch. Biochem.*, **23**, 127 (1949).
109. Zittle, C. A., and Harris, T. N., *J. Biol. Chem.*, **142**, 823 (1942).
110. Zittle, C. A., *ibid.*, **167**, 297 (1947).
111. Zittle, C. A., *Federation Proc.*, **6**, 500 (1947).
112. Zittle, C. A., *Arch. Biochem.*, **17**, 195 (1948).
113. Zittle, C. A., Smith, L. DeS., and Krejci, L. E., *ibid.*, **19**, 9 (1948).
114. Zittle, C. A., unpublished studies, 1948.
115. Zittle, C. A., and DellaMonica, E. S., *Arch. Biochem.*, **26**, 112 (1950).