These equations were put to the test for the case of serum cholinesterase (human) and its substrate acetylcholine. This enzyme is known to be competitively inhibited by choline, one of the products of the reaction, and to be completely stable during the reaction. It was also established that this reaction is not reversible to any measurable degree. Two different concentrations of acetylcholine chloride, 6×10^{-3} and 12×10^{-3} molar, were chosen. The buffer was bicarbonate–carbon dioxide at *p*H 7.8 (R_{30}),[9] the serum dilution 1:40, and the temperature 38°. The course of the hydrolysis was followed by the manometric technique. At the higher concentration of acetylcholine measurements were taken at fourteen time intervals before the reaction was about 50% complete. Because of the necessity to maintain the *p*H as constant as possible, higher values of p were not included in the calculation. The lower concentration, however, was followed to completion. Corrections were applied for the non-enzymatic hydrolysis for which first-order kinetics had been established ($k_n = 0.25 \times 10^{-3}$ per minute). Because its contribution to the over-all reactions is small, a correction factor of $m'/(m' - k_n)$ for the values of r' appears to be a reasonable approximation. (If the non-enzymatic reaction becomes more prominent, the Michaelis–Menten kinetics may not be expected to hold.) The values for r' and m' are listed in Table II; while these were determined by calculation, the graphic method gave almost identical results. Table II shows also the dissociation constants and V obtained from the parameters with equations 12 and 13 and by the conventional Lineweaver–Burk plot on the same serum. It may be seen that the agreement of the results obtained by the different methods is satisfactory.

(9) K. B. Augustinsson, *Acta physiol. Scand.*, suppl. 52, **15**, 1 (1948).

TABLE II

DETERMINATION OF K_S, K_P AND V FOR CHOLINESTERASE

a. Parameters of integrated Michaelis–Menten equation (corrected for blanks)

$S_0 \times 10^3$, moles/liter	6.0	12.0
r'	2.42	3.56
$m' \times 10^3$, per minute	11.6	6.4
$s \times 10^{3a}$	±3.3	±2.3

b. Enzyme constants

$K_S \times 10^3$, moles/liter	1.5	Eq. 12
	1.4	Eq. 13
	1.4	Lineweaver–Burk
$K_P \times 10^3$	10.7	Eq. 12
	9.1	Lineweaver–Burk
$V \times 10^3$, moles/liter serum/minute	3.4	Eq. 13
	3.3	Lineweaver–Burk

[a] Standard deviation of the function $p + f/(r' + 1)$.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, IOWA STATE COLLEGE]

Primer Specificity of Potato Phosphorylase[1,2]

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Variation in the ability of various saccharides to "prime" potato phosphorylase is shown by spraying the resolved compounds on paper chromatograms with a mixture of glucose-1-phosphate and phosphorylase, incubating in a moist atmosphere, and locating the regions in which starch synthesis has occurred by spraying with iodine solution. Primer areas appear as spots ranging from nearly colorless or yellow to deep blue, depending on the amount and nature of the saccharide. Inhibiting saccharides appear as characteristic white areas. In order to function as a primer for potato phosphorylase, an amylose chain must contain three glucose units (poor primers) or four or more glucose units (good primers). Substitution at the reducing end-group of amylose chain fragments does not qualitatively change the priming ability.

It is generally recognized that synthesis of polysaccharide from glucose-1-phosphate (G-1-P) by phosphorylase action requires the presence of co-substrate ("primer," "activator" or "catalyst") which may be starch, glycogen or oligosaccharides produced by acid or enzymatic hydrolysis of amylaceous materials.[3-8] However, glucose, maltose and other end-products of diastatic action on starch are non-priming and the cyclic Schardinger dextrins are strong inhibitors of potato phosphorylase.

Swanson and Cori[8] estimated the minimum primer size as 4–5 glucose units, while Weibull and Tiselius[6] claimed that amylotriose (maltotriose) is as effective a primer as higher saccharides. Bailey, Whelan and Peat[9] state that amylotriose is a poor primer and that amylotetraose and higher oligosaccharides are effective primers of essentially equivalent priming ability.

In the initial experiments we explored the problem of the minimum primer requirements in potato phosphorylase synthesis by examining the reverse reaction. Amyloheptaose[10] was subjected to phosphorylase action in the presence of phosphate or

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(2) Presented before the Division of Biological Chemistry, American Chemical Society, Sept., 1950. Abstracts, 51 C. Taken in part from the M.S. thesis of Gene M. Wild, Iowa State College, 1949.

(3) G. T. Cori and C. F. Cori, *J. Biol. Chem.*, **131**, 397 (1939); **135**, 733 (1940).

(4) C. S. Hanes, *Nature*, **145**, 348 (1940); *Proc. Roy. Soc. (London)*, **B129**, 174 (1940).

(5) D. E. Green and P. K. Stumpf, *J. Biol. Chem.*, **142**, 355 (1942).

(6) C. Weibull and A. Tiselius, *Arkiv Kemi, Mineral. Geol.*, **A19**, No. 19 (1945).

(7) P. H. Hidy and H. G. Day, *J. Biol. Chem.*, **152**, 477 (1944); **160**, 273 (1945); E. C. Proehl and H. G. Day, *ibid.*, **163**, 667 (1946).

(8) G. T. Cori, M. A. Swanson and C. F. Cori, *Federation Proc.*, **4**, 234 (1945); M. A. Swanson and C. F. Cori, *J. Biol. Chem.*, **172**, 815 (1948).

(9) J. M. Bailey, W. J. Whelan and S. Peat, *J. Chem. Soc.*, 3692 (1950).

(10) D. French, M. L. Levine and J. H. Pazur, THIS JOURNAL, **71** 356 (1949).

arsenate[11] and the resulting degraded oligosaccharides were examined by electrophoretic analysis.[12] Phosphorolysis gave a mixture, mostly amylotetraose and small amounts of higher oligosaccharides, whereas arsenolysis (very extensive) gave a mixture of amylotriose and amylotetraose. The limit oligosaccharides produced by phosphorolysis or arsenolysis should be the minimum primers for the synthetic reaction. In fact these preparations were found to be effective primers as judged by the rate of inorganic phosphate liberation and the production of iodine-staining polysaccharide. Amylotriose[13] itself was found to be a poor primer in that the rate of inorganic phosphate liberation was only slightly higher than in the controls without added primer.

In subsequent work we have used a method which is free from some of the ambiguities which were present in the early studies. In this method the oligosaccharides to be tested (usually a mixture of homologs) are placed on paper chromatograms together with suitable reference materials. After chromatographic separation of the oligosaccharides in the customary manner[14,15] and cutting off that part of the chromatogram containing the reference compounds, the remainder is sprayed uniformly with a mixture of G-1-P and phosphorylase. The damp chromatogram is then incubated in a damp atmosphere at room temperature so that starch synthesis may occur where suitable primers are present. The chromatogram is then dried and either sprayed with a dilute iodine solution or allowed to stand in an iodine vapor atmosphere to show the extent of phosphorylase action. There is a general light blue background (caused by traces of priming impurity in the phosphorylase and filter paper used). Priming oligosaccharides range in appearance from blue spots (for poor primers or very low concentrations of good primers) to multicolored spots with white, yellow or brown centers surrounded by purple and finally blue areas (good primers and very high concentrations of poor primers). Inhibitors show up as white patches in the otherwise blue background (see Figs. 1 and 2).

By this method, it was found that amylotetraose and all of the higher amylose homologs[16] are good primers of comparable effectiveness. Amylotriose showed greatly reduced priming activity and maltose had no discernible priming action whatsoever; in fact, at high concentrations it appeared to exert a weak inhibiting effect.

Oligosaccharides obtained by the *macerans* amylase coupling reaction[17] behaved uniformly analogous to the normal amylose series; *i.e.*, amylotriosyl derivatives were poor primers while amylotetraosyl derivatives and higher coupled products were good primers. Termination by polygalitol or D-glucoheptulose appeared to be equivalent to termination by D-glucose; *e.g.*, 4-α-amylotriopyranosylpolygalitol and higher products were good primers. Interestingly, while phlorizin is somewhat inhibitory to potato phosphorylase, phlorizin-coupled products containing 4 or more glucose units were good primers.

The phosphorylase–glucose-1-phosphate spray is the most sensitive method we have yet found for locating traces of priming carbohydrates on paper chromatograms; by this method 0.05 microgram of amyltetraose could be discerned (about one-tenth the amount necessary for detection by the ammoniacal silver nitrate spray).

We frequently use the phosphorylase spray in following the progress of enzyme action on starch. During salivary action on amylopectin, we have regularly observed the formation of branched compounds in the range of 6–10 glucose units which are primers. After action is more extensive, however, certain of the branched compounds stand out as strong inhibitors (notably singly branched heptasaccharide and doubly branched compounds in the range 8–13 glucose units). These inhibitors apparently give stable enzyme complexes, but the presence of a branching unit prevents them from functioning as co-substrates (acceptors) in starch chain synthesis.

As previously noted, the Schardinger dextrins are also inhibitors. These cyclic compounds have the fundamental amylose chain structure which is necessary for combination with the enzyme,[18] but they lack a non-reducing end-group and therefore cannot act as co-substrates.

These observations suggest a concept of the action of phosphorylase idealized in Fig. 3. A series of binding positions are present which hold the substrates in appropriate steric relationship so that other functional groups present at the enzyme surface are able to complete the reaction. If these binding positions are occupied by substances which do not have the chemical structure necessary for reaction, or if irregularities such as branches are present (thus preventing a good "fix" with the enzyme), inhibition results.

It should be possible to test certain implications of such a concept. For instance, one would expect the binding energy between enzyme and co-substrates of varying chain length to vary depending on how many of the binding sites are used. A given substrate, for example amylopentaose, might very well lead to differing binding energies depending on whether it is being used in the forward or reverse reactions (synthesis or degradation). Further, if there are multiple binding sites as suggested, it is likely that during a single encounter between

(11) J. Katz and W. Z. Hassid, *Arch. Biochem.*, **30**, 272 (1951). Owing to the equilibria in phosphorylase reactions with phosphate, it is impossible to get complete conversion to a limit saccharide unless: (a) the reaction is repeated several times, isolating the degraded saccharide each time, or (b) the G-1-P is removed from the reaction mixture, say, by dialysis against a large volume of phosphate buffer (impractical in this case) or by enzymatic decomposition. With arsenolysis, G-1-As formed decomposes spontaneously, thereby leading to eventual completion of the reaction. The arsenolysis reaction is very slow, however, in comparison with phosphorolysis.

(12) E. J. Norberg, Ph.D. thesis, Iowa State College, 1949; E. Norberg and D. French, THIS JOURNAL, **72**, 1202 (1950).

(13) D. French, M. L. Levine, J. H. Pazur and E. Norberg, *ibid.*, **72**, 1746 (1950).

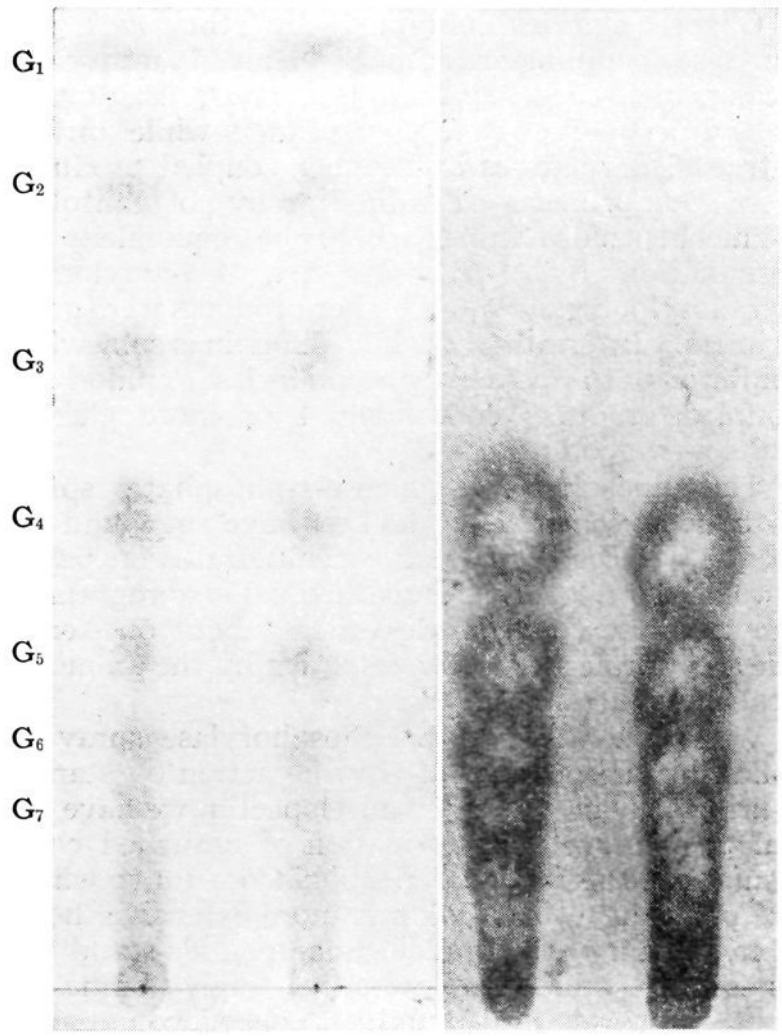
(14) A. Jeannes, C. S. Wise and R. J. Dimler, *Anal. Chem.*, **23**, 415 (1951).

(15) D. French, D. W. Knapp and J. H. Pazur, THIS JOURNAL, **72**, 5150 (1950).

(16) D. French and G. M. Wild, *ibid.*, **75**, 2612 (1953).

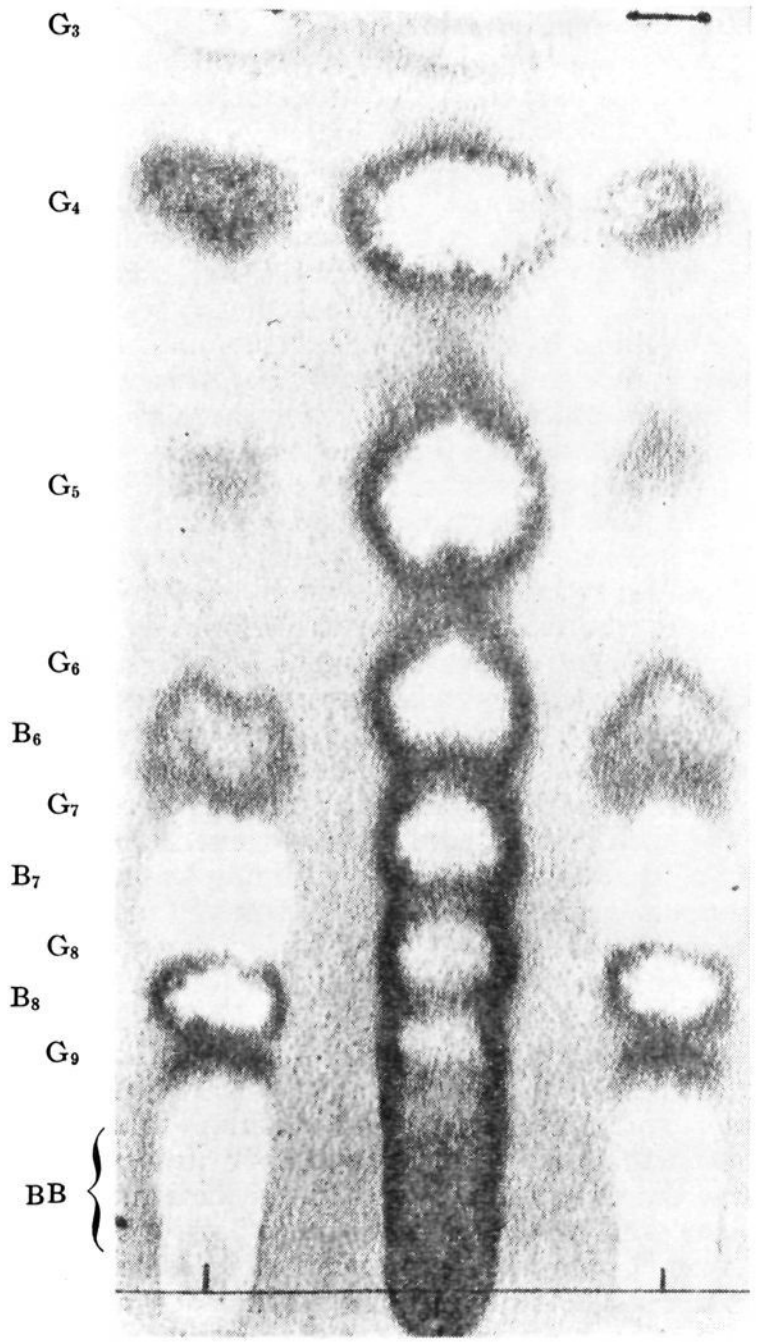
(17) D. French, J. Pazur, M. L. Levine and E. Norberg, *ibid.*, **70**, 3145 (1948).

(18) Green and Stumpf[8] conclude: "It would appear that the Schardinger dextrins and starch [the primer used] were competing for the same active group in the enzyme."



a. b.

Fig. 1.—Priming of potato phosphorylase by resolved amyloöligosaccharides: G_1, G_2, G_3, etc., represent glucose, maltose, amylotriose, etc.; a, alkaline copper, phosphomolybdic acid spray for reducing saccharides; b, phosphorylase–G-1-P, iodine spray for priming saccharides; 5 ascents.



a. b. a.

Fig. 2.—Primers and inhibitors in salivary amylase dextrins from waxy maize starch: G_1, G_2, G_3, etc., represent glucose, maltose, amylotriose, etc.; B_4, B_5, B_6, etc., represent singly branched tetrasaccharide, pentasaccharide, hexasaccharide, etc., containing one 1,6-bond per molecule; BB represents doubly branched oligosaccharides; a, dextrins from waxy maize starch; b, reference series of linear oligosaccharides (same as Fig. 1); 15 ascents.

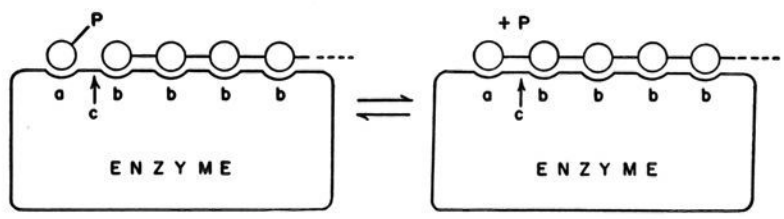


Fig. 3.—Phosphorylase–substrate relation: O—P indicates G-1-P; O—O—O—O— represents an amylase chain fragment of four or more glucose residues; a and b are binding sites for glucose units in the substrates; enzyme prosthetic groups at c effect the reversible reaction between G-1-P and the co-substrate.

enzyme and substrate, multiple attack[19–21] will occur (for either synthesis or degradation). These possibilities are now being investigated.

Experimental

The potato phosphorylase was prepared by the methods of Green and Stumpf[5] and of Barker, Bourne and Peat[23] and stored under toluene in the refrigerator. G-1-P was prepared by the method of McCready and Hassid.[24] Oligosaccharide series were prepared and resolved on paper chromatograms as given previously.[14–16] One ml. of the enzyme solution and 1 ml. of 0.1 *M* G-1-P (di-K salt) were mixed and sprayed evenly on an 8″ × 10″ resolved chromatogram. The damp paper was hung in a water-saturated atmosphere at room temperature for 10–30 min. (depending on the enzyme activity). After the paper was dried it was sprayed lightly with a dilute iodine–potassium iodide solution to stain those areas in which starch synthesis had occurred. Chromatograms treated in this way gradually fade over several weeks, but the color can be restored by spraying again with iodine solution.

(19) D. French, D. W. Knapp and J. H. Pazur, THIS JOURNAL, **72**, 1866 (1950).

(20) J. M. Bailey and W. J. Whelan, *Biochem. J.*, **51**, xxxiii (1952), give evidence that a "multi-chain" mechanisn holds for both synthesis and degradation by phosphorylase; however, their results do not exclude the possibility that multiple attack per encounter may occur.

(21) The concepts of multiple binding sites together with multiple attack per encounter (single attacking center) are alternative to the concept[22] of multiple attacking centers per enzyme molecule.

(22) J. M. Bailey, personal communication.

(23) S. A. Barker, E. J. Bourne and S. Peat, *J. Chem. Soc.*, 1705 (1949).

(24) R. M. McCready and W. Z. Hassid, THIS JOURNAL, **66**, 560 1944).

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