Competing Substrates in Enzyme Research

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OF RECENT YEARS, chromogenic substrates have found extensive use in standard methods for the assay of group-specific hydrolytic enzymes. Typical examples are phenolphthalein β-D-glucuronide, *p*-nitrophenol β-D-glucoside, and *p*-nitrophenol phosphate, used in the assay of β-glucuronidase (*1*), β-glucosidase (*2*), and phosphatase (*3*), respectively. In each of these cases, the "chromogen" liberated by enzymic hydrolysis has a readily measured characteristic color in alkaline solution, which is not given by the unchanged substrate. In other examples, the chromogen is not itself colored, but can form a colored derivative under conditions under which the parent substrate does not interfere. Compared with methods which they have superseded, such as those relying on the estimation of reducing sugar or inorganic phosphate, assay procedures employing chromogenic substrates usually have the advantages of greater specificity, accuracy, and sensitivity, and they tend to be more convenient for routine work.

As noted by Lineweaver and Burk (*4*), the case of two or more competing substrates is a special case of competitive inhibition of an enzyme. On the basis of this, the use of chromogenic substrates, with its attendant advantages, can profitably be extended, as the addition of a second, nonchromogenic, substrate during the enzyme assay will depress the liberation of the chromogen, to an extent dependent upon the relative concentrations of the two substrates and their relative affinities for the enzyme. This, the competing substrate technic, has been adopted by many workers in diverse connections, but its full possibilities when used in conjunction with chromogenic substrates do not appear to have been widely appreciated, nor, so far as we know, expounded elsewhere.

Enzyme specificity. As a screening test for possible new substrates for an enzyme, the competing substrate technic has the important advantage that the ultimate standard is the chromogenic substrate, rather than a purified preparation of the enzyme which may yet be contaminated with other enzymes. The possibility that a new substrate of low affinity for the enzyme may have been overlooked can be guarded against by repeating the test at a lower concentration of the chromogenic substrate. In such borderline cases, the conventional test for a new substrate by direct measurement of hydrolysis is often very difficult to interpret.

This technic has been employed in testing the specificity of mouse liver β-glucuronidase (*5*). Various "nonchromogenic" hexuronides were added to the enzyme in the presence of phenolphthalein β-D-glucuronide and the effect on the liberation of phenolphthalein was measured. The enzyme appeared to be specific, not just for β-glucuronides, but for β-glucopyranuronides, since methyl β-D-glucofuranuronide did not depress the hydrolysis of phenolphthalein glucuronide. This latter finding has been confirmed in direct measurements of the hydrolysis of the chromogenic substrates 2-naphthyl β-D-glucopyranuronide and β-D-glucofuranuronide (*6*); β-naphthol was liberated only from the former by β-glucuronidase.

Specificity tests by the competing substrate technic are unequivocal only when negative results are obtained. The test does not discriminate between competing substrates and true inhibitors. A study of the kinetics of inhibition (see below) quickly excludes noncompetitive inhibitors, because in this case fractional inhibition is independent of the chromogenic substrate concentration. To distinguish between a competitive inhibitor and a competing substrate, it is necessary to carry out a qualitative test for hydrolysis of the new compound by the enzyme in the absence of the chromogenic substrate. Some idea of the conditions required will have been provided by the competing substrate tests. This question arose in connection with β-glucuronidase and α- and β-glucuronic acid-1-phosphate (*5*).

Kinetics. In the presence of a fixed concentration I of a competitive inhibitor, the effect of varying the concentration S of a chromogenic substrate on its velocity of hydrolysis v can be expressed by the equation (*4*)

$$v = \frac{V \cdot S}{S + K_m + K_m(I/K_i)}$$

where V is the limiting velocity and K_m and K_i are the dissociation constants for the complexes formed by the enzyme with the substrate and inhibitor respectively; K_m is determined in absence of the inhibitor according to the equation

$$v = \frac{V \cdot S}{S + K_m}$$

and K_i is found by substitution. Provided that the inhibitory compound is shown by qualitative test to be in fact a substrate for the enzyme, K_i is equal to K_m for the new substrate. Its affinity for the enzyme is $1/K_m$. It is important that I should not have altered significantly through hydrolysis during the measurements. Lederberg (*7*) has adopted this method in studying different substrates for a bacterial β-galactosidase, using *o*-nitrophenol β-D-galactoside as the chromogenic substrate.

Unity of an enzyme. If two rather different substrates are hydrolyzed by one and the same enzyme preparation, it is not easy to decide by conventional

methods whether or not a single enzyme is responsible. The competing substrate technic can sometimes give a quick decision on this point. If there are two enzymes, the rate of hydrolysis of one substrate will be unaffected by the presence of the other, and vice versa. Ezaki (*8*) found that the liberation by emulsin of *p*-nitrophenol from its β-D-glucoside or β-D-galactoside was in each case depressed by both phenol β-D-glucoside and phenol β-D-galactoside, from which he concluded that in his emulsin preparation one and the same enzyme was responsible for the hydrolysis of β-glucosides and β-galactosides.

Quite apart from its use in conjunction with chromogenic substrates, competition between substrates has long been used as a method for establishing the unity of an enzyme (*9*). With nonchromogenic substrates, the sum of the effects of the enzyme on the two different substrates is measured and compared with the effect on each alone.

Characterization of a new compound or group. The fact that a new compound is decomposed by a known enzyme can provide valuable evidence for the chemical structure, to an extent depending upon knowledge of the specificity of the enzyme. It should not, however, be overlooked that decomposition of the new compound could be due to a contaminating enzyme in the preparation. Thus the use of a culture of *Escherichia coli* for the hydrolysis of urinary steroid conjugates (*10*) offers no proof that the enzyme responsible is β-glucuronidase. A selective enzyme inhibitor, if available, can be profitably employed in this connection (*11*). Of equal if not greater value is the use of a standard substrate for the enzyme, in competition with the compound of unknown structure.

The search for new substrates. On physiological or other grounds, there may be reason to suspect the existence in biological material of an unknown substrate for a known enzyme. Testing extracts by competition with a chromogenic substrate would seem to be a method of great promise for detecting such a new substrate and for controlling its subsequent purification and isolation. The important reservation must be made that a true inhibitor, competitive or noncompetitive, will interfere (see above). We are employing this method in seeking β-glucuronides in animal feedingstuffs (*12*), using as the test system β-glucuronidase and phenolphthalein β-D-glucuronide. It seems possible that this same test system might be applied with advantage to the fractionation of urinary steroid glucuronides.

Substrates present as impurities. While, as described above, competition between substrates can profitably be employed in enzyme research, it can also arise by accident, in which case it may be a source of difficulty.

If an enzyme preparation is contaminated with an endogenous substrate of high affinity for the enzyme, it may cause little or no decomposition of another compound which is in fact a substrate. This position may have arisen in Miwa's study of baicalinase (*13*), an enzyme from the roots of *Scutellaria baicalensis,* which according to Miwa decomposes biacalin, the glucuronide of 5,6,7-trihydroxyflavone, but not menthol β-D-glucuronide. Approximately 10 percent of the dry weight of the same root is baicalin. Preliminary work of our own suggests that baicalinase is in fact a β-glucuronidase, with rather a low affinity for menthol β-D-glucuronide. The possibility of serious contamination of an enzyme preparation by endogenous substrate would, in general, appear to be excluded if the plot of enzyme dilution against the velocity of hydrolysis of a standard substrate gives a straight line.

A similar position arises if the contaminating substrate accompanies not the enzyme, but the compound which it is proposed to hydrolyze. Thus the efficiency of β-glucuronidase added to urine as a reagent for the hydrolysis of a selected steroid glucuronide may be expected to be low due to the presence of other urinary glucuronides, steroid or not (*14*). In fact, whenever evidence is obtained of an unknown compound acting as an enzyme inhibitor, the suspicion must be entertained that it is a competing substrate.

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