# The Theoretical Basis for the Measurement of Compounds by Enzymatic Radioisotopic Displacement*

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ABSTRACT: A new approach to the measurement of compounds of biological interest has been developed. It relies upon the displacement of labeled substrate from an enzymatic reaction by the corresponding unlabeled compound. A theoretical basis for this principle is derived by incorporating the Michaelis–Menten relationship between velocity and substrate concentration into the classical isotope dilution equation. An equation is developed which defines the relative diminution of radioactive product ($P^*/P_0^*$) in terms of the concentrations of labeled and unlabeled substrate ($S^*$ and $S$, respectively) and the Michaelis constant of the enzyme involved ($K_m$), $P^*/P_0^* = (K_m + S^*)/(K_m + S + S^*)$. The validity of this relationship is experimentally confirmed and the practical limitations of the method are defined.

The measurement of minute quantities of biologically important compounds has always been a difficult problem in biochemistry. Direct chemical or physical methods are frequently not as specific or sensitive as would be desired and coupling with various enzymatic systems can complicate assays excessively.

A unique approach is that of radioimmunoassay as used for insulin and other peptide hormones (Yalow and Berson, 1959). This method, which integrates the binding specificity of the immunoproteins with the sensitivity inherent in isotopic methods, can unfortunately not be easily adapted to low molecular weight compounds.

This paper describes the theoretical basis and limitations for a parallel assay method involving the displacement of isotopically labeled substrates from an enzymatic reaction by the compound of interest. A practical description of the use of this principle for the measurement of cyclic nucleotides appears in the preceding paper (Brooker *et al.*, 1968b).

## Theory

If a system is developed in which a uniform quantity of an isotopically labeled compound can be specifically separated from a solution, the addition of the nonlabeled form of the compound will cause a proportionate decrease in the isotope content of the separated fraction. This principle, which is derived directly from classical isotope dilution methods, can be expressed in the following form

$$P^* \propto \frac{S^*}{S + S^*} \tag{1}$$

where $P^*$ is the radioactivity in the separated portion, $S^*$ is the amount of the isotopically labeled compound initially present, and $S$ is the amount of nonlabeled compound added.

In the case of immunoassays, the uniform quantity of the compound of interest is separated from the remainder by reaction with a highly specific antiserum followed by precipitation of the antigen–antibody complex. The enzymatic isotope displacement assay depends upon the formation and reaction of the enzyme–substrate complex for its specificity. This method can be defined in the same terms as those used for the immunoassay if, and only if, the addition of nonlabeled substrate does not change the reaction velocity. This ideal situation will not hold true as often as would be desirable since biologically important compounds frequently are present in tissues at concentrations in the vicinity of the Michaelis constants of the enzymes which specifically act upon them. Inclusion of a velocity factor, $v$, into the above expression yields the fundamental relationship

$$P^* \propto \frac{S^*}{S + S^*} v \tag{2}$$

Unit analysis of eq 2 indicates the necessity for a time and volume factor, $k$, which would be constant for any particular assay system and a specific radioactivity term $R/S^*$, yielding

$$P^* = k\left(\frac{S^*}{S + S^*}\right)\left(\frac{R}{S^*}\right)v \tag{3}$$

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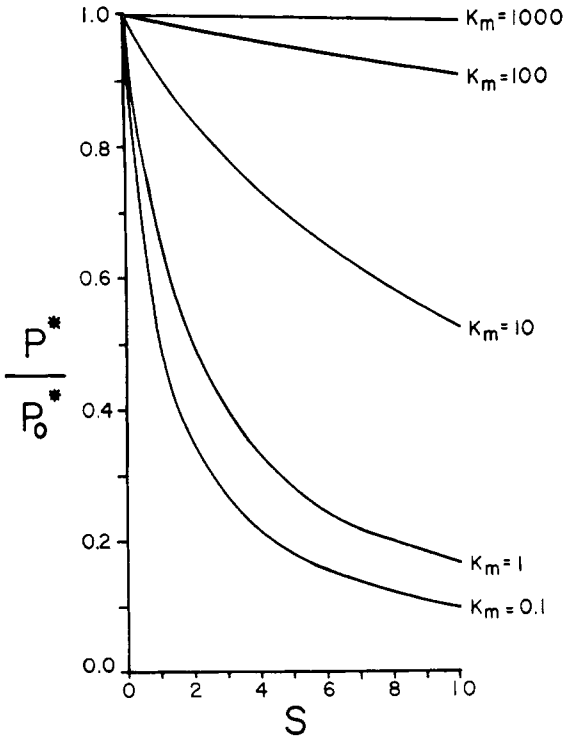


FIGURE 1: Plot of $P^*/P_0^*$ *vs.* $S$, values calculated from eq 7, $P^*/P_0^* = (K_m + S^*)/(K_m + S + S^*)$. The value of $S^*$ is held constant at 1 while $K_m$ varies as indicated.

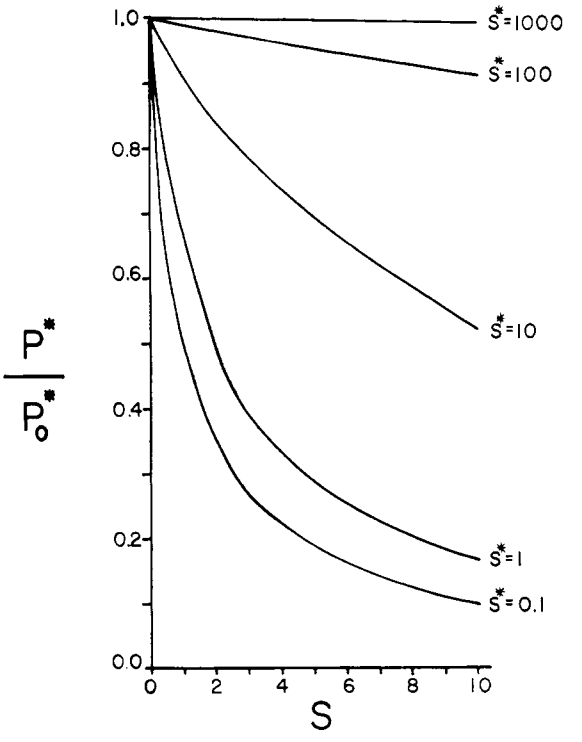


FIGURE 2: Plot of $P^*/P_0^*$ *vs.* $S$, values calculated from eq 7, $P^*/P_0^* = (K_m + S^*)/(K_m + S + S^*)$. The value of $K_m$ is held constant at 1 while $S^*$ varies as indicated.

Equation 3 is a general expression which is applicable in those situations where the velocity *vs.* total substrate concentration can be empirically determined. In the case where a well-characterized enzyme exists and it can be shown that Michaelis–Menten kinetics are appropriate, the velocity term of eq 3 can be substituted by the Michaelis equation, $v = V_m S_{total}/(K_m + S_{total})$ to

$$P^* = k\left(\frac{S^*}{S + S^*}\right)\left(\frac{R}{S^*}\right)\left(\frac{V_m(S + S^*)}{K_m + (S + S^*)}\right) \quad (4)$$

where $V_m$ is the maximum velocity of the enzyme, $K_m$ is the Michaelis constant, and $S + S^*$ is substituted for $S_{total}$. Upon simplification of terms the equation becomes

$$P^* = k\left(\frac{RV_m}{K_m + S + S^*}\right) \quad (5)$$

Equation 5 defines the radioactivity in the product, a measurable quantity, in terms of the characteristics of the enzyme, the conditions of the reaction, and the amount of substrate. If the isotopic content of the product, $P^*$, in the above equation is related to the isotopic content, $P_0^*$, in the case where there is no addition of nonlabeled substrate

$$P_0^* = k\left(\frac{RV_m}{K_m + S^*}\right) \quad (6)$$

we arrive at the ratio

$$\frac{P^*}{P_0^*} = \frac{K_m + S^*}{K_m + S + S^*} \quad (7)$$

## Results and Discussion

The value of enzymatic isotope displacement for the assay of a particular compound of biochemical interest will depend upon the characteristics of the enzymatic reaction and the relationships between the variables in eq 7. It is obviously important to have a readily available enzyme with high specificity in a preparation free of contamination by substrates, competing compounds, or competing reactions. The product must be easily separable from the substrate and the detection system must be sensitive enough to accurately register a relatively small decrease from a control value.

An analysis of eq 7 indicates that the usefulness of the method for measuring a particular concentration of the compound of interest, $S$, will depend upon the value, relative to $S$, of the variables $K_m$ and $S^*$. In Figure 1 the ratio $P^*/P_0^*$ is plotted *vs.* $S$, using a fixed concentration of $S^*$, at Michaelis constants ranging over several orders of magnitude. Optimal slopes are obtained at $K_m$ values below the highest levels of substrate which might be encountered. The above situation is reversed in Figure 2. The $K_m$ is held constant and the effect of $S$ on $P^*/P_0^*$ at various $S^*$ concentrations is plotted. Again the best results are obtained when $S^*$ is equal to or less than $S$. It is fortunate that the Michaelis constants for most enzymes are of the same order of magnitude as the normally occurring concentrations of substrates since the $K_m$ of an enzyme is not easily influenced. Modification of assay volume makes it possible to adjust the concentration of $S$ to a limited extent. The minimum concentration of $S^*$ is a function

TABLE I: Observed and Calculated Values for $P^*/P_0^*$ from the Assay of 3′5′-Cyclic GMP by Enzymatic Radioisotope Displacement Using Rat Brain Phosphodiesterase Activity.[a]

| Amt of Unlabeled 3′5′-Cyclic GMP Added ($S$) ($\mu\mu$moles) | $P^*/P_0^*$ | |
|---|---|---|
| | Calcd | Obsd |
| 0 | 1.00 | 1.00 |
| 20 | 0.95 | 0.96 |
| 40 | 0.92 | 0.94 |
| 80 | 0.86 | 0.87 |
| 160 | 0.76 | 0.77 |
| 320 | 0.62 | 0.61 |
| 640 | 0.45 | 0.41 |

[a] Assay method as described previously (Brooker *et al.*, 1968b). Calculated values are derived from eq 7 using an apparent $K_m$ of $5 \times 10^{-6}$ M and a labeled nucleotide concentration, $S^*$, of 8 $\mu\mu$moles in 150 $\mu$l.

of the specific radioactivity available and the sensitivity of the detection system for $P^*$.

The validity of the theoretical presentation of enzymatic isotopic displacement in a case which appears to follow classical Michaelis–Menten kinetics, the measurement of 3′,5′-cyclic GMP using the phosphodiesterase activity in a rat brain preparation, is shown in Table I.

There are many situations where the Michaelis relationships do not apply to the kinetics of a particular reaction; the assay of 3′,5′-cyclic AMP by brain phosphodiesterase reported in the preceding paper is a case in point. A more general theoretical expression can be derived by going directly from the relationship of the radioactive product to labeled and unlabeled substrate concentration and velocity, eq 3. to a ratio presentation similar to that in eq 7 for the classical case

$$\frac{P^*}{P_0^*} = \left(\frac{S^*}{S^* + S}\right)\frac{v}{v_0} \qquad (8)$$

where $v$ is the measured velocity in the presence of $S$ and $S^*$, while $v_0$ is the velocity with no added unlabeled substrate.

Equations 7 and 8 define the theoretical basis for the enzymatic isotope displacement assay using classical Michaelis–Menten and nonclassical kinetic systems, respectively. It must be emphasized that the application of this technique to a particular problem is not dependent upon a theoretical derivation. Reference to a standard curve will be adequate in most cases.

## Addendum

A publication has appeared during the preparation of this manuscript independently deriving this same principle (Newsholme and Taylor, 1968). While the enzyme used, nomenclature, method of derivation, and the presentation of the data differ, an identical conclusion was obtained.

The use of an inverted form of the basic equation has the advantage of yielding a linear plot in the ideal case. It might, however, be subject to misinterpretation since the reciprocal relationship places emphasis on lower values.

The analysis of Newsholme and Taylor is applicable only to those systems where any deviation from Michaelis–Menten kinetics is small, while the empirical approach derived in this paper is more general and may have wider applicability since it can be used to describe systems deviating greatly from classical kinetics.

## References

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Yalow, R. S., and Berson, S. A. (1959), *Nature 184*, 1648.