"HOMOGENEOUS" ENZYME IMMUNOASSAY. A NEW IMMUNOCHEMICAL TECHNIQUE

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Received April 17, 1972

Summary. Addition of morphine antibodies to a conjugate of morphine and lysozyme resulted in inhibition of lysozyme activity. Addition of free morphine to a mixture of the conjugate and morphine antibodies reduced the inhibition of enzyme activity in proportion to the quantity of free morphine added. As little as 1×10^{-9} M morphine could be detected in this manner. The method constitutes a powerful new immunochemical technique for the quantitative determination of haptens.

Certain naturally occurring molecules serve to regulate the activity of enzymes. For example, the binding of antibodies to cellular antigens in the presence of the family of proteins known as complement sets off a complex sequence of events culminating in the activation of esterase activity. Other examples are the numerous reversible inhibitors of enzymes (1), and the control of enzyme activity by antibodies to enzymes (2). We wish to report an example of a related artificial regulatory phenomenon in which enzyme activity can in principal be controlled by practically any desired substance. This phenomenon provides the basis for a powerful new immunoassay technique.

The extent of inhibition of enzyme activity by antibodies to an enzyme has been related to the size of the enzyme substrate. This has led to the proposal that antibodies to enzymes sterically hinder the access of the substrate to the active site of the

*Contribution No. 44

enzyme (2). Steric inhibition of an enzyme by an antibody might also be envisioned if a hapten were attached to the enzyme near its active site. In a properly selected system binding of a hapten-directed antibody to the hapten might have the same effect as binding of an enzyme-directed antibody directly to the enzyme. In such a system the enzyme activity could be regulated by the amount of antibody available for binding to the enzyme-bound hapten. The amount of free antibody could in turn be controlled by the addition of free hapten which would compete with the enzyme-bound hapten for antibody binding sites. Thus the enzymic activity of a mixture of enzyme and antibody would be directly related to the amount of free hapten introduced.

The above system possesses all the elements needed for an immunoassay. The relevant reactions are illustrated in Equations 1-2, where H is the unknown, Ab is the antibody, and Enz is the enzyme.

$$H + Ab\text{-}(H\text{-}Enz) \rightleftharpoons Ab\text{-}H + H\text{-}Enz^* \quad (1)$$

$$\text{Substrate} \xrightarrow{H\text{-}Enz^*} \text{product} \quad (2)$$

The system provides an intrinsic amplification since one molecule of free hapten frees one molecule of enzyme which in turn can catalyze the conversion of many molecules of substrate to product. We propose the term "homogeneous" enzyme immunoassay to distinguish the method from most other immunochemical methods that are "heterogeneous" and depend at some stage on the physical separation of antigen bound to antibody from unbound antigen. Such a separation is necessary when the label on the antigen is detected equally in both bound and free states; e.g., a radioactive atom (3). One other "homogeneous" immunoassay technique known as spin immunoassay has recently been described (4).

Hen egg-white lysozyme appeared to be an ideal enzyme for

this study. The natural substrate, bacterial peptidoglycan, is a high polymer which should be highly susceptible to steric effects, and also, one of the six lysine residues of lysozyme (lysine 97) is very near the active site (5) and can be modified without seriously inhibiting the enzyme (6). Carboxymethyl-morphine (CMM) (4,7) was selected as a hapten. Conjugation to lysozyme was achieved by combining lysozyme with CMM-isobutyl-chloroformate mixed anhydride (4) (2 equivalents mixed anhydride per lysine residue) in aqueous solution at pH 9.5-10.0 followed by dialysis against water. A magnetic circular dichroism spectrum of the conjugate indicated an average of four haptens per enzyme molecule.* This is consistent with the observation that four of the six lysine residues of lysozyme react readily with iodoacetate whereas the remaining two are relatively inert (8). Rabbit anti-morphine γ-globulin was prepared as described previously (4). Lysozyme activity was determined by changes in light transmission of a suspension of the substrate, Micrococcus luteus, by a method similar to that of Shugar (9).

Anti-morphine γ-globulin did not affect the activity of native lysozyme. However addition of morphine antibodies to CMM-lysozyme resulted in up to 98% inhibition of enzyme activity. Thus the antibody specifically inhibits hapten-labelled enzyme. The result of titration of the antibody with CMM-lysozyme is given in Figure 1. The CMM-lysozyme concentrations were estimated from the absorption of stock solutions at 280 nm (10). As expected, with no antibody present enzyme activity was proportional to enzyme concentration. With a fixed amount of antibody present the activity increased proportionately only at

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*We wish to thank Dr. G. Barth, Dept. of Chemistry, Stanford University for this measurement.

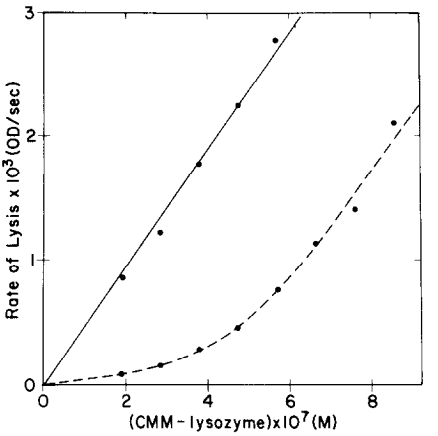

Figure 1. Effect of CMM-lysozyme on the rate of lysis of M. luteus (Miles) at pH 6.0 in the presence..... and absence _____ of anti-morphine γ-globulin (4.2×10^{-7}M binding sites).

high CMM-lysozyme concentrations where nearly all the antibody binding sites were saturated. The horizontal distance between the resulting two parallel lines yields the concentration of inhibited enzyme. Based on the antibody titer as determined by spin immunoassay (4) the data require that about two antibody binding sites per enzyme molecule must be occupied to completely inhibit activity.

Free morphine competes with the enzyme for binding sites causing an increase in enzyme activity. Figure 2 shows the effect of morphine on the enzyme activity of a solution that contained an antibody binding site/enzyme ratio of 1.7 and was initially 96.5% inhibited. By following the enzyme kinetics for 10 sec at 30° the minimum morphine concentration detectable in the assay mixture was approximately 3×10^{-8}M. Fifty fold lower concentrations of the reagents and a 90 minute measurement permitted detection of as little as 1×10^{-9}M morphine.

The "homogeneous" enzyme immunoassay technique offers the virtues of simplicity, reagent stability and quantifiability.

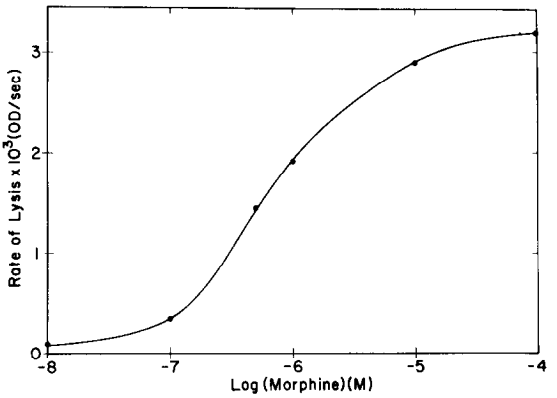

Figure 2. Effect of morphine on the rate of lysis of *M. luteus* by 1.11×10^{-7}M CMM-lysozyme containing 1.9×10^{-7}M antibody binding sites at pH 6.0.

Although the present assay employs lysozyme which can be detected above about 10^{-10}M, the use of other more readily detected enzymes should permit the technique to rival even radioimmunoassay (3) in sensitivity. Moreover the new method requires no physical separation of antibodies and antigens which are drawbacks both to radioimmunoassay and a recently reported "heterogeneous" enzyme immunoassay utilizing an uninhibitable enzyme-labelled antigen (11). Preliminary studies have demonstrated that other enzymes such as amylase and horseradish peroxidase can also be employed and may provide increased sensitivity. In addition the technique has been found applicable to other haptens. Details of these studies will be reported elsewhere.

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