3.1.2 Criteria for the Study of Mechanism-Based Inactivators. In addition to the requirements described above for an affinity label, a mechanism-based inhibitor should also demonstrate the following:

1. Occurrence of a catalytic step. The major difference between the mechanism of inactivation of mechanism-based inactivators vs. that of any other type of inhibitor is the obligate involvement of a catalytic step, that is, step 2 in Equation 17.46. Initially, the mechanism-based inhibitor binds reversibly to form the E . I complex. The enzyme then starts its normal catalytic cycle, resulting in the conversion of the inhibitor into a reactive species (I^r). If the reactive species is electrophilic, it may react with an active-site nucleophile, much like an affinity label. If the reactive species is nucleophilic, it may react with an electrophilic species on the enzyme, probably an oxidized cofactor. Finally, a radical species may be generated that has the potential to react with an enzyme radical, or generate one by hydrogen atom abstraction. The experiments necessary to provide evidence for a catalytic step are obviously strongly dependent on the individual catalytic mechanism involved. The experiments may include spectrophotometric detection of oxidized or reduced cofactor, observing C–H bond cleavage by monitoring the release of tritium, or the detection of some component of cleaved inhibitor (such as fluoride ion as in some examples shown below).
2. No release of the activated species before enzyme inactivation. For a mechanism-based inactivator to retain its high specificity during inactivation, release of the reactive species from the active site must not be part of the normal mechanism of inactivation. A time-dependent increase in the rate of inactivation points to the release of an activated species before inactivation. This increase in the rate of inactivation is brought about by the accumulation of free reactive species in solution. Inhibitors generated in this manner have been termed metabolically activated affinity labels (171). In these cases, as with affinity labels, nonspecific covalent modification of residues other than those located in the active site cannot be excluded. A second test for a metabolically activated affinity label is to add an additional aliquot of fresh enzyme to the incubation buffer. The fresh enzyme should be inactivated at a higher rate than that of the first equivalent of enzyme because there is more reactive species present in solution. By contrast, the mechanism-based inhibitor should show no difference in rate until the concentration of inhibitor is depleted. It should also be noted that the observation of such rate increases necessitates that the reactive species is relatively stable and is not immediately quenched by the incubation buffer.

   Additional tests such as the addition of nucleophilic scavengers (e.g., thiols such as dithiothreitol or β-mercaptoethanol) can provide further evidence for the presence of a free, reactive electrophilic species. The scavengers should quench all of the free reactive species, thereby protecting the enzyme from inhibition. Unfortunately, this method cannot exclude the possibility that a nucleophilic thiol may even attack the bound reactive species at the active site of the enzyme (which would also give rise to protection from inactivation). However, the use of a bulky thiol, such as reduced glutathione, should limit that possibility. An alternative scenario occurs wherein the released reactive species returns and reacts faster with an active-site nucleophile than with the added thiol. Clearly this is a complex problem and, consequently, it is advisable to use several different tests to avoid misleading conclusions.
3. Partition ratio. The partition ratio is the ratio of product release to enzyme inactivation and is a measure of the efficiency of the mechanism-based inhibitor. Formally, it refers to the ratio k_4/k_3 (Equation 17.46). The most efficient inactivators will have partition ratio of zero. In those cases, theoretically, every enzymatically processed inhibitor molecule will result in the inactivation of a molecule of enzyme. Even though the partition ratio is independent of