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ERRORS AND ARTIFACTS IN COUPLED SPECTROPHOTOMETRIC ASSAYS OF ENZYME ACTIVITY

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Key Word Index—Enzyme assay; coupled spectrophotometric assay; phosphofructokinase; pyrophosphate:fructose 6-phosphate phosphotransferase; contaminant.

Abstract—This paper considers problems associated with the accurate determination of enzyme activity using coupled spectrophotometric assays. Criteria for establishing optimum assay conditions and ensuring that the coupled assay accurately reflects enzyme activity are presented. The susceptibility of such assays to interference is illustrated by five instances in which contamination of specific assay components has produced misleading estimates of phosphofructokinase and pyrophosphate:fructose 6-phosphate phosphotransferase activity. Such artifacts have resulted in publication of spurious biochemical and physiological conclusions. These examples suggest that problems associated with contaminants are likely to be widespread in coupled spectrophotometric assays, and are likely to confound interpretation of the measurements. Strategies for identifying artifacts resulting from contaminants in coupled assays are proposed.

INTRODUCTION

Coupled spectrophotometric assays based on the differential absorbance of reduced and oxidized forms of nicotinamide adenine nucleotides are one of the most popular methods of measuring enzyme activity. Such assays are most commonly applied to the wide range of enzymes for which the provision of substrate or removal of product can be linked to reduction of $NAD(P)^+$ or oxidation of NAD(P)H through auxiliary enzymes. The popularity of this approach is due to its simplicity, versatility and the commercial availability of a sufficient range of purified enzymes to ensure that most enzymes of intermediary metabolism can be assayed using this technique [1].

Although technically simple, this approach for determining enzyme activity cannot be applied uncritically. Many of the problems that arise during coupled spectrophotometric assays are common to all types of enzyme assays. These have been considered recently by Tipton [2] in an authoritative review which examines the potential sources of blank rates obtained in the absence of added substrates, as well as the possible causes of bursts and lags in progress curves, and considers the principal reasons why enzymes may apparently depart from the simple hyperbolic behaviour predicted by the Michaelis–Menten equation. Here I wish to examine, in detail, some of the problems specifically associated with coupled spectrophotometric assays. The potential pitfalls in this technique are illustrated by difficulties encountered in the assay of enzymes catalysing the conversion of fructose 6-phosphate to fructose 1,6-bisphosphate in plants. On the basis of these examples, I argue that components of such assays are frequently contaminated with compounds that may confound the precise measurement of enzyme activity.

SPECTROPHOTOMETRIC ASSAY OF ENZYME ACTIVITY

The standard assays for phosphofructokinase (EC 2.7.1.11) (PFK) and pyrophosphate:fructose 6-phosphate 1-phosphotransferase (EC 2.7.1.90) (PFP) are typical continuous coupled assays in which one, or more, additional enzymes are used to catalyse a reaction involving one of the products to yield a compound that can be detected directly. This is by far the most commonly used spectrophotometric assay technique. For PFK and PFP the production of fructose 1,6-bisphosphate is coupled to the oxidation of NADH through the series of reactions involving aldolase, triose phosphate isomerase and glycerol 3-phosphate dehydrogenase as depicted in Fig. 1 [3]. An alternative coupled assay for PFK links ADP production to NADH oxidation through the action of pyruvate kinase and lactate dehydrogenase. However, this assay is undesirable because phosphoenolpyruvate, the substrate for pyruvate kinase, is a potent allosteric inhibitor of PFK from higher plants [4–6].

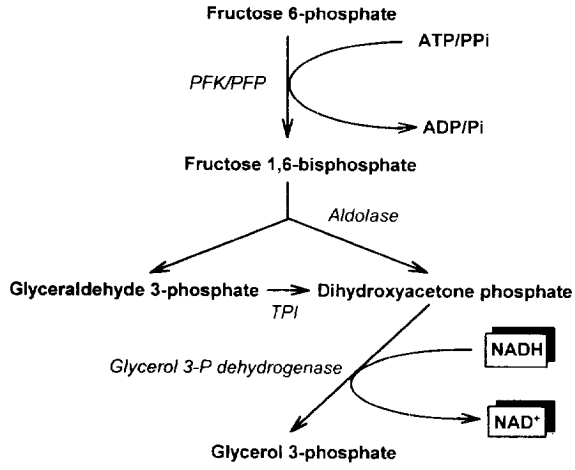

Fig. 1. Coupled assay for PFP and PFP based on linking fructose 1,6-bisphosphate production to oxidation of NADH through the following auxiliary enzymes: aldolase (EC 4.1.2.13), TPI (triose phosphate isomerase, EC 5.3.1.1) and glycerol 3-phosphate dehydrogenase (EC 1.1.1.8).

In general, enzyme assays should be performed under conditions that yield maximum activities. Indeed, the instructions for authors for *Phytochemistry* explicitly demand that enzyme measurements be made under specified optimum conditions [7]. The practical benefits of optimizing the assay are two-fold. One benefit is that the greater enzyme activity achieved under such conditions is often easier to measure. The other is that these are the conditions under which enzyme activity is least sensitive to slight variations in the concentration of the components of the assay, and thus will give the most reproducible measurements. Optimum conditions are achieved by systematically altering the pH of the assay and the concentration of each component in the assay medium. With respect to the last point, it is important to note that enzymes are frequently inhibited at high levels of one or more substrates [6, 8], and so uncritical use of high substrate concentrations will not necessarily produce maximum activity. In addition, it is expedient to vary the buffer in the assay and the counter-ion used to adjust the pH of the assay buffer since both may influence enzyme activity [5]. However, three problems can arise in attempting to optimize assay conditions. First, the reaction mixtures for most coupled enzyme assays are complex. Varying the concentration of each component of the assay systematically may require a large number of measurements. Unfortunately there is no way to avoid this task if the assay is to be optimized. An appropriate strategy is, initially, to determine the pH optimum of the assay under standard conditions, next, to confirm that activity does not increase when the concentration of each component of the reaction mixture is doubled or halved (if it is, a more comprehensive range of concentrations must be tested to determine the optimum), and finally, to establish the pH optimum at the optimized concentrations of the other assay components. Sometimes there is no substitute for hard work! A second complication is that in a coupled enzyme assay the optimized conditions necessarily represent a compromise between the properties of the enzyme being assayed and those of the auxiliary enzymes(s). Normally this does not present much of a problem since auxiliary enzymes are usually present in vast excess and sub-optimal conditions for the coupling system can be compensated for by increasing the amounts of auxiliary enzymes in the assay (but see following section). The third complication is that plant extracts often contain two or more isozymes that possess different kinetic properties. Establishing optimum conditions for measuring the maximum catalytic activity of all the isozymes may be difficult. Although not unique to coupled assays, this problem may be exacerbated in the relatively more complex assay mixtures found in coupled assays. In some instances the kinetic properties of isozymes are sufficiently different to allow the individual forms to be measured separately. Alternatively, the optimum conditions may be sufficiently broad to allow maximum activities of both isozymes to be measured. The latter can almost certainly be achieved for plastidic and cytosolic isozymes of PFK from spinach leaves [5, 9] and castor bean endosperm [6]. In other instances compromise may be necessary.

Notwithstanding the difficulties described above, it is important to spend time establishing optimum assay conditions since these are critical in estimating maximum catalytic activity. The latter is crucial for at least two reasons. One is that comparisons between the activities of different enzymes in the same tissue, or between the same enzyme in different tissues, are only meaningful if they are accompanied by adequate evidence that the measurements represent the maximum capacity of the tissue to catalyse the particular reaction being considered [10]. The second is that quantitative theories of control analysis which are being applied increasingly to plant metabolism are based on the determination of maximum catalytic activities [11]. Criteria for establishing that measurements represent maximum catalytic activities have been proposed [10] and reiterated [11, 12] but sadly, are frequently ignored. If the conditions used to assay enzyme activity are not optimized, the measurements that are obtained are of limited value.

ERRORS ASSOCIATED WITH COUPLED SPECTROPHOTOMETRIC ASSAYS

For a coupled assay such as that commonly used to measure PFK and PFP to accurately reflect the activity of the enzyme being studied, it is essential that the measurements are not limited by the activity of the auxiliary enzymes. This can be checked by confirming that the measured activity is not increased by increasing the amount of the auxiliary enzymes present, and that it is proportional to the amount of PFK or PFP present at each substrate concentration and under all assay conditions that are used. Generally, such conditions are achieved by having a vast excess of the auxiliary enzymes. However, there are at least three disadvantages in uncri-

tical application of excessive activities of auxiliary enzymes. First, this practice wastes reagent and may be expensive. Secondly, it may lead to unnecessary complications because of side-reactions. For example, glucose 6-phosphate dehydrogenase can use glucose as a substrate instead of glucose 6-phosphate [13]. Consequently, in a coupled enzyme assay for hexokinase involving glucose 6-phosphate dehydrogenase, high concentrations of glucose can produce an observable blank rate in the absence of hexokinase that is exacerbated by increasing the amount of the auxiliary enzyme. Thirdly, excessive amounts of auxiliary enzymes increase the likelihood that contaminants in the enzyme preparations may interfere with the assay. The consequences of such contamination on the measurement of PFK and PFP activities are considered in detail below.

There are several methods for calculating the amount of auxiliary enzyme required for an accurate coupled enzyme assay [14]. The most rigorous theoretical treatment of this problem is that developed by Storer and Cornish-Bowden [15]. Their approach estimates the amount of auxiliary enzyme necessary to establish a defined fraction, say 0.99, of the authentic reaction rate and specifies the length of the lag-phase before the final linear rate is obtained. This treatment can be extended to assays containing two, or more, auxiliary reactions by defining the upper and lower limits for the time required for the measured rate to reach a defined fraction of the actual rate of the enzyme being assayed [15]. The validity of this approach has been confirmed by comparing the predicted and experimentally determined time-course of measurements of glucokinase activity coupled to $NADP^+$ reduction using glucose 6-phosphate dehydrogenase. These tests revealed two further important practical points. First, an apparently linear chart trace following an initial lag-phase may considerably underestimate the actual enzyme activity. To obtain a reliable measure of the limiting rate the apparently linear period should be at least 10 times the length of the period of obvious acceleration. In my experience, few plant biochemists are this rigorous. Secondly, the calculated amounts of auxiliary enzymes used must be based on their activities under the conditions encountered in the assay. A seemingly massive excess of auxiliary enzyme activity may be inadequate, especially if the activity is inhibited by a component of the assay. This problem is particularly pertinent in PFK and PFP assays in which aldolase may be inhibited by fructose 6-phosphate [16]. Moreover, glycerol 3-phosphate dehydrogenase is known to be inhibited by excess NADH [17]. Failure to appreciate this problem has led to the erroneous conclusion that PFK from sheep and rat liver are inhibited by NADH, and the suggestion that this inhibition may contribute to metabolic control of glycolysis [18, 19]. Checking for such inhibition of auxiliary enzymes must be performed carefully. It is inappropriate to check the activity of the auxiliary enzymes by adding a large amount of the metabolite whose production is being monitored since this will mask the effects of any competitive inhibition of an auxiliary enzyme. Consequently, the inhibition of glycerol 3-phosphate dehydrogenase referred to above is only apparent at low levels of the reaction intermediates, and cannot be detected if the activity of the auxiliary enzymes are measured by supplementing the assay mixture with 25 μM fructose 1,6-bisphosphate [17].

ERRORS ARISING FROM CONTAMINATION OF METABOLITES

Contamination of fructose 6-phosphate by fructose 2,6-bisphosphate

Fructose 2,6-bisphosphate is a potent activator of PFP from higher plants. Early reports suggested that in the absence of this activator, the enzyme from potato tuber [20] and spinach leaf [21] exhibited sigmodial kinetics with respect to fructose 6-phosphate. In these studies fructose 2,6-bisphosphate increased the maximum activity of PFP about two-fold, and increased the affinity of the enzyme for fructose 6-phosphate by altering the response from sigmoidal to hyperbolic. In contrast, the enzyme for mung bean hypocotyl [22] and castor bean endosperm [23] displayed hyperbolic kinetics in the absence of fructose 2,6-bisphosphate. For PFP from the latter sources, the activator decreased K_m for fructose 6-phosphate by a factor of 10–50 and increased maximum activity 15- to 20-fold. Subsequent studies revealed that the discrepancy between these two sets of reports could be explained by the presence of fructose 2,6-bisphosphate in some commercial preparations of fructose 6-phosphate [24]. The first indication of this was that the kinetic properties of purified PFP varied depending on the source of fructose 6-phosphate. Two lines of evidence demonstrated that the contaminant was fructose 2,6-bisphosphate. First, activation was completely abolished by incubation of the substrate of pH 3.0 for 30 min. This extreme acid-lability is characteristic of fructose 2,6-bisphosphate. Neither glucose 1,6-bisphosphate nor fructose 1,6-bisphosphate, which may also activate PFP at high concentrations, were affected by this treatment. Secondly, during anion exchange chromatography, the activator eluted in the position of authentic fructose 2,6-bisphosphate. The apparent sigmoidal kinetics of PFP for fructose 6-phosphate encountered in some studies were artifacts caused by inadvertently increasing the concentration of fructose 2,6-bisphosphate in concert with that of fructose 6-phosphate. Following removal of fructose 2,6-bisphosphate from the substrate, hyperbolic kinetics were obtained with PFP that had previously displayed a sigmoidal response to fructose 6-phosphate. Based on the extent of activation of PFP by authentic fructose 2,6-bisphosphate, the batches of commercial fructose 6-phosphate were estimated to contain between 0.03 and 1.3 μmol fructose 2,6-bisphosphate mol^{-1} fructose 6-phosphate.

Contamination of ATP by inorganic pyrophosphate

As part of a study on the relationship between PFK and PFP in plants, Buchanan and coworkers [25] described an anomalous kinetic behaviour of PFK. In the

Table 1. Contamination of components of enzyme-linked phosphofructokinase assay

Component	Contaminant	Level of contamination (%)	Reference
Fructose 6-phosphate	Fructose 2,6-bisphosphate	0.00013	24
ATP	PPi	0.27	26
Imidodiphosphate	PPi	1.8	28
Glycerol 3-phosphate dehydrogenase/triose phosphate isomerase	UDPglucose pyrophosphorylase	0.37	30
Glycerol 3-phosphate dehydrogenase/triose phosphate isomerase	Adenylate kinase	0.007	32

Levels of contamination are expressed as mol% for metabolites and activity% with respect to glycerol 3-phosphate dehydrogenase for enzymes.

presence of fructose 2,6-bisphosphate this enzyme exhibited an initial rapid burst of activity which declined to a lower, constant rate after a few minutes. This kinetic response formed the principal evidence for the proposal that fructose 2,6-bisphosphate mediates the conversion of PFP to PFK in plants. However, the apparent hyperactive burst of PFK was later shown to be an artifact resulting from the metabolism of small amounts of PPi present in the ATP preparations by PFP in the plant extracts [26]. The dependence of the apparent hyperactive phase on fructose 2,6-bisphosphate was due to the pronounced activation of PFP by this metabolite. Again, the first indication of this contamination was the observation that both the initial activity and the length of hyperactive phase were dependent on the source of ATP. Subsequently, we demonstrated that the activity of PFP in the plant extract was sufficient to account for the initial burst of activity, that the ATP preparation used in the study was contaminated with 2.73 mmol PPi mol^{-1} ATP, and that removal of PPi from the ATP preparation by inorganic pyrophosphate completely abolished the initial rapid reaction rate associated with fructose 2,6-bisphosphate. Consistent with this view, purified PFK containing no detectable PFP activity did not exhibit the apparent hyperactive behaviour. Moreover, we were able to mimic the initial burst of activity by including purified PFP in the assay.

Contamination of imidodiphosphate by inorganic pyrophosphate

Neuhaus and Stitt have recently analysed the kinetic response of PFP to imidodiphosphate as part of an investigation of pyrophosphate turnover in leaves [27]. Their conclusion that PFP does not contribute to the metabolism of pyrophosphate produced during sucrose synthesis was based primarily on the apparent ability of PFP to use imidodiphosphate as a phosphoryl donor for the production of fructose 1,6-bisphosphate. However, more detailed studies have revealed this to be an artifact [28]. The measured activity is due to the metabolism by PFP of a small quantity of PPi that is a contaminant of imidodiphosphate. The first indication of this was that in assays containing low concentrations of imidodiphosphate the rate of reaction declined after a few minutes. When the reaction was allowed to run to completion, the amount of NADH oxidized in the assay was only a small fraction of that predicted from the amount of fructose 1,6-bisphosphate that could have been formed from the substrates available in the reaction. Treatment of the imidodiphosphate preparation with inorganic pyrophosphatase completely abolished the oxidation of NADH in this assay. The identity of the contaminant was confirmed by ^{31}P NMR. The sample of imidodiphosphate contained a resonance that was coincident with authentic PPi. Treating the sample with inorganic pyrophosphatase abolished this resonance and resulted in a corresponding increase in the peak associated with Pi.

Subsequent kinetic analysis of PFP has revealed that, when contaminating PPi is taken into consideration, imidodiphosphate is a mixed inhibitor with respect to both fructose 6-phosphate and PPi [28]. The affinity of PFP for imidodiphosphate is sufficient for the enzyme to be significantly inhibited by levels of imidodiphosphate that affect sucrose synthesis, and there is strong evidence that PFP is inhibited *in vivo* under such conditions [28]. The correlation between PFP inhibition, PPi accumulation and decrease in the rate of sucrose production in the presence of imidodiphosphate suggests that PFP contributes to the removal of PPi generated during sucrose synthesis by catalysing the PPi-dependent conversion of fructose 6-phosphate to fructose 1,6-bisphosphate.

ERRORS ARISING FROM CONTAMINATION OF AUXILIARY ENZYMES

Contamination of triose phosphate isomerase/glycerol 3-phosphate dehydrogenase by UDPglucose pyrophosphorylase

In an early comparison of PFK and PFP, Buchanan and coworkers suggested that PFK may be converted to PFP in the presence of UDPglucose [25, 29]. This idea

arose from the observation that addition of UDPglucose to PFK preparations resulted in a PPi-dependent production of fructose 1,6-bisphosphate. Subsequently, we demonstrated that the auxiliary enzymes used in assaying both PFK and PFP are contaminated by UDPglucose pyrophosphorylase, and that the UDPglucose-dependent production of fructose 1,6-bisphosphate from fructose 6-phosphate and PPi can be explained by the following scheme [30]:

UDPglucose + PPi → Glucose 1-P + UTP

Fructose 6-P + UTP → Fructose 1,6-P_2 + UDP.

The initial observation that led to this scheme was that the assay for apparent PFP activity exhibited a lag period of 4–7 min if the reaction was started by adding PPi, but not if either fructose 6-phosphate or PFK was used to begin the reaction. The lag-phase was abolished only if UDPglucose, PPi and triose phosphate isomerase plus glycerol 3-phosphate dehydrogenase were pre-incubated before starting the assay. We demonstrated that apparent PFP activity was dependent on the amount of auxiliary enzymes used in the assay. Furthermore, we confirmed that UTP was a suitable phosphoryl donor for the PFK used in our study, and we showed that the contaminating UDPglucose pyrophosphorylase activity was sufficient to account for the observed rates of fructose 1,6-bisphosphate production.

Contamination of triose phosphate isomerase/glycerol 3-phosphate dehydrogenase by adenylate kinase

Surendranathan and Nair [31] have reported that ADP may replace ATP as the phosphoryl donor for partially purified PFK from ripening banana. Such an activity would have profound implications for our understanding of the control of glycolysis and, more generally, on our concepts of energy metabolism in plants. However, our recent studies suggest that this activity is an artifact arising from contamination of the triose phosphate isomerase plus glycerol 3-phosphate dehydrogenase mixture by adenylate kinase [32]. In combination with PFK, this contaminant can produce an apparent ADP-dependent PFK activity as described in the following scheme:

ADP + ADP → ATP + AMP

Fructose 6-P + ATP → Fructose 1,6-P_2 + ADP.

The presence of this contaminant was initially suggested by the observation that apparent ADP-dependent PFK activity was dependent on the reagent used to start the reaction, and on the length of time prior to beginning the assay. Significant ADP-dependent activity was only obtained if ADP and the auxiliary enzymes were combined before the start of the assay. Neither fructose 6-phosphate nor PFK were required in the preincubation, although no ADP-dependent PFK activity was detectable if either component was omitted from the subsequent assay. We demonstrated that apparent ADP-dependent PFK activity was directly proportional to the duration of preincubation of the assay mixture and the amount of auxiliary enzyme present. Moreover, we established that the triose phosphate isomerase plus glycerol 3-phosphate dehydrogenase mixture contained sufficient adenylate kinase to account for the apparent ADP-dependent PFK activity observed in our studies. As predicted by our explanation, we have been able to measure significant apparent ADP-dependent activity in every PFK preparation we have studied, including PFK purified to near homogeneity from rabbit muscle [32].

DISCUSSION

The contaminants that have been shown to distort the measurement of PFK and PFP activity in plant extracts are summarized in Table 1. There are three important features of this information. First, the contaminants are not confined to a single component of the assay, but are found in a variety of compounds. Secondly, the contaminants are often present at extremely low levels. However, even these seemingly trivial amounts are sufficient to have a profound influence on the enzyme assay. Thirdly, this is not an exhaustive list of contaminants in the assay components. In addition to PPi and Pi, the ^{31}P NMR spectrum of imidodiphosphate reveals at least two other contaminants with resonances of 0.33 and 5.06 ppm (relative to 85% orthophosphoric acid) that are present at 0.35 and 0.6%, respectively [28]. The identities of these components are unknown. In a separate study on PFP, the synthetic substrate μ-monothiopyrophosphate was shown to be contaminated by 0.3% PPi [33]. Moreover, there is good circumstantial evidence that fructose 2,6-bisphosphate is present as a contaminant of commercial preparations of fructose 1,6-bisphosphate [34]. Finally, a recent commercial batch of triose phosphate isomerase plus glycerol 3-phosphate dehydrogenase contained 40 mkat PFK kat^{-1} glycerol 3-phosphate dehydrogenase (Clasper and Kruger, unpublished observation).

It is important to know about contaminants such as those discussed above since they will have profound effects on the quantitative analysis of PFK and PFP activity. In many instances, if the nature and quantity of a contaminant is known, its effects can be taken into consideration during the kinetic analysis [28, 33]. However, the influence of the contaminants described in this paper extends far beyond a distortion of the kinetic analysis of enzymes. On an analytical level, appreciation that fructose 6-phosphate from some sources is contaminated by fructose 2,6-bisphosphate has led to a 10-fold increase in the sensitivity of the PFP-based bioassay for fructose 2,6-bisphosphate. Elimination of the apparent sigmoidal response of PFP to fructose 6-phosphate allows a higher concentration of this substrate to be used. This enables as little as 50 fmol fructose 2,6-bisphosphate to be measured reliably in a 250 μl assay (Scott, Rowntree and Kruger, unpublished results). At a biochemical level, contamination of both metabolites and auxiliary enzymes has led to the spurious proposal of a novel form of enzyme regulation by metabolite-mediated inter-

conversion of activities [25, 29]. Similar contamination of auxiliary enzyme led to the claim of the existence of a new enzyme and prompted the suggestion that ADP may be an important phosphoryl donor in plants [31]. Finally, at a physiological level, contamination of an inhibitor of PFP by pyrophosphate resulted in potentially misleading conclusions about the pathway of PPi metabolism during sucrose synthesis [27].

Although PFK and PFP catalyse an important step in carbohydrate metabolism, there is little reason to suspect that the measurement of these activities is any more prone to artifacts than that of the vast majority of enzymes. Of the five contaminants described in this review, only contamination of fructose 6-phosphate by fructose 2,6-bisphosphate is related to allosteric properties of either PFK or PFP. The remainder are directly associated with substrates and auxiliary enzymes. We must therefore accept that similar artifacts are likely to be widespread and not necessarily confined to the assay of enzymes possessing complex regulatory properties.

There is no general strategy for avoiding artifacts in enzyme assays. Since the problems that are encountered tend to be specific to particular combinations of enzyme preparations and reagents, each must be dealt with in its own way. However, the examples discussed in this review suggest three approaches that may identify potential problems. The simplest is to check the requirement for each component of the assay system. Both artifacts arising from contamination of auxiliary enzyme preparations were identified by the anomalous response of the assay to changes in the amount of the auxiliary enzyme in the assay. A second approach is to acknowledge and identify the cause of any significant differences between enzyme activity measured using metabolites from different sources. A third approach is to check that the reaction does not stop before the depletion of the limiting substrate or cofactor. The latter two approaches may highlight contamination of substrates or allosteric effectors in the assay. However, even in combination, these tests are not guaranteed to detect all potential artifacts. Consequently, the researcher should question any unusual kinetic behaviour, since this may result from the presence of a contaminant in the assay and confound attempts to determine enzyme activity accurately.

Faced with the situation described above, the response of some researchers is to criticize the suppliers of biochemicals. I believe such a reaction is inappropriate. The compounds provided by the manufactures are used in a wide range of biochemical assays. It is impractical for a supplier to test the suitability of a compound for all foreseeable uses. Rather, the onus is on the individual researcher to confirm the reliability of an assay technique–this includes ensuring that the materials used in the assay do not significantly interfere with the measurement of enzyme activity. The examples discussed in this review serve to highlight the care that must be taken when using coupled assays. Only if each of us performs rigorous checks to validate the assay procedures used will we be able to have confidence in the results that are published.

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