# EXTENDING ENZYME CLASSIFICATION WITH METABOLIC AND KINETIC DATA: SOME DIFFICULTIES TO BE RESOLVED

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## ABSTRACT

Classification of enzymes according to the reaction(s) catalysed is a relatively straightforward procedure, as it deals with more-or-less factual data. However, attempting to add meaning to those data by adding metabolic or kinetic information takes one into the field of parameters rather than absolutes. Thermodynamic data have been assembled for a number of reactions, but the direction in which a reaction is favoured in isolation does not necessarily mean that that will be the direction of the reaction in cellular metabolism; there are many metabolic examples of enzyme reactions proceeding in the thermodynamically less-favoured direction. Attempts to predict "missing enzymes" from metabolic pathways should also be treated with caution, since there are several cases where such guesses have proven to be wide of the mark. Incorporation of kinetic data requires the definition of standard conditions, which should ideally bear some relevance to the physiological situation in which the enzyme operates. However, not all enzymes operate under the same physiological conditions and there are, as yet, no universally accepted standard conditions, or sets of conditions, of temperature, pH, ionic strength etc. for the collection of such data.

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## INTRODUCTION

The International Union of Biochemistry and Molecular Biology (IUBMB) Enzyme List classifies enzymes in terms of the reactions they catalyse (see [1, 2] for definitive versions). It is restricted to classification and recommendations on nomenclature. As such, the data contained within it are, as far as possible, strictly factual and should provide a system for the unambiguous identification of the enzyme(s) being studied. Thus, it should provide a solid basis for the incorporation of data on enzyme behaviour, compartmentation etc. to facilitate studies on the behaviour of biological systems. However, the value of such reconstructive approaches will depend on the quality of the data incorporated.

This brief account will consider the structure of the Enzyme List (see [3,4] for fuller details) and its limitations. Then some of the problems and pitfalls in attempts to assign metabolic and kinetic data will be considered with particular reference to the wide variations in assay conditions that have been used in the literature. The examples used are far from comprehensive and are largely drawn from systems with which we are familiar from our own work.

## PRINCIPLES OF ENZYME CLASSIFICATION

Unlike many other classification systems, where classification is based on structure or function, enzymes are classified according to the reactions they catalyse, with each enzyme being assigned a four-digit number, called the EC (Enzyme Commission) number. An EC number takes the form w.x.y.z, where w, x, y and z represent the class, subclass, sub-subclass and serial number, respectively. At present, there are six enzyme classes, each of which covers a different type of reaction, as summarized in Table 1.

The subclass normally provides information about the type of compound or group involved.

For example, in EC 1.x.-.-, the subclass number, x, indicates the group oxidized, with 1 indicating a CH-OH group, 2 an aldehyde or oxo group, 3 a CH-CH group, 4 a CH-$NH_2$ group etc.

The sub-subclass further specifies the type of reaction involved, often the "other" substrate.

For example, in EC 1.-.y.-, the sub-subclass (y) provides information on the group reduced, with 1 indicating $NAD(P)^+$, 2, a cytochrome, 3, $O_2$, 4, S-S…99, others. The forth digit, z, is a serial number that identifies individual enzymes within a sub-subclass.

**Table 1.** Enzyme classes

| Class | Name | Reaction catalysed |
|---|---|---|
| 1 | Oxidoreductases | $AH_2 + B = A + BH_2$<br>*or* $AH_2 + \mathbf{2}\ B^+ = A + \mathbf{2}\ B + \mathbf{2}\ H^+$ |
| 2 | Transferases | AX + B = A + BX |
| 3 | Hydrolases | $AB + H_2O = AH + BOH$ |
| 4 | Lyases | A=B + X–Y = A–B<br>\| \|<br>X Y |
| 5 | Isomerases | A = B |
| 6 | Ligases | A + B + NTP* = A–B + NDP + P<br>*or* A + B + NTP = A–B + NMP + PP |

*NTP = nucleoside triphosphate

Sometimes an enzyme might fit into more than one class, e.g. EC 1.14.12.1, anthranilate 1,2-dioxygenase (deaminating, decarboxylating), which catalyses the reaction:

$$\text{anthranilate} + \text{NAD(P)H} + H^+ + O_2 + 2\ H_2O = \text{catechol} + CO_2 + \text{NAD(P)}^+ + NH_3$$

might also be classed among the deaminases (EC 3.5.-) or the decarboxylases (EC 4.1.1.-). In such cases, the general rule is that the lower EC class number takes precedence.

Entries within the Enzyme List have a standardized format, although not all enzymes will contain each of the fields described below.

**Common name**

This is often the most commonly used name for the enzyme, provided that it is neither misleading nor ambiguous. Some generic words indicating reaction type are used in common names, but not in the systematic names, e.g. dehydrogenase, reductase, oxidase, peroxidase, kinase, tautomerase, deaminase and dehydratase. The common name may indicate the direction in which the reaction is perceived to operate (either thermodynamically or in vivo), e.g., use of the term 'reductase' in the common name indicates that the reaction occurs in the opposite direction to that written (see Reaction).

**Reaction**

This shows the actual reaction catalysed, written, where possible, in the form of a 'biochemical' equation, for example:

**EC 2.6.1.1** (aspartate transaminase)

L-aspartate + 2-oxoglutarate = oxaloacetate + L-glutamate.

Sometimes, when an enzyme has wide specificity, the reaction is written in terms of the general type of reactant, for example:

**EC 1.1.1.1** (alcohol dehydrogenase)

an alcohol + $NAD^+$ = an aldehyde + NADH + $H^+$

and sometimes as a description, for example:

**EC 3.2.1.1** (α-amylase)

Endohydrolysis of 1,4-α-D-glucosidic linkages in polysaccharides containing three or more 1,4-α-linked D-glucose units.

It must be stressed that the reaction as written is not meant to indicate the preferred equilibrium of the reaction or the direction in which some may believe the enzyme to operate *in vivo*. In any given sub-subclass, the direction chosen for the reaction is the same for all enzymes. Systematic names are based on this written reaction. Frequently, such biochemical equations are not charge-balanced.

**Other name(s)**

This field contains other names that have been used for the same enzyme, and is as comprehensive as possible to facilitate searching. It should be noted that the inclusion of a name in this list does not mean that its use is encouraged. In some cases where the same name has been given to more than one enzyme, or when the common name is misleading, this may be indicated.

**Systematic name**

This attempts to describe in unambiguous terms the reaction that the enzyme actually catalyses, and consists of two parts. The first contains the name of the substrate or, in the case of a bimolecular reaction, of the two substrates, separated by a colon. The second part, ending in *-ase*, indicates the nature of the reaction.

A number of generic words indicating a type of reaction are used: *oxidoreductase*, *oxygenase*, *transferase* (with a prefix indicating the nature of the group transferred), *hydrolase*, *lyase*, *racemase*, *epimerase*, *isomerase*, *mutase* and *ligase*. Where additional information is needed to make the reaction clear, a word or phrase indicating the reaction or a product is added in parentheses after the second part of the name, e.g. (*ADP-forming*), (*dimerizing*) or (*CoA-acylating*).

**Comments**

This field may contain information on the nature of the reaction catalysed, possible relationships to other enzymes, species differences, metal-ion and cofactor requirements, etc.

**References**

Key references on the identification, nature, properties and function of the enzyme are listed. It is important to note that the characterization of an enzyme is a prerequisite to its inclusion in the Enzyme List.

## CONVENTIONS

The way in which the reaction and systematic name are presented follow certain conventions. These are designed for consistency and in order to make it easier for proposers of new enzymes to suggest valid entries. Each class has its own specific conventions.

**(a) The oxidoreductases (class 1)**

For most enzymes in this class, the reaction is written in the general form:

$$XH_2 + \text{acceptor} = X + \text{reduced acceptor}.$$

Where the acceptor may be $NAD(P)^+$, a cytochrome, oxygen, a disulfide etc. The exceptions are those enzymes where two acceptors are involved. These include the subclass EC 1.6 where NAD(P)H is regarded as the oxidized substrate, as for example in the case of NAD(P)H oxidase (EC 1.6.3.1), where the reaction is written as:

$$NAD(P)H + H^+ + O_2 = NAD(P)^+ + H_2O_2$$

Those enzymes that use peroxide as an acceptor (EC 1.11; the peroxidases) also depart from the normal formulation when the other substrate is NAD(P)H. For example the reaction catalysed by NADH peroxidase (EC 1.11.1.1) is presented as:

$$NADH + H^+ + H_2O_2 = NAD^+ + \mathbf{2}\ H_2O.$$

The subclass EC 1.14, which includes enzymes acting on paired donors, with incorporation or reduction of molecular oxygen, is also treated differently. For example, the reaction for salicylate 1-monooxygenase (EC 1.14.13.1) is written as:

$$salicylate + NADH + H^+ + O_2 = catechol + NAD^+ + H_2O + CO_2$$

The systematic names are written in terms of these directions and substrate orders.

**(b) Enzymes catalysing transfer reactions (class 2)**

For these reactions the donor is written first, in the general form:

$$AX + B = A + BX\ .$$

Examples are:

$$AdoMet + X = AdoHcy + methyl\text{-}X$$

$$ATP + X = ADP + X\text{–}P.$$

Note that the order is preserved in the products (e.g. AdoHcy before methyl-X and ADP before X–P).

Two enzymes involved in the synthesis of sucrose illustrate this convention.

(1) UTP-glucose-1-phosphate uridylyltransferase (EC 2.7.7.9)

$$UTP + \alpha\text{-D-glucose 1-phosphate} = diphosphate + UDP\text{-glucose}$$

and has the systematic name UTP:α-D-glucose-1-phosphate uridylyltransferase.

(2) The reaction catalysed by sucrose synthase (EC 2.4.1.13) has the reaction:

$$NDP\text{-glucose} + D\text{-fructose} = NDP + sucrose$$

and the systematic name NDP-glucose:D-fructose 2-α-D-glucosyltransferase.

In all of the above examples there is little difficulty in deciding which is the donor substrate and which is the acceptor substrate. The aminotransferases (subclass 2.6) catalyse the general reaction:

amino acid + oxoacid = oxoacid + amino acid.

Since the reaction could be written in either direction, by convention, when 2-oxoglutarate or an unspecified oxoacid is involved, this is usually treated as the acceptor. For example, the reaction catalysed by aspartate transaminase (EC 2.6.1.1; L-aspartate:2-oxoglutarate aminotransferase) is written as:

L-aspartate + 2-oxoglutarate = oxaloacetate + L-glutamate

and that for alanine-oxo-acid transaminase (EC 2.6.1.12; L-alanine:2-oxo-acid aminotransferase) is:

L-alanine + a 2-oxo acid = pyruvate + an L-amino acid.

**(c) The hydrolases (class 3)**

These are generally straightforward, with the reaction written as:

substrate + $H_2O$ = products.

For example, the choline-sulfatase (EC 3.1.6.6) reaction is written as:

choline sulfate + $H_2O$ = choline + sulfate.

The systematic name, choline-sulfate sulfohydrolase, indicates both the substrate and the group removed by hydrolysis.

**(d) The lyases (class 4)**

These differ from other enzymes in that two substrates are involved in one direction and only one in the other. The reaction is written in the direction of less to more. Thus, malonyl-CoA decarboxylase (EC 4.1.1.9; malonyl-CoA carboxy-lyase) catalyses the reaction:

malonyl-CoA = acetyl-CoA + $CO_2$

and the reaction catalysed by argininosuccinate lyase (EC 4.3.2.1; *N*-(L-argininosuccinate) arginine-lyase) is written as:

$N^{\omega}$-(L-arginino)succinate = fumarate + L-arginine.

**(e) The isomerases (class 5)**

These are straightforward, one substrate, one product reactions. The reaction catalysed by alanine racemase (EC 5.1.1.1) is, for example,

L-alanine = D-alanine.

Similarly the reaction for glucose-6-phosphate isomerase (EC 5.3.1.9; D-glucose-6-phosphate ketol-isomerase) is written as:

D-glucose 6-phosphate = D-fructose 6-phosphate.

**(f) The ligases (class 6)**

These enzymes catalyse the joining of two molecules with the concomitant hydrolysis of a diphosphate bond in ATP or a similar triphosphate. The reactions are normally written in the order:

XTP + A + B = XDP + P (or XMP + diphosphate) + A–B.

Thus, the reaction catalysed by alanine-tRNA ligase (EC 6.1.1.7; L-alanine:$tRNA^{Ala}$ ligase (AMP-forming)) is written as:

ATP + L-alanine + $tRNA^{Ala}$ = AMP + diphosphate + L-alanyl-$tRNA^{Ala}$.

The pyruvate carboxylase reaction (EC 6.4.1.1; pyruvate:carbon-dioxide ligase (ADP-forming)) is, likewise, written as:

ATP + pyruvate + $HCO_3^-$ = ADP + phosphate + oxaloacetate.

## PEPTIDASES AND RESTRICTION ENZYMES ARE TREATED DIFFERENTLY

The group of enzymes that catalyse the hydrolysis of peptide bonds in proteins and peptides are grouped together under the hydrolases as EC 3.4.11.*z* – EC 3.4.25.*z* according to the type of reaction catalysed or the catalytic type of the enzyme involved. For example, EC 3.4.11 contains the aminopeptidases and EC 3.4.13 contains the dipeptidases, whereas EC 3.4.21 and EC 3.4.22 contain those endopeptidases where serine and cysteine residues, respectively, are involved in the catalytic process. Similarly, EC 3.4.24 contains the metalloendopeptidases. A further sub-subclass EC 3.4.99 is reserved for endopeptidases for which the catalytic mechanism is not yet known.

Where necessary, the amino acids in the peptide substrate are represented by P1...P*n*, numbered towards the N-terminus and P1'...P*n*', numbered towards the C-terminus. The peptide bond cleaved (the scissile bond) is indicated by the symbol ↯.

-P3-P2-P1 ↯ P1'-P2'-P3'.

This departure from the normal logic of the Enzyme List is a result of the demand for classification of a large number of peptidases with similar substrate specificities. If the reaction catalysed were used as the sole basis of classification, there would be rather few peptidases classified. Another difference concerns the naming of the peptidases. There are no systematic names, since the overlapping specificities would make it impossible to assign unique systematic names to each of them. Whereas in the remainder of the Enzyme List, it was decided to adopt the most commonly used name as the common name of an enzyme, even though such a name would not be one that might have been chosen as an adequate description of the reaction catalysed (e.g., catalase, pyruvate kinase), the absence of a systematic name necessitates the recommendation of a unique name for each peptidase. Thus, in this case, names like trypsin, pepsin A, pepsin B and renin are recommended names. Some examples are given in Table 2.

**Table 2.** The peptidases - some examples

| |
|---|
| **EC 3.4.16.2** |
| **Recommended name:** lysosomal Pro-X carboxypeptidase |
| **Reaction:** Cleavage of a -Pro↯Xaa bond to release a C-terminal amino acid |
| **EC 3.4.21.1** |
| **Recommended name:** chymotrypsin |
| **Reaction:** Preferential cleavage: Tyr↯, Trp↯, Phe↯, Leu↯ |
| **EC 3.4.22.2** |
| **Recommended name:** papain |
| **Reaction:** Hydrolysis of proteins with broad specificity for peptide bonds, but preference for an amino acid bearing a large hydrophobic side chain at the P2 position. Does not accept Val in P1'. |

**Table 2.** continued

**EC 3.4.23.1**

**Recommended name:** pepsin A

**Reaction:** Preferential cleavage: hydrophobic, preferably aromatic, residues in P1 and P1' positions. Cleaves $Phe^{1}$↯Val, $Gln^{4}$↯His, $Glu^{13}$↯Ala, $Ala^{14}$↯Leu, $Leu^{15}$↯Tyr, $Tyr^{16}$↯Leu, $Gly^{23}$↯Phe, $Phe^{24}$↯Phe and $Phe^{25}$↯Tyr bonds in the B chain of insulin

**EC 3.4.23.38**

**Recommended name:** plasmepsin I

**Reaction:** Hydrolysis of the -$Phe^{33}$↯Leu- bond in the α-chain of hemoglobin, leading to denaturation of the molecule

**EC 3.4.21.10**

**Recommended name:** acrosin

**Reaction:** Preferential cleavage: Arg↯, Lys↯

The restriction deoxyribonucleases also constitute a large family of enzymes with specificities that sometimes overlap. However, in this case, they can be divided into three families, as shown in Table 3. The Enzyme List directs users to the Restriction Enzyme Database (REBASE) [5] for further information about what is currently known about individual enzymes in these sub-subclasses.

**Table 3.** The restriction deoxyribonuclease types

**EC 3.1.21.3**

**Common name:** type I site-specific deoxyribonuclease

**Reaction:** Endonucleolytic cleavage of DNA to give random double-stranded fragments with terminal 5'-phosphates; ATP is simultaneously hydrolysed

**Other name(s):** type I restriction enzyme; deoxyribonuclease (ATP- and *S*-adenosyl-L-methionine -dependent); restriction-modification system; deoxyribonuclease (adenosine triphosphate-hydrolyzing); adenosine triphosphate-dependent deoxyribonuclease; ATP-dependent DNase

**Table 3.** continued. Comments on EC 3.1.21.3

**Comments:** This is a large group of enzymes which, together with those now listed as EC 3.1.21.4 (type II site-specific deoxyribonuclease) and EC 3.1.21.5 (type III site-specific deoxyribonuclease), were previously listed separately in sub-subclasses EC 3.1.23 and EC 3.1.24. They have an absolute requirement for ATP (or dATP) and *S*-adenosyl-L-methionine. They recognize specific short DNA sequences and cleave at sites remote from the recognition sequence.

They are multifunctional proteins that also catalyse the reactions of EC 2.1.1.72 [site-specific DNA-methyltransferase (adenine-specific)] and EC 2.1.1.73 [site-specific DNA-methyltransferase (cytosine-specific)], with similar site specificity. A complete listing of all of these enzymes has been produced by R.J. Roberts and is available at http://rebase.neb.com/rebase/rebase.html.

**EC 3.1.21.4**

**Common name:** type II site-specific deoxyribonuclease

**Reaction:** Endonucleolytic cleavage of DNA to give specific double-stranded fragments with terminal 5'-phosphates

**Other name(s):** type II restriction enzyme

**Comments:** This is a large group of enzymes which, together with those now listed as EC 3.1.21.3 (type I site-specific deoxyribonuclease) and EC 3.1.21.5 (type III site-specific deoxyribonuclease), were previously listed separately in sub-subclasses 3.1.23 and 3.1.24. They require only $Mg^{2+}$. They recognize specific short DNA sequences and cleave either within, or at a short specific distance from, the recognition site. A complete listing of all of these enzymes has been produced by R.J. Roberts and is available at http://rebase.neb.com/rebase/rebase.html .

**EC 3.1.21.5**

**Common name:** type III site-specific deoxyribonuclease

**Reaction:** Endonucleolytic cleavage of DNA to give specific double-stranded fragments with terminal 5'-phosphates

**Other name(s):** type III restriction enzyme; restriction-modification system

**Comments:** This is a large group of enzymes which, together with those now listed as EC 3.1.21.3 (type I site-specific deoxyribonuclease) and EC 3.1.21.4 (type II site-specific deoxyribonuclease), were previously listed separately in sub-subclasses EC 3.1.23 and EC 3.1.24. They have an absolute requirement for ATP but do not hydrolyse it; *S*-adenosy-L-methionine stimulates the reaction, but is not absolutely required. They recognize specific, short DNA sequences and cleave a short distance away from the recognition sequence.

**Table 3.** continued. Comments on EC 3.1.21.5

| |
|---|
| These enzymes exist as complexes with enzymes of similar specificity listed under EC 2.1.1.72 [site-specific DNA-methyltransferase (adenine-specific)] or EC 2.1.1.73 [site-specific DNA-methyltransferase (cytosine-specific)]. A complete listing of all of these enzymes has been produced by R.J. Roberts and is available at http://rebase.neb.com/rebase/rebase.html. |

## RESTRICTIONS/LIMITATIONS OF THE PRESENT CLASSIFICATION SYSTEM

There are a number of limitations associated with the classification system used for enzymes. These are listed below.

### (a) The same EC number may be assigned to many different proteins

For example, any isoenzymes and species differences come under the umbrella of the single enzyme alcohol dehydrogenase (EC 1.1.1.1) as they all perform the same reaction, i.e., convert an alcohol to an aldehyde and in the process reduce $NAD^+$. Only when there is a clear difference in specificity is an enzyme assigned a different EC number and name. Thus there are other enzymes classified that catalyse the dehydrogenation of alcohols, as shown in Table 4.

**Table 4**. Some alcohol dehydrogenase enzymes

| EC number | Common Name | Reaction |
|---|---|---|
| EC 1.1.1.1 | alcohol dehydrogenase | an alcohol + $NAD^+$ = an aldehyde or ketone + NADH + $H^+$ |
| EC 1.1.1.2 | alcohol dehydrogenase ($NADP^+$) | an alcohol + NAD(P)+ = an aldehyde + NAD(P)H + $H^+$ |
| EC 1.1.1.71 | alcohol dehydrogenase [$NAD(P)^+$] | an alcohol + $NAD(P)^+$ = an aldehyde + NAD(P)H + $H^+$ |
| EC 1.1.99.8 | alcohol dehydrogenase (acceptor) | a primary alcohol + acceptor = an aldehyde + reduced acceptor |
| EC 1.1.1.192 | long-chain-alcohol dehydrogenase | a long-chain alcohol + **2** $NAD^+$ + $H_2O$ = a long-chain carboxylate + **2** NADH + **2** $H^+$ |
| EC 1.1.1.194 | coniferyl-alcohol dehydrogenase | coniferyl alcohol + $NADP^+$ = coniferyl aldehyde + NADPH + $H^+$ |
| EC 1.1.1.21 | aldehyde reductase | alditol + $NAD(P)^+$ = aldose + NAD(P)H + $H^+$ |
| EC 1.1.1.184 | carbonyl reductase (NADPH) | R-CHOH-R' + $NADP^+$ = R-CO-R' + NADPH + $H^+$ |

**(b) Different EC numbers may be assigned to the same protein**

For example, the tryptophan synthase complex may be a single protein containing some of the following [EC 2.4.2.18 (anthranilate phosphoribosyltransferase), EC 4.1.1.48 (indole-3-glycerol-phosphate synthase), EC 4.1.3.27 (anthranilate synthase), EC 4.2.1.20 (tryptophan synthase) and EC 5.3.1.24 (phosphoribosylanthranilate isomerase)] (see [1,2]). The Enzyme List also indicates that 6-phosphofructo-2-kinase (EC 2.7.1.105) may be part of the same protein as fructose-2,6-bisphosphate 2-phosphatase (EC 3.1.3.46). Other examples include: two distinct domains on the NadR protein *Haemophilus influenzae* allow it to function both as a nicotinamide-nucleotide adenylyltransferase (EC 2.7.7.1) and a ribosylnicotinamide kinase (EC 2.7.1.22) [6], and human maleylacetoacetate isomerase (EC 5.2.1.2) is also a glutathione transferase (EC 2.5.1.18) zeta isoenzyme [7].

**(c) The reaction equations and systematic names do not necessarily indicate the direction in which the reaction may be perceived to operate *in vivo***

In many cases, such as many of the enzymes of glycolysis, the reaction direction depends upon the cellular metabolic conditions, whereas in some other cases the direction is not known.

**(d) Whereas the reaction equations are generally mass-balanced, they are not necessarily charge-balanced**

This is because they are written as pH-independent equations. For example, the reaction catalysed by fructose-bisphosphatase (EC 3.1.3.11) is written as:

D-fructose 1,6-bisphosphate + $H_2O$ = D-fructose 6-phosphate + phosphate

with no attempt to indicate the charges on the phosphates or whether metal-ion complexes are involved. In fact it would only be possible to indicate these if the reaction conditions, e.g., pH and metal cation composition, were specified. Similarly hydrogen ions are generally omitted from reactions which might be expected to produce them at physiological pH values, e.g. carboxylesterase (EC 3.1.1.1)

a carboxylic ester + $H_2O$ = an alcohol + a carboxylate.

An exception to this is NAD(P) in oxidoreductase reactions where these oxidized forms are, by convention, written as $NAD(P)^+$ and the reduced forms as $NAD(P)H + H^+$.

It has been recognized that this is a somewhat arbitrary departure from the rule that charges are generally omitted from the biochemical equations used (see [8, 9]) and, indeed, NADP, for example does not have a net positive charge at physiological pH values. However, the convention is too well embedded amongst the biochemical community and the suggestion [10] that it would be more appropriate to represent the redox pairs as NAD(P) and NAD(P)$H_2$, or NAD(P) and reduced NAD(P), did not meet with favour. Furthermore, these alternative formulations would not make it easy to represent the NAD radicals that occur in some reactions.

Where it is convenient, charges may also be used to represent radical species, e.g., the reaction catalysed by superoxide dismutase (EC 1.15.1.1) is written as:

$$\mathbf{2}\ O_2^{\cdot -} + \mathbf{2}\ H^+ = O_2 + H_2O_2$$

**(e) The Enzyme List does not provide information on the mechanism of a reaction**

Only the overall reaction catalysed is considered, although some information on the mechanism may be provided in the comments. For example, fructose-bisphosphate aldolase (EC 4.1.2.13), which catalyses the reaction:

D-fructose 1,6-bisphosphate = glycerone phosphate + D-glyceraldehyde 3-phosphate,

contains enzymes that operate by very different chemical mechanisms. This is indicated in the Comments' section, where it is stated that "The yeast and bacterial enzymes are zinc proteins. The enzymes increase electron-attraction by the carbonyl group, some (Class I) forming a protonated imine with it, others (Class II), mainly of microbial origin, polarizing it with a metal ion, e.g. zinc".

**(f) Since the Enzyme List is based on the overall reaction catalysed, immediate reaction products that rapidly and spontaneously convert to a more stable form may not be indicated**

Thus the pyruvate kinase (EC 2.7.1.40) reaction is written as:

ATP + pyruvate = ADP + phospho*enol*pyruvate.

However, the product of this reaction, which operates in the direction of ADP phosphorylation in mammalian cells, is enolpyruvate which immediately isomerizes to the *keto* form.

On the other hand, if the product isomerizes rather slowly, such that the immediate product can be detected without recourse to rapid-reaction techniques, it is the immediate product that is shown, as in the case of the reaction catalysed by maltose phosphorylase (EC 2.4.1.8)

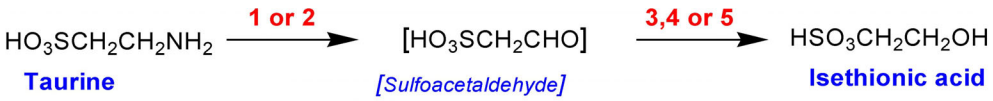
maltose + phosphate = D-glucose + β-D-glucose 1-phosphate.

**(g) The Enzyme List does not provide any information on any nonenzymic functions**

An increasing number of enzymes are recognized to have additional nonenzymic functions (see [11] for discussion). Such functions are not included in the Enzyme List.

## ATTEMPTS TO PREDICT MISSING ENZYMES

While it is tempting to predict the existence of an enzyme based on a 'gap' in a metabolic pathway, where it could be argued that an enzyme must exist to convert the product of one reaction into the substrate of another, this can lead to incorrect assumptions. For example, in the case of taurine metabolism, it would be reasonable to presume that the reaction from the substrate taurine to the product isethionic acid should proceed as follows:




with 1 being an amine oxidase, 2 an aminotransferase, 3 an aldehyde oxidase, 4 an aldehyde dehydrogenase and 5 an alcohol dehydrogenase. Indeed, such pathways do occur in some bacteria and fungi. However, this does not appear to be what happens in mammalian systems, where taurine is first converted spontaneously to taurine chloramine in the presence of hypochlorous acid and it is taurine chloramine and not taurine that takes part in the enzyme-catalysed reaction, shown in Fig. 1 [12].

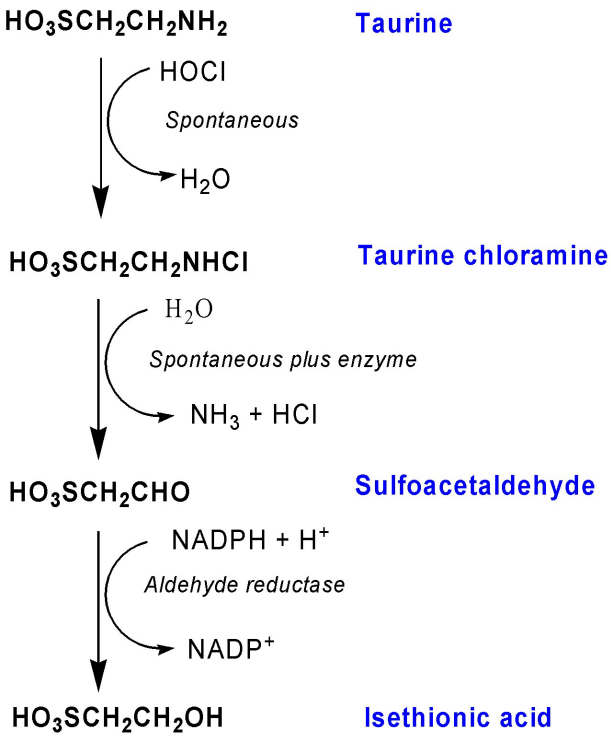



**Figure 1.** Breakdown of taurine in rat. Taurine reacts non-enzymically with hypochlorous acid (HOCl) to form *N*-chlorotaurine (taurine chloramine) and this is then converted to sulfoacetaldehyde and isethionic acid (see [12] for further details).

## NEW ENZYMES

The purpose of the Enzyme List is, as far as possible, to provide unambiguous data on enzymes and the reactions they catalyse. Such data are then used by a variety of other databases (see Table 5 for examples). The criteria for the addition of a new enzyme are simple but strict. The proposed new enzyme must be shown actually to catalyse a reaction that is significantly different from those catalysed by enzymes already listed. Forms for submitting new enzymes or corrections/updates to existing entries are available on-line [1, 13].

**Table 5.** Some databases that use the EC classification system

| Database | URL |
|---|---|
| BRENDA | http://www.brenda.uni-koeln.de/ |
| CarBank | http://bssv01.lancs.ac.uk/gig/pages/gag/carbbank.htm |
| Database Enzyme (UK HGMP Resource Centre) | http://www.hgmp.mrc.ac.uk/Bioinformatics/Databases/enzyme-help.html |
| Directoryof p450-containing systems | http://www.icgeb.trieste.it/~p450srv/ |
| EcoCyc | http://ecocyc.org |
| EMP Database of enzymes and metabolic pathways | http://wit.mcs.anl.gov/WIT2/EMP/ |
| Enzyme information and structure database | http://restools.sdsc.edu/biotools/biotools12.html |
| Enzyme Nomenclature | http://www.chem.qmul.ac.uk/iubmb/enzyme/ |
| Enzyme Structures Database | http://www.biochem.ucl.ac.uk/bsm/enzymes/ |
| ExPasy | http://ca.expasy.org/enzyme/ |
| GTD (thermodynamics of enzyme catalysed reactions) | http://wwwbiotech.nist.gov:8030/enzyme/ |
| HUGO | http://www.gene.ucl.ac.uk/nomenclature/ |
| KEGG (Kyoto Database of Genes and Genomes) | http://www.genome.ad.jp/kegg/ |
| Klotho | http://www.biocheminfo.org/klotho/ |
| LIGAND | http://www.genome.ad.jp/dbget/ligand.html |
| MaizeDB | http://www.maizegdb.org/ |
| MEROPS | http://merops.sanger.ac.uk/ |
| PDB | http://www.rcsb.org/pdb/ |
| Phosphoprotein database | http://www.lecb.ncifcrf.gov/phosphoDB/ |
| PROMISE | http://metallo.scripps.edu/PROMISE/ |
| REBASE | http://rebase.neb.com/rebase/rebase.html |
| UMBBD (Biocatalysis/Biodegradation) | http://umbbd.ahc.umn.edu/ |
| WIT | wit.mcs.anl.gov/WIT |
| Worthington Enzyme Manual | http://www.worthington-biochem.com/index/manual.html |

## APPLLICATION OF THERMODYNAMIC DATA

Because the Enzyme List is restricted to providing factual data, it is the function of other databases to provide activity and thermodynamic data that may be used for simulating metabolic processes, reconstructing systems, determining control properties etc. Thermodynamic data for many enzymes can be found in the GTD Thermodynamics of Enzyme-catalysed Reactions database (see Table 5) and kinetic data are included in the BRENDA and WIT databases (see Table 5). However, for these to be meaningful, it is necessary that such data refer to 'physiologically relevant conditions' (see [9]).

Attempts to use thermodynamic data for isolated enzyme-catalysed reactions to predict the direction of flux *in vivo* should be treated with extreme caution, since enzymes usually form parts of metabolic systems. As a simple example, the equilibrium constants for the reaction catalysed by alcohol dehydrogenase are shown in Table 6. From the value at neutral pH, it is quite clear that we should not be very good at metabolizing ethanol. However, we have no great problem with this in the tissues because the acetaldehyde produced is rapidly converted to acetate by aldehyde dehydrogenase ($NAD^+$)(EC1.2.1.3), which has a very low $K_m$ value for that substrate and catalyses a reaction that is essentially irreversible.

**Table 6.** Equilibrium constants for the alcohol dehydrogenase (EC 1.1.1.1) reaction

$$\text{ethanol} + NAD^+ = \text{acetaldehyde} + NADH + H^+$$

$$K_{eq} = ([\text{ethanol}]\,[NAD^+])/([\text{acetaldehyde}]\,[NADH]\,[H^+])$$

| pH | ($K_{eq}$) |
|---|---|
| 7.0 | $1.1 \times 10^{-4}$ |
| 8.0 | $7.1 \times 10^{-4}$ |
| 9.0 | $1.05 \times 10^{-2}$ |
| 10.0 | $9.0 \times 10^{-2}$ |

Temperature 293,15°K

Source GTD - see Table 5

## ASSAY CONDITIONS

Comparison of enzyme activities and kinetic parameters and the reconstruction of metabolic systems require data to be obtained under comparable conditions. However, even a brief survey of the literature will indicate that this is far from the case. Even with what is apparently the same enzyme, different laboratories often assay under different conditions and the assay conditions used for different enzymes in the same metabolic pathway can differ markedly. Some recommendations have been formulated (see [14]), but these are somewhat imprecise.

This section will not deal with the assay procedures, importance of determining initial rates or the essential controls for progress-curve analysis, since these have been dealt with in detail elsewhere [15, 16]. Despite this, there have been attempts to define standard assay conditions to facilitate comparison of data.

**a) Temperature**

Originally many studies were conducted at 'room temperature', which could, of course, vary widely between laboratories. It had been recommended that enzymes should be assayed at 25°C, which was regarded as a standard 'room temperature'. However, not all laboratories were able to meet this requirement and the standard assay temperature was raised to 30°C. Even this gradual thermal inflation does not satisfy those studying human enzymes, who would regard a temperature of 37°C as being more appropriate for most tissues. However, this definition of physiological temperature for a mammalian system would not be appropriate, for example, for a thermophilic bacterium or a poikilotherm.

Perhaps the only logical way of dealing with this issue would be to specify recommended assay temperatures for specific organisms, or groups of organisms, remembering that to stick to a single temperature for the study of organisms that might be subjected to quite large temperature fluctuations could lead to the loss of important information.

**b) pH value**

The recommended pH value for enzyme assays is also not very helpful. Although it has been suggested that the assay pH should "where practicable, be optimal", this is not a great deal of help. Optimal pH is to some extent part of a circular argument since this may depend on the choice of substrate, the substrate concentrations, buffer, temperature and ionic strength and there are no strict recommendations for any of these. Furthermore, the optimum pH may be far removed from the pH at which an enzyme is perceived to operate *in vivo*. For example, the optimum pH for arginase (EC 3.5.3.1) is reported to be about pH 10 in horse, pH 9.8 in rat and pH 11 in *Bacillus brevis*. Those working with mammalian systems might favour an assay pH of about 7.2, which is believed to be around the physiological pH within the cell, but clearly this would be unphysiological for gastrointestinal enzymes, such as pepsin and trypsin, or for lysosomal enzymes. Thus it would not be helpful to recommend a standard pH value that would be appropriate for all systems. An alternative might be to devise individual standards for each organism, organ and organelle to be studied.

A further complication that must be borne in mind is that it is sometimes not easy to assay an enzyme at the desirable pH. For example, as discussed above, the equilibrium constant for the reaction catalysed by alcohol dehydrogenase is so far towards acetaldehyde at neutral pH values that it is difficult to assay the enzyme in the direction of ethanol oxidation.

Because of this, many studies of that reaction have utilized a high assay pH value and also, sometimes, included an aldehyde-trapping reagent, such as semicarbazide.

**c) Substrates and substrate concentrations**

Naturally it would be appropriate to use physiological substrates for enzyme assays and, in the case of enzymes that use more than one substrate that compete with one-another for the enzyme, it would be necessary to study each in turn. In reality, however, many studies have used non-physiological substrates for ease of manipulation and assay. For example, acetylthiocholine is frequently used to assay acetylcholinesterase (EC 3.1.1.7) because the thiocholine produced can be readily detected by reaction with sulfhydryl reagent 5,5'-dithiobis-2-nitrobenzoate ($Nbs_2$), releasing a yellow-coloured compound the formation of which can be followed at 412 nm [17]. Other examples include the use of pyroglutamyl-histidyl-prolylamido-4-methyl coumarin instead of the physiological substrate thyroid-stimulating hormone (pyroglutamyl-histidyl-prolylamide) to assay pyroglutamyl-peptidase II (EC 3.4.19.6) [18], the use of esters rather than peptides to assay a number of peptidases, the use of dyes as electron acceptors in oxidoreductase assays and the use of *p*-nitrophenyl phosphate to assay alkaline phosphatase (EC 3.1.3.1). The demand for higher assay sensitivity and high-throughput procedures has resulted in the development of an increasing number of chromogenic and fluorogenic substrates and, in some cases, it is difficult to find the structures of these compounds. Clearly, in such cases, a considerable amount of work would be necessary to show that the enzyme behaves identically towards such substrates as it does towards its physiological substrates.

Unless the $K_m$ and $V_{max}$ values are to be determined, the substrate concentration used in an assay will, of course, affect the activity values obtained. It is often recommended that saturating substrate concentrations should be used (i.e. > 10 $K_m$,) for all substrates. This, of course, assumes that the $K_m$ value has already been determined. Furthermore, this might not always be practicable because of factors such as solubility, the occurrence of high-substrate inhibition or a high absorbence of the assay mixture affecting the behaviour of optical assays (see [15, 16]). Furthermore, it should be remembered that any change in the assay conditions (e.g., pH, temperature, ionic strength) may affect the $K_m$ values.

Although the above considerations indicate the desirability of performing a thorough kinetic study to determine the $K_m$ and $V_{max}$ values for all physiological substrates, there are many situations where simple activity level comparisons are sufficient, as, for example, in comparing the activities of plasma enzymes in the diagnosis of different pathological conditions. In such cases, one may be able to use non-physiological substrates and assay conditions, provided these are fully specified to allow the results to be replicated by others.

**d) Buffers and ionic strength**

The buffers and ionic strengths used in enzyme assays vary widely and are often far from physiological. It might be helpful if it were possible to recommend a simple standard buffer for use in enzyme assays. Unfortunately, this goal appears to be unobtainable, because at least some enzymes are unhappy in one or other of the common buffers. Furthermore, buffers that contain physiologically occurring compounds can be problematical in that they are likely to be substrates for some enzymes. For example, inorganic phosphate is a substrate for several enzymes and citrate is a substrate for enzymes such as aconitate hydratase (EC 4.2.1.3). Assay of such enzymes in a buffer based on these anions would of course preclude studies at varying substrate concentrations.

Phosphate buffer is widely used for enzyme studies but it can act as a product inhibitor for some enzymes that release phosphate. It is also, for example, an inhibitor of arylsulfatase (EC 3.1.6.1) (see [14]). Tris buffer (commonly known as Tris-HCl) is hardly physiological and also interferes with the assay of aldehyde dehydrogenase, and glutamate dehydrogenase (EC 1.4.1.2) from some sources is unstable in this buffer (see [19]). Pyrophosphate buffer can inhibit some enzymes because it is a metal-ion chelator and is also a product inhibitor of several other enzymes. It is, however, the buffer in which some isoenzymes of aldehyde dehydrogenase are most active and has commonly been used in assays for those enzymes (see e.g., [20]). Citrate is a chelator and therefore inhibits several enzymes that require metal ions for activity. The range of so-called Good buffers fare no better, for example, some of these inhibit the amine oxidases (EC 1.4.3.4 & EC 1.4.3.6) and HEPES and Tris inhibit carbamoyl-phosphate synthase (ammonia) (EC 6.3.4.16) [21].

From the above examples, it should be clear that it is unlikely that a universal buffer medium, suitable for all enzymes, will easily be found.

Perhaps the answer will lie in more complex mixtures, including proteins, as buffers, that more closely mimic the *in vivo* environments of groups of enzymes. At present, it appears that specifying the buffer and its components might be the only alternative. Even then, present usage is often too imprecise. It is common to read statements such as 0.1 M phosphate buffer pH 7.2, with no information as to its precise composition or whether sodium or potassium phosphate was used.

The ionic strength of assay media is seldom stated, although this can be calculated if the full composition of the assay mixture is given, which is not always the case. Several enzymes are sensitive to inhibition by high ionic strengths and altering the concentrations of charged substrates and the pH of the buffer may also affect the ionic strength. It would be helpful if all authors were required to state the ionic strength of their assay mixtures.

**e) Other additives**

Assay mixtures often contain additional components to stabilize, protect or activate the enzyme. Each of these has to be assay-specific, since compounds that facilitate some enzyme assays may act as substrates for some other enzymes or inhibit them.

Some enzymes that contain reactive sulfhydryl groups that are essential for activity (e.g. papain (EC 3.4.22.2), aldehyde dehydrogenase and glycoprotein *N*-palmitoyltransferase (EC 2.3.1.96) are assayed in the presence of reducing agents, such as cysteine, glutathione, ethanethiol (2-mercaptoethanol) or dithiothreitol (DTT). These compounds inhibit some other enzymes. Since glutathione and cysteine are physiological compounds, they are substrates for several other enzymes. Dithiothreitol is also a substrate for enzymes, the vitamin-K-epoxide reductases (EC 1.1.4.1 & EC 1.1.4.2) and can replace reduced thioredoxin and glutathione in the reactions catalysed by methionine-*S*-oxide reductase (EC 1.8.4.5) and adenylyl-sulfate reductase (glutathione) (EC 1.8.4.9), respectively. Ethanethiol can act as a substrate for thioether *S*-methyltransferase (EC 2.1.1.96) [22]. Such compounds may also interfere with some enzyme-assay procedures, for example, assays based on sulfhydryl-group detection, such as that for acetylcholinesterase, referred to above.

In some cases, a reducing agent is added to protect the substrate from oxidation, such as the addition of ascorbate, which is a substrate for some other enzymes, to solutions of adrenaline for monoamine oxidase (EC 1.4.3.4) assays.

Clearly the alternative of preventing non-enzymic adrenaline oxidation by working anaerobically is not possible for that oxygen-requiring enzyme.

The addition of a chelating agent such as EDTA is common for the assay of some enzymes that are sensitive to inhibition by traces of heavy-metal ions, such as papain and fructose bisphosphatase (EC 3.1.3.11). Chelating agents are also sometimes used to buffer the free concentrations of divalent cations in solution. However, they can also inhibit a number of metal-ion-requiring enzymes, such as aryldialkylphosphatase (EC 3.1.8.1), diisopropyl-fluorophosphatase (EC 3.1.8.2) and some metallopeptidases (e.g. EC 3.4.11.6, EC 3.4.17.10, EC 3.4.24.29).

Many enzymes require the addition of a metal cation for activity, either because the substrate for the enzyme is a metal chelate, as is, for example, the case with many reactions involving ATP, or because a metal ion is an essential activator of the enzyme. In some cases both of these factors may operate as, for example, in the case of pyruvate carboxylase (EC 6.4.1.1), where the reaction involves the binding of Mg-ATP to an enzyme-Mg complex [23]. In many cases, the metal ion may be tightly bound to the enzymes, but in other cases it dissociates readily. For example, it is necessary to add $Fe^{2+}$ to cytoplasmic aconitate hydrolase (EC 4.2.1.3), to replace that lost in extraction and purification, in order to detect activity [24].

The specificity for metal ions can be high, for example, $Mg^{2+}$ is required for adenylate cyclase (EC 4.6.1.1) activity but the enzyme is inhibited by $Ca^{2+}$ [25]. Useful listings of metal and buffer ions as inhibitors of specific enzymes have been compiled by Zollner [26]. In addition, several enzymes where the true substrate is the metal-substrate complex are inhibited by high concentrations of uncomplexed metal ion and/or substrate.

Other factors are necessary for specific enzymes. The activator *N*-acetyl-L-glutamate is included in assays for carbamoyl-phosphate synthase (ammonia) (see [21]), pyruvate carboxylase has very little activity in the absence of acetyl-CoA (see [23]), ADP is frequently added, as an activator, for the assay of glutamate dehydrogenase [$NAD(P)^+$] (EC 1.4.1.3) (see [10]) and one form of glutaminase (EC 3.5.1.2) has little activity in the absence of phosphate (see e.g., [27]). All such compounds are inhibitors and/or substrates of other enzymes.

**f) The enzyme**

The question of whether studies of isolated enzymes can provide data that are relevant to cellular metabolism is almost as old as enzymology. It is often posed and just as often ignored because, despite many wishful claims to the contrary, the available technology does not offer a viable alternative. Sometimes even the simplest steps to ensure that the enzyme preparation is adequate are not taken. It is common to find that proteolysed preparations are used, either by design or accident, with the assumption that if the enzyme preparation has activity, it must be satisfactory. However, there is a considerable amount of evidence that this may not be a valid assumption. Removal of 4 or 5 amino-acid residues from the N-terminus of glutamate dehydrogenase, which can readily occur during extraction and purification, has been shown to affect its regulatory properties [28]. Similarly, proteolytic cleavage of fructose bisphosphatase affects its pH optimum and allosteric regulation (see [29]). Despite such 'cautionary tales' an increasing number of studies have been conducted with preparations that are truncated, contain tags such as poly-His, lack glycosylation or are suspended in some odd detergent. The relevance of such studies is not clear.

Since the enzyme is a catalyst and usually present at concentrations very much lower than those of the substrates, one would expect the initial velocity of the reaction to be proportional to the enzyme concentration and this is true in the majority of cases. Situations in which this proportionality does not apply, which may be a result of assay artefacts, impurities in the enzyme preparation or the assay mixture, or as a result of dissociating systems that may be of physiological significance, have been discussed in detail elsewhere [15,16]. However, they indicate that it is essential that such proportionality is checked experimentally and that the causes of any departures are investigated. The often-quoted assertion that Michaelis-Menten kinetics do not apply if the concentration of the substrate is much less than that of the enzyme, which arises from the failure to distinguish between total substrate concentration (used in test-tube experiments) and free substrate concentration, which is the only valid parameter within the cell (see [22]), can easily be shown to be misleading. If the latter is maintained constant, owing to steady-state or equilibrium conditions in a metabolic system, there is no departure from the Michaelis-Menten equation when [S] << [Enzyme].

## CONCLUSIONS

The necessity of fully describing the assay mixtures used should not need stressing. Neither should the necessity for more care in ensuring that the enzyme preparation used corresponds to that existing *in vivo*. Certainly, temperature and pH might be more standardized, where appropriate. However, the above discussion indicates that it would be counter-productive to attempt to develop a universal assay mixture for the assay of all enzymes, since not all enzymes share the same environments. Even if satisfactory buffer mixtures were developed for the study of groups of enzymes in discrete systems, the necessity to have other additives in some assays that may be inimical to others will prevent the development of universal assay cocktails.

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