Corrective Metabolism: An Appreciation

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Abstract: In intermediary metabolism, enzymes are mainly for the specific provision of one small molecule from another, B from A, for its further use. But there are also decades of work relating to another evolved purpose, which is to prevent untoward effects of odd compounds of various origins, and of ordinary intermediates, whose improper concentrations may be avoided by hydrolases and other activities of metabolic correction.

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Introduction: Many individual small molecules, M, in cells – the metabolites – have specific enzymatic formation and use, as shown in the usual metabolic map by the shorthand of two black arrows, Fig. 1A. For certain small molecules, M', though, the representation might use colored arrows for a special origin (blue), possible toxicity (red) and repair (green), Fig. 1B. There, blue may stand for a spontaneous or an enzymatic reaction, red for inhibition or chemical change of target(s), and green for enzymatic corrections or removal of the toxic compounds by their conversion to normal or innocuous ones.

Four long-studied examples of metabolism in the mode of Fig. 1B concern non-canonical compounds with a pyrophosphate linkage (Bessman et al., 1996); reactive oxygen species (Imlay, 2013); methylglyoxal (Sousa Silva et al., 2013, briefly considered below); and 2-phosphoglycolate in plants (Bauwe et al., 2010) – whose phosphatase is a major player in the discussion which follows. The subject of metabolite damage and repair is a broad one with catchy words like proofreading, housecleaning, damage control, metabolic frustration, safety valves, orphan, messy, fuzzy, shadow, sloppy, underground and so on (Bessman et al. 1996; Galperin et al. 2006; Linster at al. 2013; de Lorenzo et al. 2015). Recent work is exemplified by a wonderful paper by Collard et al. (2016), which stimulated this piece.

"Phosphoglycolate phosphatase"

For purposes of presentation, the paper of Collard et al. (2016) might be said to implicitly address the origin of an unusual compound occasionally revealed in clinical samples, the 4-carbon sugar acid, erythronate. In the route shown in Fig. 2, left hand side, a three-step sequence starts with a familiar intermediate of the pentose-P pathway, erythrose-4-P, and the first two reactions are examples of minor activities of major high activity enzymes, in this case two successive reactions of the glycolytic pathway. The first of those reactions, inferred from Ishii et al. (1964), would be glyceraldehyde-3-P dehydrogenase (Gap) acting on erythrose-4-P instead of glyceraldehyde-3-P, to give erythronate-1,4-P_2 (and reduced NAD). The second one would be its further conversion, by the next enzyme of the usual pathway, phosphoglycerate kinase (Pgk), but now acting on the erythronate-1,4-P_2 to give erythronate-4-P (and ATP). In the paper, both reactions are demonstrated *in vitro*, and in Fig. 2 they are given blue arrows to denote minor formation of a non-traditional product. (For clarity, Figs. 2 and 3 use names of compounds; some structures are collected in Fig. 4.)

Since erythronate-4-P is an *in vitro* inhibitor of gluconate-6-P dehydrogenase in the pentose-P pathway (Pastii et al., 2003), Collard et al. (2016) speculated that the third reaction might be a phosphatase for its removal to avoid such an effect. So in Fig. 2 its putative phosphatase activity is given a green arrow.

It was a stretch that the proposed activity might be the same one discovered in plants, where 2-phosphoglycolate results from misincorporation of O_2 instead of CO_2 by ribulose bisphosphate carboxylase (rubisco), the main enzyme of CO_2 assimilation, and which is handled by photorespiration, a multistep process starting with dephosphorylation of the 2-phosphoglycolate by a phosphoglycolate phosphatase (Bauwe et al., 2010). Other than plants, P-glycolate is best known as an intermediate from DNA damage (Stubbe and Kozarich, 1987). But the most detailed earlier studies of its phosphatase (e.g., Rose et al., 1986), were related to an *in vitro* effect of 2-P-glycolate on the synthesis of the hemoglobin ligand, glycerate-2,3-P_2, in mammalian red cells, which have neither photosynthesis nor DNA.

Like the plant enzyme, the red cell phosphoglycolate phosphatase was known to be almost inactive on familiar glycolytic intermediates with the same substituents, like glycerate-3-P and phosphoenolpyruvate. However, Rose et al. (1986) found that the red cell enzyme acted also on L-lactate-2-P. The latter compound had

been reported to be made *in vitro* by the glycolytic enzyme pyruvate kinase, acting on L-lactate plus ATP in reverse of its normal direction (Ash et al., 1984), and to inhibit that same enzyme (Nowak and Mildwan, 1970). And Rose et al. (1986) speculated that if L-lactate-2-P were made *in vivo* by pyruvate kinase from blood L-lactate, the phosphatase might be an enzyme for its removal. Accordingly, in Fig. 2, formation of L-lactate-2-P is shown as a blue reaction of pyruvate kinase, and its disposal by phosphoglycolate phosphatase, a green one.

Collard et al. (2016) indeed report that purifications of phosphatases from human red cells, as done either according to activity on erythronate-4-P or on 2-P-glycolate, appear to give the same enzyme which, cloned from mouse DNA and overexpressed, has high and similar activity on those two compounds, as well as on L-lactate-2-P, but almost none with common phosphorylated intermediates.

And knockout of the phosphatase in a mammalian cell line causes accumulation of both erythronate-4-P and lactate-2-P, as well as a high level of gluconate-6-P in line with the *in vitro* inhibition of its dehydrogenase by erythronate-4-P. 2-P-glycolate itself was not detected in the cell line and circumstances. (Ironically, in view of attention to the phosphatase in red cells, the presence of 2-P-glycolate in those cells has been uncertain, Knight et al., 2011.)

The cell line mutant was also partially impaired in glucose use and product formation, effects which, in turn, might fit with a known strong *in vitro* inhibition of the glycolytic enzyme phosphoglucose isomerase by gluconate-6-phosphate (Noltmann, 1972, Fig. 2). However, high activity reversible reactions like phosphoglucose isomerase are uncommon regulatory targets, and assessment of glycolytic metabolites pointed instead to a possible impairment *in vivo* of the next reaction of the glycolytic pathway, phosphofructokinase, Pfk-1.

The latter enzyme has several effectors, and the authors focus on an activator in eukaryotic cells, fructose-2,6-P_2 (Hers and van Schaftingen, 1982), whose synthesis from fructose-6-P uses the enzyme Pfk-2, a reaction which is inhibited *in vitro* by phosphoenolpyruvate. Speculating that the L-lactate-2-P accumulating in the phosphatase mutant might act at Pfk-2 (Fig. 2) like a non-disposable phosphoenolpyruvate analog, the authors find that fructose-2,6-P_2 is decreased in the mutant. They also show L-lactate-2-P inhibition *in vitro* for the Pfk-2 isozymes of the particular cell line.

Together, the results speak to normal formations of erythrose-4-P and L-lactate-2-P in higher cells, to a role of the phosphatase in affecting their levels, and to possible inhibitory targets should they not be removed. Their effects are not suggested as necessarily being regulatory in the usual sense, distinguishing them from traditional ligands like fructose-2,6-P_2, whose synthesis and degradations employ dedicated and controlled activities.

The paper also deals with yeast. Phosphoglycolate phosphatases belong to the HAD (haloacid dehalogenases) family of proteins, many of which have unknown specific function (Kuznetsova et al., 2006, 2015). They include yeast Pho13, which was first recognized as a phosphatase for the generic substrate *p*-nitrophenyl-phosphate, and where – hinting a role in metabolism - a *pho13* mutant had been obtained in a screen for improved use of D-xylose (Ni et al., 2007). Collard et al. (2016) show purified Pho13 to be a phosphatase with similar specificity to the mammalian phosphoglycolate phosphatase. And they find that *pho13* knockout in the microbe causes *in vivo* increases in both 2-P-glycolate and erythronate-4-P (as well as gluconate-6-P), again implying their normal formation and destruction. Lactate-2-P was not detected in the yeast mutant, a result fitting with yeast not making lactate as a fermentation product.

It may be interesting to also address bacteria. In *E. coli*, for example, of 21 encoded HAD proteins with small molecule phosphatase activity, only one acted on 2-P-glycolate (Kuznetsova et al., 2006; erythronate-4-P not yet tested). That enzyme had already been assigned as a specific 2-P-glycolate phosphatase, gene *gph*, in a DNA repair pathway, and providing glycolate for further metabolism (Pellicer at al., 2003).

However, unlike higher cells or yeast, in *E coli* and certain other bacteria erythronate-4-P is an intermediate in a normal pathway, the one to cofactor pyridoxal-5-P. There, biochemistry appears to show that the erythronate-4-P is made by a single step non-phosphorylating erythrose-4-P dehydrogenase (Epd in Fig. 2) with very low activity on glyceraldehyde-3-P (Boschi-Muller et al., 1997). Epd was first recognized by sequence and named GapB to distinguish it from the glycolytic enzyme GapA; gapB mutants, however, did not require pyridoxine on glucose, and tests for a possible role for GapA in synthesis of the cofector have been contradictory (see Yang et al., 1998). To further muddy the waters, the

phosphoglycolate phosphatase Gph of *E coli* is reported to interact with GapA (Ferreira et al., 2013).

Perhaps L-lactate-2-P formation and fate in bacteria also merit testing: L-lactate is made by many bacteria (but not *E. coli*) and is abundant, and pyruvate kinase is another near universal enzyme. And although bacteria do not have a Pfk-2 to be inhibited, phosphoenolpyruvate is a prototypical inhibitor of the bacterial glycolytic enzyme Pfk-1 (Blangy et al., 1965).

Other examples in perturbed and ordinary metabolism.

Fig. 1B implies that abnormal metabolite M' differs from the normal one M, and that it is M' which may be both toxic and corrected. Although that scheme fits examples above, elsewhere such a distinction between normal and abnormal may too narrow, because, as suggested (Bessman et al., 1996), sanitizing activities may prevent the unbalanced accumulation of a normal metabolite and, as emphasized in fascinating reviews (Lerma-Ortiz et al., 2013; de Lorenzo, et al., 2015), toxicity may be a consequence of the spontaneous chemical reactivity of many normal metabolites or intermediates in enzyme catalysis. Indeed, in some situations it may be that the ordinary metabolite itself, in inappropriately high amount but without special reactivity, which interferes with other processes. Hence Fig. 1C might be preferred, with (M) standing for either the normal compound or closely related derivatives of whatever origin.

Impairments nominally related to excess of normal metabolites include the traditional findings of geneticist-induced stress in microbes, where the appropriate block in a catabolic pathway may cause the substrate to be inhibitory to growth and, in certain cases, even lethal (Fukasawa and Nikaido, 1959), or where mere constitutivity may confer substrate toxicity (Horuichi et al., 1962). And, perhaps relating to correction, are cases of the destruction of piled up intermediates in a perturbed pathway (e.g., the excretion of dephosphorylated intermediates in histidine mutants, Ames and Mitchell, 1955).

Even in metabolic perturbations, though, details of corrective processes have sometimes been elusive. Thus, decades of investigations of glucose uptake in *E. coli*, employing various mutants or analogs, and with accumulation of the phosphorylated substrates, toxicities, and removals, specific implication of a likely corrective activity, YigL, is relatively recent (Papenfurt et al., 2013) – Yig being an

HAD phosphatase for certain hexose-P's (Kuznetsova et al., 2006). A recent example of metabolic perturbation engaging hydrolytic correction comes in a revisiting of controls in *E. coli* pyrimidine biosynthesis (Reaves et al., 2013), where exogenous addition of an intermediate, orotate, to bypass the normal inhibition at an earlier step (Fig. 3A), has revealed an overflow phenomenon of uridine-5-P accumulation and the appearance of free uracil, which is not a normal intermediate. There, sequence analysis, biochemistry and genetics implicate two nucleotide phosphatases.

The following brief examples, though, are for corrections in ordinary metabolism unperturbed by known chemical or genetic stress. Frelin et al. (2015) deal with riboflavin biosynthesis where, Fig. 3B, intermediates I and 2 are known as highly reactive because of their particular glycosidic linkage, which differs from that in ordinary nucleotides like the GTP which starts the pathway. They show that a previously unidentified protein domain, variously located in different organisms, is a hydrolase for the special linkage, and suggest that it preempts toxicity of the intermediates in a biosynthetic pathway which, unlike many others, lacks control of flux by end product inhibition. In this example, deletion or overexpression of the hydrolase are without clear effects on growth, and changes in substrate level are small.

Another example in biosynthesis concerns transaminations, a place in metabolism where ordinary enzyme specificity is broad with, often, either glutamate or aspartate being effective donors, the 2-ketoproducts (2-ketoglutarate and oxaloacetate, respectively) being recycled by familiar reactions. For the usually weaker donor in common transaminations, glutamine, its 2-ketoglutaramate product, Fig. 3C, is recycled by the Nit2 reaction, its ω-position deamidation restoring 2-ketoglutarate (Cooper et al., 2016).

Unlike glutamine, the tripeptide glutathione is not readily demonstrable even as an *in vitro* donor in transaminations. However, Peracchi et al. (2017) have shown that a homolog of Nit2, called Nit1, is a specific amidase acting on 2-ketoglutathione, thus also restoring 2-ketoglutarate (Fig. 3C) – as if glutathione might, to some degree, be a transamination donor *in vivo*. 2-Ketoglutathione accumulates in Nit1 mutants and, as known also for 2-ketoglutaramate, it can cyclize, with potential loss to reuse, or even toxicity. Hence the Nit enzymes are speculated as being corrective, and the formation of 2-ketoglutathione is offered

as an example of the promiscuity of the normal transaminases occasionally using the exceptionally abundant metabolite, glutathione.

Of course, if many compounds are potentially toxic in excess, ordinary black arrow metabolism could be thought of as being corrective by its nature and special designation inappropriate (or, as put in an unattributed quip, the purpose of enzymes [anyway] is to reduce the concentrations of their substrates). Two examples, again from intermediary carbohydrate metabolism, illustrate the ambiguities, and the limitations of color coding. The first one concerns the product of the glucose-6-P dehydrogenase reaction in the pentose-P pathway, δ-gluconolactone-6-P (Fig. 2 and Fig. 3D), whose spontaneous hydrolysis, of the lactone ring, might provide the next intermediate, gluconate-6-P, but where there is an enzyme anyway (Pgl in *E coli* or, in some organisms, a domain of, e.g., glucose-6-P dehydrogenase, Stover et al., 2011).

There is much evidence for toxic reactivities of δ-gluconolactone-6-P (Adhya and Schwartz. 1971; Thompson et al., 1997; Miclett et al., 2001; Aon et al., 2008), and Galperin et al. (2006) suggested that although spontaneous hydrolysis might be adequate, the Pgl enzyme (lessening substrate concentration and ill effects) might a case where a normal enzyme deserved special assignment as "housecleaning, " i.e., corrective. Accordingly, Fig. 3B has the Pgl reaction green (and there, if only to indicate its unusual nature, the spontaneous reaction is shown blue).

Complicating this example is that the actual contribution of spontaneous hydrolysis of δ-gluconolactone-6-P to gluconate-6-P formation *in vivo* is unknown. For in an *E. coli pgl* mutant there was also formation (and then use) of gluconate, apparently by dephosphorylation of δ-gluconolactone-6-P followed by hydrolysis of the free lactone (Kupor and Fraenkel, 1970), as indicated by the second green arrow at δ-gluconolactone-6-P in Fig. 3D. It is interesting in the present context that in *pgl* mutants of *L monocytogenes*, which also make gluconate from glucose, the most overexpressed gene is for an HAD protein, which the authors thought might a phosphatase for δ-gluconolactone-6-P (Crimmins et al., 2009).

A last example is to revisit methylglyoxal. Glyoxylase, discovered a century ago, converts methylglyoxal (+ H_20) to lactate. Glucose metabolism often gives lactate, and into the 1930's methylglyoxal was considered a possible normal glycolytic intermediate (Fig. 3E(i)). Very comsiderable work, however (see Sousa Silva et al,

2013, and Rabbani and Thornalley, 2014), would place it as an iconic accidental metabolite in the mode of Fig. 1B and shown that way in Fig. 3E(ii) where, with the trunk glycolytic route indicated by the two black arrows, it is formed from the triose-phosphates as an inevitable low level side product (the blue arrow); is toxic by advanced glycation reactions (the red arrow); and glyoxylase – which turned out to make D-lactate rather than the more common L-lactate of higher cells - is an enzyme of its removal (the green arrow).

Yet in bacteria, which have both glyoxylase-dependent and other routes for methylglyoxal (see, e.g, for *E. coli,* Ozyamak et al*.,* 2010), and where a variety of stresses – chemical, genetic, metabolic – which, likely through increased triose-P, cause methylglyoxal accumulation and even killing (Entner and Doudoroff, 1952; Cooper, 1984; Weber et al. 2005), methylglyoxal formation seems to use a dedicated enzyme, MgsA acting on dihydroxyacetone-P (Fig. 3E(iii)). And, supporting a role for this enzyme in methylglyoxal formation and toxicity, is that methylglyoxal was not detected in an MgsA mutant of *E. coli* and nor was there killing by a metabolic stress (Tötenmeyer et al., 1998).

Normal MgsA function has been variously put to phosphate homeostasis, to the relieving of other consequences of excess (and hence, in Fig. 3E(iii), red arrows at both triose-P and methylglyoxal), or the provision of a route to D-lactate for reuse. Roles in unstressed metabolism have not been emphasized, nor even mutant studies. In any case, the last sentence in the review by the discoverer of MgsA (Cooper, 1984). “that it should not take another 50 years to determine the proper place of methylglyoxal in cell metabolism,” may have been optimistic.

Also to note (i) That physiological details of correction reactions are not yet widely explored, and, implicit in some of the examples, it may be – as elsewhere in metabolism – that certain of them are constitutive activities primarily governed by concentration of their substrates.

(ii) How, for corrective reactions, the findings of previously unrecognized small molecules in null mutants – an example of the sensitivity of metabolites to what may appear to be silent changes (Raamsdonk et al., 2001) - have served not only to identify ORF’s but are contributions to basic knowledge of what cells make, i.e., to the parts list itself.

(iii) How, although these remarks have focused on corrective reactions (green), analysis of formation reactions (blue) of toxic compounds has special interests. For minor reactions of major enzymes, enzymology has been a fount of knowledge, the enzymes are sometimes essential, and altering specificity may be difficult (Tcherkez et al, 2006). Addressing spontaneous formation of toxic compounds has other challenges.

(iv) That, nonetheless, toxicities (red) may be the hardest to figure out. Thus, although the erythronate mentioned earlier is one of several markers in human transaldolase deficiency (Engelke et al., 2010), its relevance to the pathology may be remote. Even in microbial systems where there may be overt effects on growth, mechanisms can be several, partial, and idiosyncratic: the example of valine toxicity in wild type *E. coli* had already fifty years' attention (Umbarger, 1996). Similar complexities are seen in models of inherited disease using microbial systems (e.g., galactose toxicity, de Jongh et al., 2008; Gibney et al., 2018), and issues of reactivities in normal catalysis can likewise be baroque (e.g. pyridoxal 5'-phosphate dependent enzymes, Downs and Ernst, 2015).

Toxic effects range from lethal to inapparent, and in some of the examples mentioned it is only inferred – albeit strongly – from the prevalence and specificity of the apparent corrections. Modified cofactor NADHX, not mentioned above, is telling in this regard, reactions of formation and removal being observed in the 1950's but *in vivo* toxicities only recently (see Becker-Kettern et al., 2018).

(v) And, last, is that including minor reactions and compounds extends and complicates the metabolic network. To some extent this is about background noise, but the facts of observable effects and evolved preemptive mechanisms show that noise matters. It points to our knowing only what we know or, as AV Golubev put it (1996), that "metabolic maps are what shows how it is thought to be."

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Legends to Figures. Fig. 1A. A normal metabolite, M, of intermediary metabolism, made and used as indicated by arrows in black type. Here, each arrow might be multiple (branch points, isozymes), and one or the other might represent import or export or incorporation. Fig. 1B. An accidental metabolite made, blue arrow; toxic, red arrow; and disposed of, green arrow. Again, reactions can be multiple. Fig. 1C. Fig. 1C represents how many normal metabolites or their close derivatives may also have, in certain circumstances, ill effects and be subject to preemption, red and green arrows, respectively. These are cartoons: other situations are mentioned in the text and some of them, as emphasized, are ambiguous.

Fig. 2. Central metabolism and phosphoglycolate phosphatase, slightly modified from Fig. 6 in Collard *et al.* (2016). For simplicity, cofactors are not shown, and nominal regulatory interactions are only those mentioned in their paper. The enzyme abbreviations are Pgi (phosphoglucose isomerase); Pfk-1 and Pfk-2 (fructose 6-P 1-kinase and 2-kinase); Gap (glyceraldehyde-3-P dehydrogenase); Pgk (phosphoglycerate kinase); Pyk (pyruvate kinase). Pgp (2-phosphoglycolate phosphatase). Zwf (glucose-6-P dehydrogenase); Pgl (phosphogluconolactonase), Gnd (gluconate-6-P dehydrogenase); and Epd (*E coli*, erythrose-4-P dehydrogenase) (While many of the reactions are almost universal, exceptions, see text, are (i) that the reaction of pyruvate reduction (by NADH) to L-lactate is not found in yeast or *E. coli*; (ii) bacteria do not have Pfk-2; and (iii) Epd is not found in eukaryotes.)

Fig. 3. Some other examples. Fig. 3A, Pyrimidine overflow. Fig. 3B, Riboflavin biosynthesis. Fig. 3C. Transamination. Fig. 3D, δ-Gluconolactone-6-P metabolism. Fig. 3E, Methylglyoxal metabolism.

Fig. 4. Some structures, listed alphabetically ("Ph" is used for the group $-PO_3H_2$).

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Fig. 1A

Fig. 1B

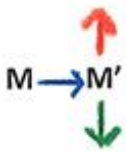


Fig. 1C

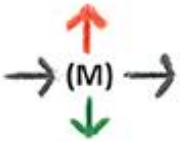
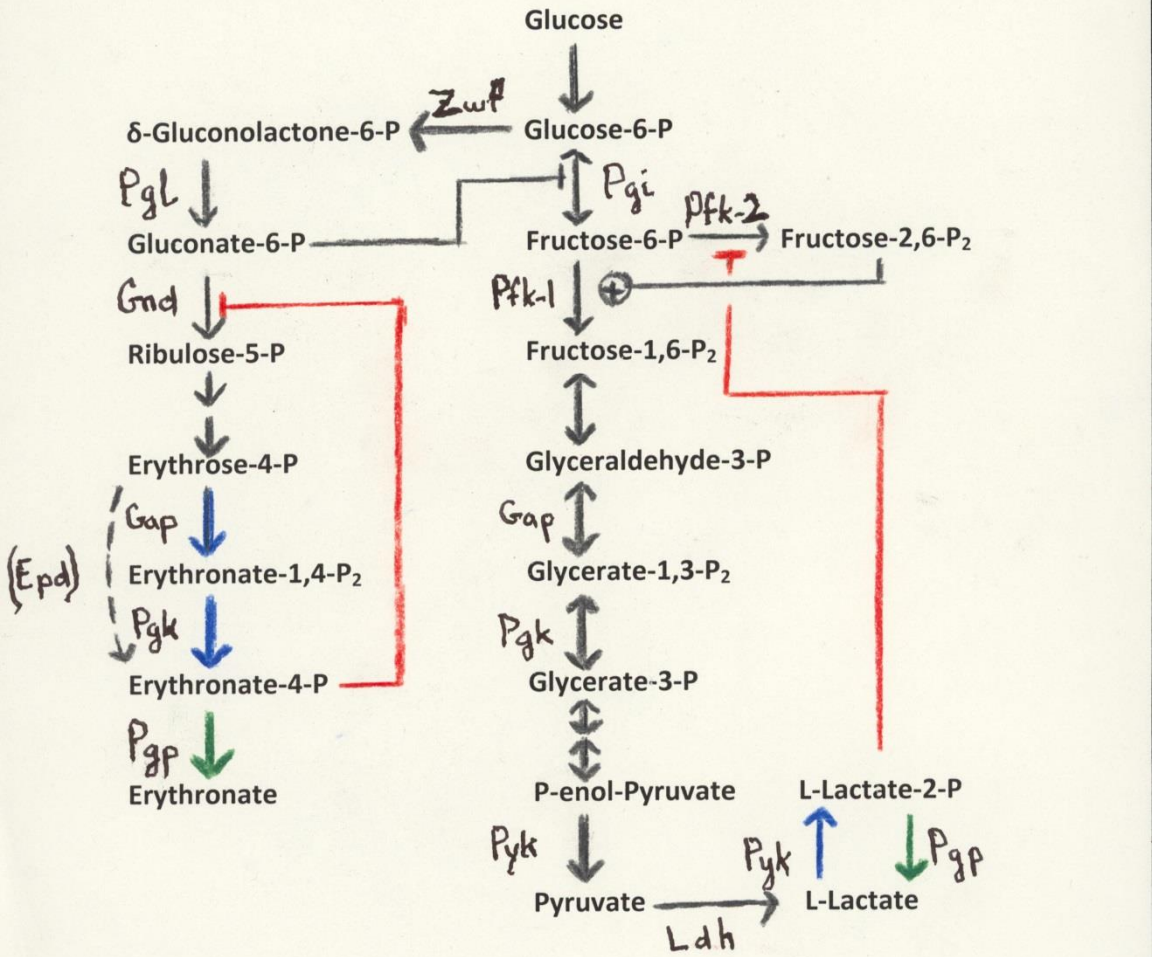


Fig. 2



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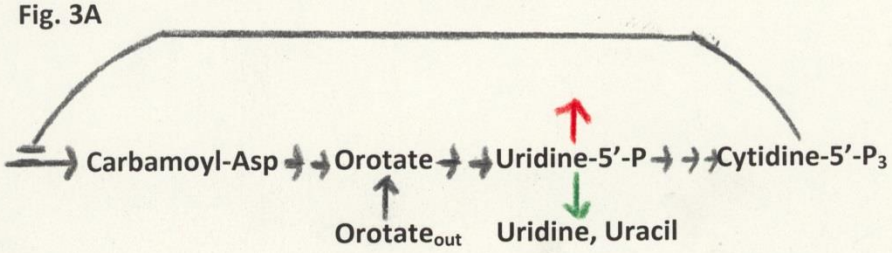
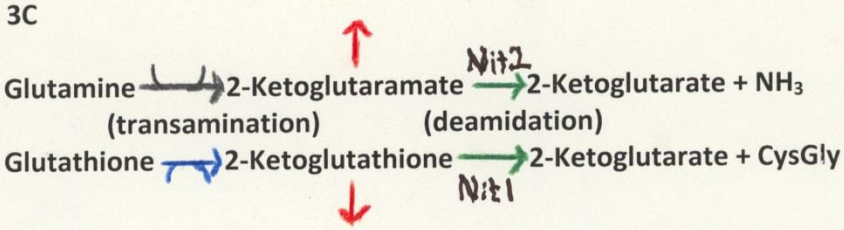


Fig. 3B

Fig. 3C



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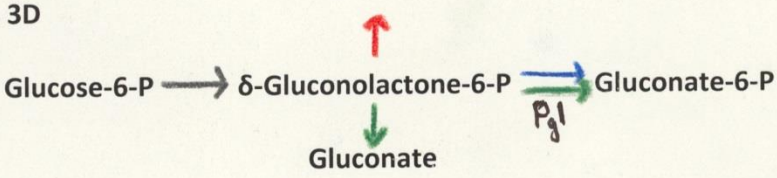

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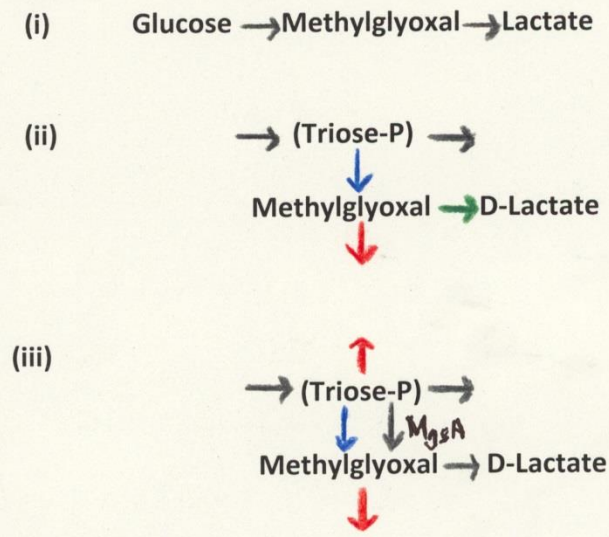
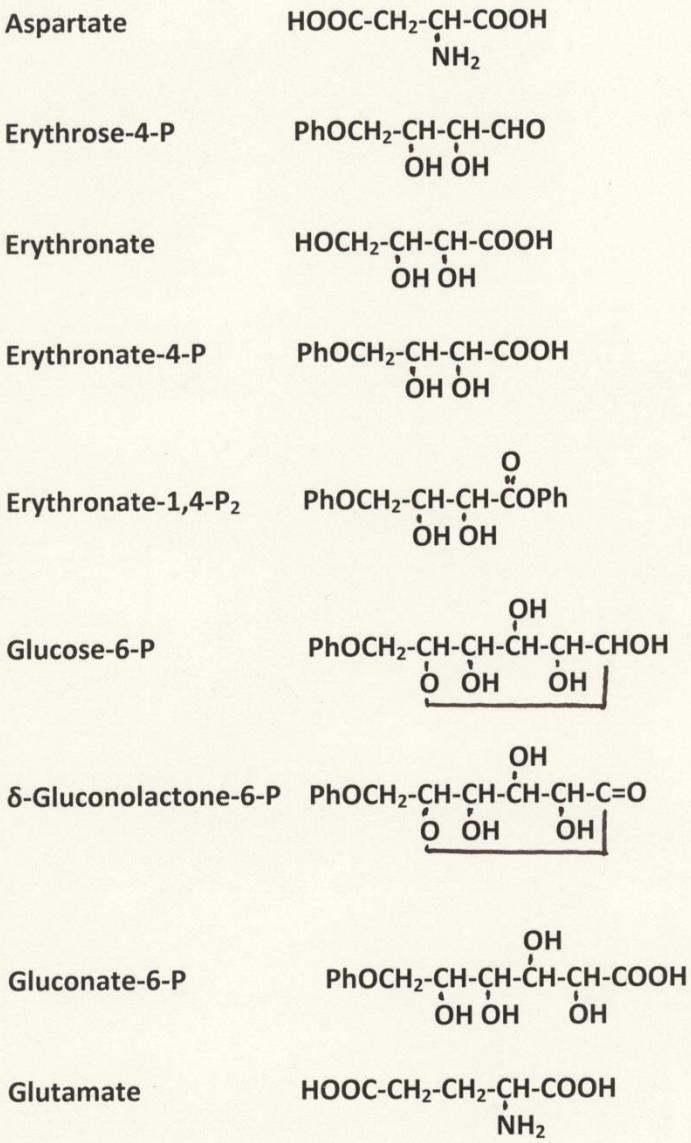
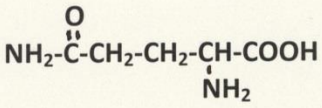
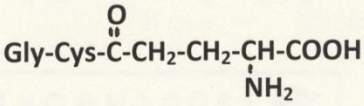
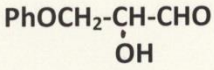


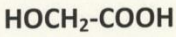
Fig. 4

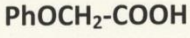


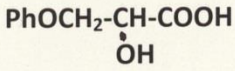
Glutamine 

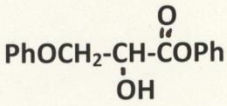
Glutathione 

Glyceraldehyde-3-P 

Glycolate 

2-P-Glycolate 

Glycerate-3-P 

Glycerate-1,3-P_2 

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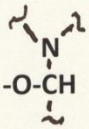
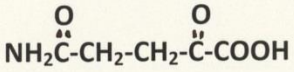
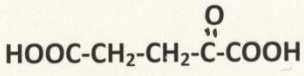
(N-glycosidic linkage) 

2-Ketoglutaramate 

2-Ketoglutarate 

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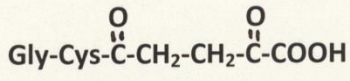

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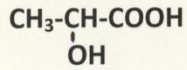

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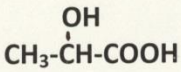

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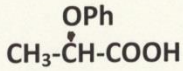
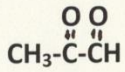


Methylglyoxal



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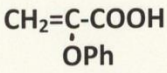

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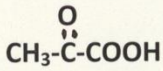

Riboflavin intermediates 1 and 2
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