Review Letter

FINE CONTROL OF THE CONVERSION OF PYRUVATE (PHOSPHOENOLPYRUVATE) TO OXALOACETATE IN VARIOUS SPECIES

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Received 10 February 1978

1. Introduction

Net synthesis of oxaloacetate from pyruvate or phosphoenolpyruvate provides one of the mechanisms which permits the tricarboxylic acid cycle to fulfil a biosynthetic as well as energy-generating role [1,2]. This reaction is catalyzed by pyruvate carboxylase (reaction 1) in vertebrates, invertebrates, fungi and certain bacteria:

$$\text{Pyruvate} + \text{MgATP}^{2-} + \text{HCO}_3^- \xrightleftharpoons{Mg^{2+}, K^+} \text{Oxaloacetate} + \text{MgADP}^- + \text{P}_i \quad (1)$$

by PEP carboxylase (reaction 2) in other bacteria:

$$\text{PEP} + \text{HCO}_3^- \xrightarrow{Mg^{2+}} \text{Oxaloacetate} + \text{P}_i \quad (2)$$

and by PEP carboxytransphosphorylase (reaction 3) in the Propionibacteria and a parasitic protozoon:

$$\text{PEP} + \text{HCO}_3^- + \text{P}_i \xrightleftharpoons{Mg^{2+}} \text{Oxaloacetate} + \text{PP}_i \quad (3)$$

In differentiated organisms it is possible to assign more definitively a metabolic role for the net synthesis of oxaloacetate. For example, in rat adipose tissue it has been suggested that pyruvate carboxylase is predominantly involved in an anabolic cycle (the pyruvate/citrate cycle) which transports acetyl residues from the mitochondrion to the cytosol during lipogenesis [3,4] while in mammalian brain a role has been proposed in the synthesis of L-glutamate and γ-aminobutyrate from glucose [5]. In both cases the role of pyruvate carboxylase appears correlated with a biosynthetic function although no clear distinction can be drawn between this and the more classical anaplerotic view [2]. However, in insect flight muscle oxaloacetate synthesis by pyruvate carboxylase has been suggested [6] to elevate and/or maintain the levels of the tricarboxylic acid cycle intermediates when a massive increase in ATP demand occurs as for example on the initiation of flight. No biosynthetic role for the enzyme is apparent in this tissue. A similar postulate might explain the presence of a low, but significant, level of pyruvate carboxylase in mammalian heart [7], and would provide an example of a situation in which the role of pyruvate carboxylase is purely anaplerotic, since its proposed function relates only to the rate of cycle flux. A requirement for activation by acetyl-CoA has been demonstrated for pyruvate carboxylases obtained from these and other tissues [5,6]. However, little attempt has yet been made to examine the enzymes for tissue-specific modes of regulation except in the case of the locust flight muscle pyruvate carboxylase [8], although such tissue specificity in regulation appears predictable on the basis of the differences in the proposed metabolic roles.

More data are however available which describe the mechanisms that may regulate the enzymes responsible for net synthesis of oxaloacetate from pyruvate or PEP in various species and from which some preliminary conclusions can now usefully be drawn. With the exception of PEP carboxytransphosphorylase which does not appear, on the basis of data now available, to be a regulated enzyme [9], the characteristic effectors which regulate net synthesis of oxaloacetate are either activation by an acyl-derivative of coenzyme A and/or inhibition by a dicarboxylic acid [2,10]. However, this generalisation

disguises a very wide diversity of potential regulatory effects which have been observed for these enzymes in different species. It is the purpose of this review letter to examine this diversity and its possible implications.

2. Enzymes which are activated by acyl derivatives of coenzyme A and related compounds and also inhibited by dicarboxylic acids

2.1. *Regulatory effects*

Activation by acetyl-CoA is well recognised as a mechanism which may regulate the net synthesis of oxaloacetate. The arrangement whereby the substrate of the tricarboxylic acid cycle regulates the rate of synthesis of the cycle intermediate which is its co-substrate for entry into the cycle is readily rationalised and hence widely accepted, although provision of evidence that such a mechanism actually operates in vivo has been extremely difficult especially in vertebrates [11]. Inhibition by dicarboxylic acids, although equally attractive on the grounds of classical regulation theory is less well recognised as a mechanism of control at this step. This latter phenomenon was first described for *Saccharomyces cerevisae* pyruvate carboxylase [12] and for *Escherichia coli* PEP carboxylase [13] with L-aspartate as the most potent inhibitor.

Examination of the specificity of regulatory activation and inhibition for several pyruvate and PEP carboxylases however demonstrates the wide range which exists in these properties in different species within the general framework outlined above. An example of such differences is provided by fig.1 which summarises the relationship between the properties of activation and the acyl chain length for activation of pyruvate carboxylases from rat liver and a thermophilic Bacillus, and of PEP carboxylase from *E. coli* by acyl derivatives of coenzyme A. A more detailed account of the specificity of activation of pyruvate carboxylases from chicken liver and *S. cerevisiae* by acyl derivatives of coenzyme A and related compounds, and of the possible implications of these data, may be found reviewed in [18].

More interestingly from our point of view clear relationships can be discerned between various pyruvate and PEP carboxylases on the basis of these studies as illustrated in table 1. The type I enzymes, which are exemplified by pyruvate carboxylases purified from the livers of higher vertebrates, are activated by acetyl-CoA, but not by long chain acyl-CoAs, and are inhibited by L-glutamate but not by L-aspartate. For some enzymes, e.g., pyruvate

Table 1
Regulatory properties of various pyruvate carboxylases

Type	Examples	Effectiveness of activation[a]	Effectiveness of inhibition[b]	Ref.
I	Pyruvate carboxylase (chicken liver) (rat liver)	Acetyl-CoA >> palmityl-CoA = 0	L-glutamate >> L-aspartate = 0	[14,19,20]
II A	Pyruvate carboxylase (thermophilic Bacillus)	Acetyl-CoA > palmityl-CoA	L-aspartate > α-oxoglutarate > L-glutamate = 0	[16]
B	Pyruvate carboxylase (*S. cerevisiae*) PEP carboxylase (*E. coli*)	Palmityl-(oleyl-)CoA > acetyl-CoA	L-aspartate > α-oxoglutarate L-glutamate = 0	[12,15, 17,21]

[a] Based on consideration of both app. K_a and V_{max}
[b] Based on consideration of $[I]_{0.5}$

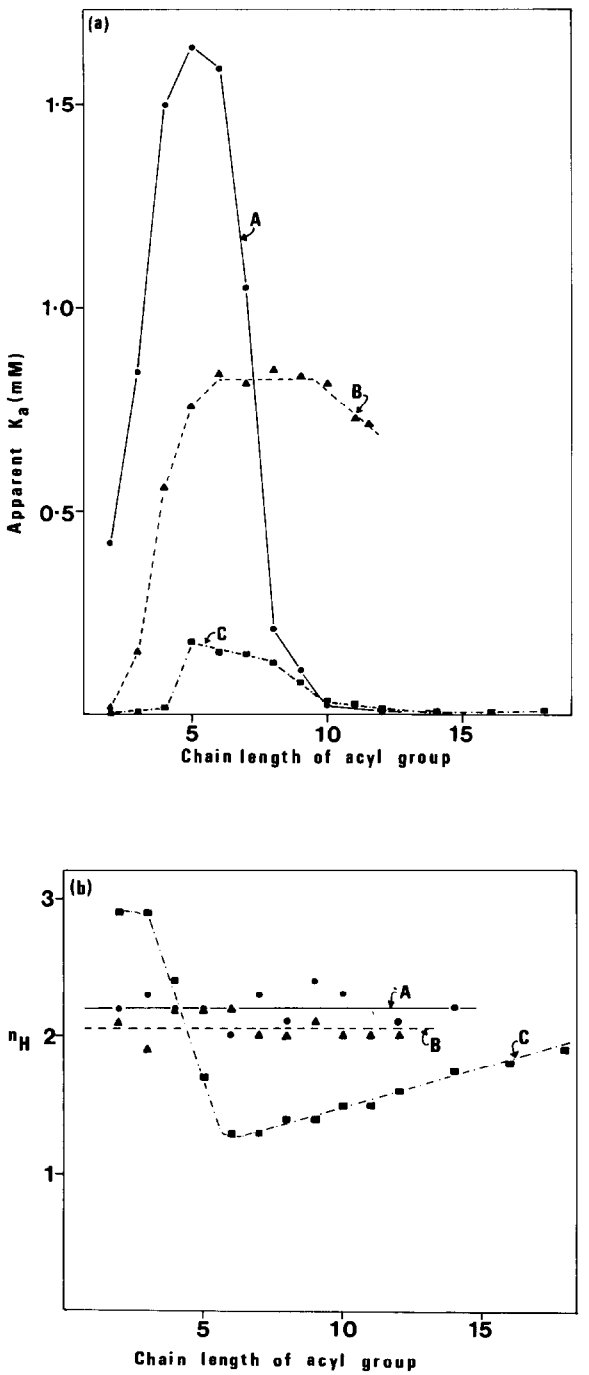

Fig.1. Variation of app. K_a (a) and n_H (b) with the chain length of the acyl group for PEP carboxylase from *E. coli* (A), pyruvate carboxylases from rat liver (B) and a thermophilic Bacillus (C). The data were taken from [14–17].

carboxylase from chicken liver, α-oxoglutarate is also effective as a regulatory inhibitor but the data obtained suggests that this effect results from occupancy of a different site [20]. Other dicarboxylic acids are ineffective with the exception of citrate and L-malate. The properties of inhibition by L-malate suggest that it is interacting at the catalytic site as a product analogue while that of citrate may result from removal of Mg^{2+} which is an essential activator of all pyruvate carboxylases, although a more direct action cannot be excluded [20].

The type II enzymes, which are all of bacterial or fungal origin, are activated by both long and short chain acyl-CoAs and are inhibited by L-aspartate, and less effectively by α-oxoglutarate, but are insensitive to L-glutamate. The group is sub-classified on the basis of whether acetyl-CoA, or a long chain acyl-CoA, is the most effective activator. In all cases, it has been established that the effects of the long chain acyl-CoAs are reversible and are therefore due to specific binding rather than to a non-specific detergent effect [16,17,21].

Although activation by acetyl-CoA and inhibition by L-aspartate has also been described for pyruvate carboxylase from *Arthrobacter globiformis* [22] and PEP carboxylases from *Salmonella typhimurium* [23,24], *Azotobacter vinelandii* [25], *Pseudomonas citronellolis* [26] and *Thiobacillus thioxidans* [38], detailed studies of the specificity of interaction have not been reported.

2.2. *Implications*

The above classification highlights several points. First a strong correlation is apparent between the properties of interaction of metabolites at the regulatory activator and inhibitor sites. Several other observations suggest such a relationship. In all cases an apparently competitive relationship is observed in kinetic studies in which initial rate is measured as a function of acyl-CoA concentration at a series of dicarboxylic concentrations, or vice versa [20,27]. Furthermore, for the pyruvate carboxylases a thiolester of CoA with a non-carboxylic acid which can be regarded as an analogue of acetyl-CoA, e.g., methanesulphonyl-CoA, acts as an inhibitor of the type I, but as an activator of the type II, enzymes [16]. In this case however PEP carboxylases do not appear to conform since the noncarboxylic thiolester

inhibits the enzyme obtained from *E. coli* [28].

The relationship between the regulatory activator and inhibitor sites which is indicated by all these observations cannot however be explained by competition of the various metabolites for portions of the same binding site. Thus incubation of all acyl-CoA-activated pyruvate carboxylases with lysyl group-specific reagents causes specific loss of sensitivity of acyl-CoA activation and has no effect on the properties of the inhibition of these enzymes by L-aspartate (*S. cerevisiae,* thermophilic Bacillus) [16,29], α-oxoglutarate (chicken liver) [20] or L-glutamate (rat liver) [28]. Conversely, for PEP carboxylase from *E. coli* selection of appropriate mutants [30] or incubation with histidine group-specific reagents [28,31] causes partial or complete loss of sensitivity to inhibition by L-aspartate without a concomitant alteration in the properties of activation by acetyl-CoA. Binding studies using PEP carboxylase from *Sal. typhimurium* have directly demonstrated the independence of the sites for acetyl-CoA and L-aspartate on this enzyme and have shown that the binding properties for L-aspartate are not significantly modified by the presence of acetyl-CoA (and vice versa) unless a third effector, e.g., fructose-1,6-diphosphate or GTP is also present [32]. And finally, the presence of Blue Dextran 2000 at concentrations in the nM range causes specific and reversible loss of sensitivity of *E. coli* PEP carboxylase to both activation by acetyl-CoA and inhibition by L-aspartate in accord with the relationship noted above, but only L-aspartate is capable of dissociating the enzyme–Blue Dextran 2000 complex as indicated by the ability to elute the enzyme from a Blue Dextran 2000–Sepharose column [17].

Second, some suggestions may be made as to the likely metabolic significance of these differences in regulatory properties illustrated in table 1. For example, the activation properties of the type I and, to a lesser extent the type IIA, enzymes suggest a predominant functional linkage to the tricarboxylic acid cycle in these species in accord with the anaplerotic concept [2]. This postulate is supported for the type I enzymes by their mitochondrial localisation within the cell [33,34], and for the type IIA enzymes by the observation of properties indicative of association with the cell membrane which is suggested as the site of localisation of the tricarboxylic acid cycle in certain prokaryotes [35]. The activation properties of the type IIB enzymes appear however to be more consistent with a relationship between lipid metabolism and oxaloacetate synthesis. This relationship may take the form of a feed-forward activation whereby the product of fatty acid biosynthesis rather than the substrate of the tricarboxylic acid cycle, increases the rate of net conversion of pyruvate to oxaloacetate. Such a linkage would be advantageous if the oxaloacetate formed is predominantly utilised for biosynthetic purposes rather than for an enhancement of cycle flux, and could therefore provide a mechanism whereby a balance can be achieved in the use of glucose (or lactate) carbon in the synthesis of lipids and of amino acids and pyrimidines (fig.2). In accord with this postulate current evidence suggests that in *S. cerevisiae* pyruvate carboxylase is localised in the cytosol [36], rather than in the mitochondrion as is the case in vertebrates and invertebrates [6,33,34]. *E. coli* PEP carboxylase fails to show the characteristic transient activation on exposure to long chain acyl-CoAs which has been taken to indicate a membrane association in the case of pyruvate carboxylase from a thermophilic Bacillus [28]. The difference in inhibitor specificity between the type I and type II enzymes is also generally consistent with these ideas. Inhibition by L-asparate is a logical indicator for the rate of anabolic metabolism since this amino acid

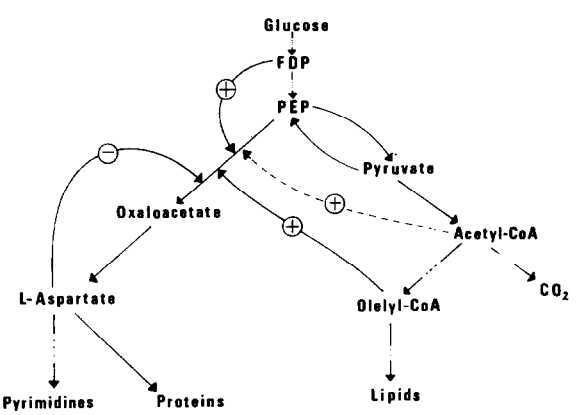

Fig.2. Mechanisms for regulation of PEP carboxylase in *E. coli* during growth on glucose (+) indicates activation and (–) indicates inhibition. The dashed line indicates the suggested lesser importance of this effect.

serves as a precursor of proteins, purines and pyrimidines. The situation is less clear in the case of inhibition of the type I enzymes by L-glutamate since although the rate of pyruvate carboxylation must be related to the level of the tricarboxylic cycle intermediates there is no obvious rationale for the choice of this amino acid as the inhibitor. The proposal is however supported by the studies [37] which have shown that in isolated rat hepatocytes the mitochondrial L-glutamate concentration is in the same range as the K_i for this metabolite observed in in vitro studies on purified rat liver pyruvate carboxylase [14]. Furthermore the concentration of L-glutamate decreases to a non-inhibitory level under conditions in which gluconeogenic flux is increased. The classification employed here does disguise an important difference in inhibitor specificity for the type I enzymes. Inhibition by α-oxoglutarate is observed for avian, but not for mammalian, liver pyruvate carboxylases [20]. It has been suggested that the regulatory inhibition of avian liver pyruvate carboxylases by α-oxoglutarate is related to the predominantly mitochondrial localisation of PEP carboxykinase in these species which may require that the rate of mitochondrial oxaloacetate synthesis to reflect at least in part the GTP/GDP ratio in this organelle [20].

3. Enzymes which are reported to be either activated by acyl derivatives of coenzyme A or inhibited by dicarboxylic acids

Although pyruvate and PEP carboxylases obtained from many organisms are sensitive to regulation by both acyl derivatives of coenzyme A and dicarboxylic acids, the enzymes obtained from certain species appear to be sensitive to only one of these effectors. Since none of the enzymes in this group has yet been purified and studied in detail and many of the observations described have been obtained using cell-free extracts, further studies are required in all cases before any definitive conclusions can be drawn. In particular it is questionable whether certain of the inhibitory effects reported are truly regulatory.

3.1. *Only activation by acyl derivatives of coenzyme A*

This may characterise the pyruvate carboxylases purified from frog liver and locust flight muscle [8] which are insensitive to inhibition by L-aspartate and L-glutamate [7,8,39].

3.2. *Only inhibition by dicarboxylic acids*

Several examples of this situation have been fairly well established. For example, L-aspartate at mM concentrations, inhibits PEP carboxylase from *Acetobacter suboxydans* [40] and pyruvate carboxylase from *Aspergillus niger* [41] and *Aspergillus nidulans* [42] but both these enzymes appear to be fully active in the absence of acetyl-CoA. For the enzyme from *Asp. nidulans* the absence of activation by acetyl-CoA has been demonstrated under conditions in which pyruvate carboxylase from *S. cerevisiae* shows little activity in the absence of this activator [42]. However, inhibition of this enzyme by L-aspartate can be prevented by addition of either acetyl-CoA or palmityl-CoA [42]. These results suggest that *Asp. nidulans* pyruvate carboxylase may carry a site for acyl-derivatives of CoA for which interaction with the catalytic site is expressed only in the presence of the regulatory inhibitor, thus establishing a relationship with the type II pyruvate carboxylases (table 1). Acyl-CoA-independent PEP carboxylases have been obtained from *Acetobacter xylinium* and *Euglena gracilis* which are inhibited by succinate, or by citrate and isocitrate, respectively, but are unaffected by L-aspartate or L-glutamate [43,44].

4. Regulation by effectors other than acyl derivatives of coenzyme A and dicarboxylic acids

Other effectors have been described for various PEP carboxylases, including hexose phosphate, nucleotides and NADH. However, with the exception of NADH which has been reported to be a weak inhibitor of pyruvate carboxylase from *S. cerevisiae* [45] none of these metabolites appear to influence the activity of any of the pyruvate carboxylases examined thus far.

4.1. *Activation by hexose phosphates*

Fructose-1,6-diphosphate and other hexose phosphates at mM concentrations activate PEP carboxylases obtained from *E. coli* [28], *Sal. typhimurium* [24] and *Brevibacterium flavum* [46] but fructose-1,6-diphosphate is ineffective as an activator of the

acyl-CoA activated enzymes obtained from *Thb. thioxidans* [38] or *Ps. citronellolis* [26].

In addition, it has been reported that various hexose phosphates activate PEP carboxylases obtained from a range of plant tissues although the extent of activation is never greater than 2-fold and mM concentrations are required [47–49]. In certain plants (C_4 species) PEP carboxylase catalyses the primary CO_2 fixation and plays a key role in a mechanism (the Hatch-Slack pathway) [50] which traps CO_2 as malate in the monophyll cell. The malate produced is then transported to the bundle sheath cell for decarboxylation and utilisation of the CO_2 thus released by the Calvin cycle. The hexose phosphates are therefore produced in a different cell type from that which contains PEP carboxylase in this tissue. Primary control appears to be exerted on the activity of NADP-malate dehydrogenase and pyruvate dikinase in the mesophyll cell which are subject to 'light activation' [51]. Control of the availability of PEP may then regulate the rate of CO_2 fixation. In this situation it is hard to visualise a role for activation of PEP carboxylase by hexose phosphates even if these do gain access to the enzyme. A rise in hexose phosphate concentration would signal an imbalance between the rate of synthesis by the Calvin cycle and the rate of incorporation of these intermediates into cellular materials and other products. Hence it would be reasonable to expect an effect which would decrease the rate of hexose phosphate synthesis under these conditions, e.g., inhibition of PEP carboxylase by hexose phosphates. The significance of the activation observed in C_4 plants is therefore uncertain. The similar effects observed for PEP carboxylases obtained from other (C_3) plants are difficult to evaluate since no role for the enzyme has been clearly defined in these species.

4.2. *Nucleotides*

At mM concentrations certain nucleotides activate some of the bacterial PEP carboxylases. For example, PEP carboxylases obtained from *E. coli* and *Sal. typhimurium* are activated by CDP, CTP and GTP [52,53] whereas the enzyme from *Ps. citronellolis* and *Az. vinelandii* [26,28] is activated by ADP. In the case of the enzyme from *Sal. typhimurium* data obtained in studies of the effect of GTP on the interaction between the binding of acetyl-CoA and L-aspartate [32] suggest that a unique regulatory nucleotide site exists on this enzyme.

4.3. *NADH*

When *Pseudomonas* MA is grown on carbon sources such as sucrose, succinate or acetate this organism is found to contain a PEP carboxylase which is subject to activation by acetyl-CoA and inhibition by L-aspartate, and appears similar to the enzyme present in related species. However, when this microorganism is grown on one-carbon substrates, e.g., methylamine, methanol, a similar level of PEP carboxylase activity is present but the enzyme is now insensitive to acetyl-CoA, L-aspartate and purine or pyrimidine nucleotides and instead is activated specifically by NADH (and less effectively by NADPH) [54]. Assimilation of the one-carbon growth substrate occurs by the serine/hydroxypyruvate pathway and the activation by NADH serves as a plausible mechanism for maintenance of balance between flux in the pathways of energy production and of carbon assimilation. A similar role for NADH has been suggested for integration of oxidative and gluconeogenic flux in vertebrate liver but no direct effect of NAD or NADH has been demonstrated on the catalytic properties of pyruvate carboxylase. Although inhibition by acetoacetyl-CoA and activation by β-hydroxybutyryl-CoA has been suggested as a mechanism which provides indirect control of chicken liver pyruvate carboxylase by the NAD/NADH ratio, the concentrations of the acyl-CoAs required to achieve the effects appear to exclude a physiological role [55].

5. Pyruvate and PEP carboxylases which appear insensitive to metabolic effectors

Pyruvate carboxylases have been obtained from at least two microorganisms (*Ps. citronellolis* and *Az. vinelandii*) which appear insensitive to any of the characteristic metabolic effectors described thus far for this enzyme [56–58]. Since both these organisms also contain a PEP carboxylase which is regulated by acetyl-CoA and L-aspartate [25,26] and since the pyruvate carboxylase content of the cells is a function of the carbon source used for growth [26,58], it seems reasonable to conclude that 'fine' control is not involved in regulation of pyruvate carboxylation in these species. This conclusion is supported by the

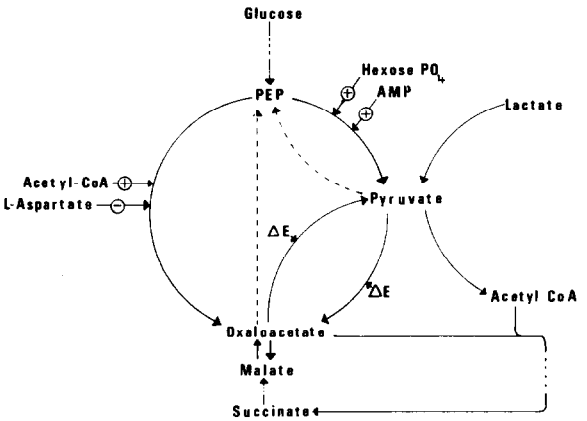

Fig.3. Interrelationships in the metabolism of pyruvate, PEP and oxaloacetate in *Az. vinelandii*. The figure is drawn on the basis of data in [25] and [58]. The symbols indicating fine control are as for fig.2. ΔE indicates that the maximal catalytic capacity of the enzyme changes during growth on different carbon sources. Dashed lines indicate enzymes not yet demonstrated in *Az. vinelandii* or where there is uncertainty regarding the presence of the enzyme. It is however apparent that in order to grow on lactate this organism must possess either one of the enzymes capable of converting pyruvate to PEP or PEP carboxykinase.

observation that the subunit structure of the *Ps. citronellolis* pyruvate carboxylase differs from that characteristic of pyruvate carboxylases which exhibit regulatory properties [59]. The existence of multiple pathways of pyruvate metabolism as shown in fig.3 for *Az. vinelandii*, raises interesting problems of control in this area of metabolism, and in several areas notably between pyruvate and oxaloacetate it is not clear how metabolite recycling is regulated.

The situation in respect to PEP carboxylases is less well-defined. Originally it was considered that most, if not all, plant PEP carboxylases were insensitive to fine control (cf. [60]) but more recent studies have suggested that many of these enzymes may show effects which are labile and which would in principle be regulatory in character [47]. However, as noted above for species in which the metabolic role of PEP carboxylase is well understood the regulatory properties do not appear consistent with the proposed role. No convincing reports have appeared which suggest that the level of this enzyme alters markedly in response to a changed growth environment except in the case of *Pseudomonas* MA where the overall level of PEP carboxylase activity remains the same but different isoenzymes are synthesised in response to a change in growth substrate [54].

6. Conclusions

This analysis of the regulatory properties of pyruvate and PEP carboxylases in different species has revealed a very wide spectrum of regulatory effects including important differences which have only been documented by detailed examination of these properties. The analysis is still far from complete since many of the enzymes have not been properly characterised and further studies are indicated especially for those enzymes which appear to respond to activation by an acyl-CoA but to show no response to addition of a dicarboxylic acid, or vice versa. The results obtained to date do however indicate the potential value of such studies in assessing the metabolic role of pyruvate (or PEP) carboxylase. For example whereas the regulatory properties and intracellular localisation of mammalian and avian liver pyruvate carboxylases seem consistent with the functional relationship to the tricarboxylic acid cycle which is implicit in the anaplerotic concept, this is not the case in *S. cerevisiae*. In this latter microorganism similar studies suggest that the replenishment function has little, if any, significance and that the enzyme functions predominantly as the initial step in a pathway which commits carbon from hexose, or three-carbon growth substrates, to biosynthesis of cell constituents. In the case of this eukaryotic organism the absence of pyruvate carboxylase from the mitochondrial fraction is the most significant observation although the greater efficiency of activation by long chain acyl-CoA as compared with acetyl-CoA is consistent with this conclusion since, as shown in fig.2, activation by long chain acyl-CoA is one component of a mechanism which may balance carbon flow in the synthesis of various cellular materials. Thus in this organism, and possibly also for PEP carboxylase in *E. coli*, the regulatory mechanisms may relate primarily to the biosynthetic function and may have little direct relationship to flux in the tricarboxylic acid cycle.

In the case of *S. cerevisiae* the location of pyruvate carboxylase and the tricarboxylic acid cycle in

different intracellular compartments has further implications. For example in respect to fatty acid synthesis it seems clear that the pyruvate/citrate cycle for acetyl-group translocation as proposed for mammalian liver and adipose tissue cannot operate in *S. cerevisiae*. It remains to be determined whether this function is carried by another translocation system or whether in this organism the acetyl-CoA required for fatty acid synthesis is produced directly from glucose in the cytosol. Furthermore the necessity for synthesis of amino acids derived from L-glutamate seems to imply that an indirect relationship between pyruvate carboxylase and the tricarboxylic acid cycle must exist although the nature of this relationship which could be fulfilled, e.g., by cytosolic → mitochondrial transport of oxaloacetate, or some metabolite derived from it, e.g., L-aspartate, is not yet clear.

The relationships which have been revealed by these studies of the properties of the regulatory activator and inhibitor sites may therefore be of considerable significance in indicating the predominant metabolic fate of oxaloacetate synthesised by pyruvate or PEP carboxylases. Although further studies are necessary to test the validity of this postulate the data presently available are adequate to demonstrate the utility of comparative studies of this type when undertaken with an awareness of the physiological context in which they are expressed. It seems that the regulation of the net synthesis of oxaloacetate from pyruvate or PEP (and very probably of other key reactions as well) is finely tuned to the particular metabolic pattern of the organism. If this view is correct appropriately designed studies of regulatory mechanisms in different tissues and species should provide valuable information relating to the precise metabolic role of such enzymes.

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