APPENDIX

Computer simulation of the effects of α -cyano-4-hydroxycinnamate on gluconeogenesis from L-lactate in rat liver cells

Basic assumptions

(1) It is assumed that the formation of pyruvate from L-lactate is at dynamic equilibrium and that once formed pyruvate can be metabolized, as indicated in Scheme 1. The mechanism by which oxaloacetate crosses the mitochondrial membrane is ignored, the assumption being made that the transaminase steps involved are at equilibrium (see Rognstad, 1980).

(2) Only mitochondrial pyruvate transport is assumed to be readily reversible and to be inhibited by α -cyano-4-hydroxycinnamate. The pH gradient is set at zero, allowing the further assumption that the $V_{\max}$ and K_m for the forward and back reactions are identical.

(3) All steps are assumed to obey Michaelis-Menten kinetics and the K_m values are estimated by using the sources cited in Denton & Halestrap (1979). Hormonally induced changes in activity are represented by changes in $V_{\max}$, with the exception of pyruvate kinase, where K_m changes are used (see Engström, 1980). For simplicity the Hill coefficient for this enzyme is set to be 1.

(4) Values for the $V_{\max}$ of the various enzymes are estimated from references given in Denton & Halestrap (1979). Pyruvate transport rates are calculated from data of Halestrap *et al.* (1980). The actual $V_{\max}$ values used are somewhat arbitrary,

greater importance being given to the relative values and to the final rate of gluconeogenesis calculated.

(5) The cytoplasmic pyruvate concentration remains constant at about 0.35 mM.

(6) All the oxaloacetate formed by pyruvate carboxylase is utilized by phosphoenolpyruvate carboxykinase under gluconeogenic conditions, except for a small proportion which is lost by other minor pathways such as recycling through the malic enzyme.

(7) Gluconeogenesis from phosphoenolpyruvate to glucose is assumed to have no rate-limiting steps and to act as a single 'black box' with one $V_{\max}$ and K_m .

Derivation of formulae used

Let the subscripts PC, PK, PD, PT and GN refer to pyruvate carboxylase, pyruvate kinase, pyruvate dehydrogenase, pyruvate transport and gluconeogenesis respectively. V , v and K represent the $V_{\max}$, actual velocity and K_m of these enzymes while K_i is the K_i of pyruvate transport for α -cyano-4-hydroxycinnamate. The concentration of pyruvate, phosphoenolpyruvate and α -cyano-4-hydroxycinnamate are shown as [PYR], [PEP] and [I] respectively. All rates are given as nmol of pyruvate/min per mg of cell protein. v'_{PT} represents the carrier-independent rate of pyruvate entry into the mitochondria and F the proportion of pyruvate carboxylase flux participating in gluconeogenesis.

For pyruvate transport:

$$v_{PT} + v'_{PT} = v_{PC} + v_{PD} \quad (1)$$

$$v_{PT} = v_{PT}^{\text{in}} - v_{PT}^{\text{out}}$$

$$= \frac{1}{(1 + [I]/K_i)} \cdot \left(\frac{V_{PT}}{1 + \frac{K_{PT}}{[\text{PYR}]_{\text{out}}}} - \frac{V_{PT}}{1 + \frac{K_{PT}}{[\text{PYR}]_{\text{in}}}} \right) \quad (2)$$

where in and out refer to concentrations inside and outside the mitochondria respectively.

For pyruvate carboxylase and pyruvate dehydrogenase:

$$v_{PC} = \frac{V_{PC}}{\left(1 + \frac{K_{PC}}{[\text{PYR}]_{\text{in}}}\right)} \quad (3)$$

Scheme 1. The enzymic steps considered by the computer simulation.

Table 1. *Parameter values used for simulation and the computed values of variables*

The values quoted only included those parameters varied in Fig. 1. The following values were held constant: K_{PT} (200 μM), K_{PC} (100 μM), K_{PDH} (25 μM), K_{GN} (750 μM), V_{PK} (250 nmol/min per mg of protein), V_{PT} (5 nmol/min per mg of protein) and F (0.9).

Condition	Values used						Values calculated at zero inhibitor					
	(nmol/min per mg of cell protein)				(μM)		(nmol/min per mg of cell protein)				(μM)	
	V_{PT}	V_{PC}	V_{PD}	V_{GN}	K_{PK}	K_i	v_{PC}	v_{GN}	v'_{GN}	v_{PK}	[PEP]	[Mitochondrial pyruvate]
(a) i	45	300	5	300	800	40	28.46	7.51	1.19	11.77	29.4	11.0
ii	500	40	5	300	800	5	28.03	7.40	0.91	11.59	28.9	274.6
(b) i	500	500	5	17.5	800	30	178.53	5.91	0.15	165.4	974	65.0
ii	500	1000	5	17.5	800	30	225.87	7.41	0.15	209.4	2340	33.2
iii	500	500	5	25	800	30	179.18	8.27	0.21	160.8	905	65.0
iv	500	500	5	17.5	1500	30	178.78	6.92	0.26	163.4	1661	65.0
v	1000	500	5	17.5	800	30	244.01	7.97	0.15	226.3	4124	116.5
(c) i	125	150	5	75	800	40	49.73	5.82	0.49	36.8	100	55.9
ii	125	500	5	75	800	40	67.14	7.85	0.53	49.7	143	17.0
iii	150	200	5	75	1000	40	62.06	8.26	0.59	43.7	152	50.3
iv	125	150	5	75	1500	40	50.29	8.41	0.75	31.6	155	55.9
v	250	150	5	75	800	40	69.96	8.17	0.49	51.8	150	101.9
vi	250	60	25	75	800	40	33.17	3.90	0.22	24.5	64	149.1

$$v_{PD} = \frac{V_{PD}}{\left(1 + \frac{K_{PD}}{[\text{PYR}]_{\text{in}}}\right)} \quad (4)$$

But [PEP] is unknown.

However, using assumption (7):

$$v_{GN} = \frac{V_{GN}}{1 + \frac{K_{GN}}{[\text{PEP}]}} \quad (9)$$

Thus substituting eqns. (2), (3) and (4) into eqn. (1) and re-arranging:

$$\frac{V_{PC}}{1 + \frac{K_{PC}}{[\text{PYR}]_{\text{in}}}} + \frac{V_{PD}}{1 + \frac{K_{PD}}{[\text{PYR}]_{\text{in}}}} - \left[\frac{1}{1 + [I]/K_i} \cdot V_{PT} \left(\frac{1}{1 + \frac{K_{PT}}{[\text{PYR}]_{\text{out}}}} - \frac{1}{1 + \frac{K_{PT}}{[\text{PYR}]_{\text{in}}}} \right) \right] - v'_{PT} = 0 \quad (5)$$

By setting the values of all parameters except $[\text{PYR}]_{\text{in}}$, the value of $[\text{PYR}]_{\text{in}}$ may be determined by reiteration.

Rate of gluconeogenesis

$$v_{GN} = v_{PC} \cdot F - v_{PK} \quad (6)$$

Now

$$v_{PC} = \frac{V_{PC}}{1 + \frac{K_{PC}}{[\text{PYR}]_{\text{in}}}} \quad (7)$$

This allows v_{PC} to be calculated.

Also

$$v_{PK} = \frac{V_{PK}}{1 + \frac{K_{PK}}{[\text{PEP}]}} \quad (8)$$

Re-arranging eqn. (9):

$$[\text{PEP}] = \frac{K_{GN}}{\left(\frac{V_{GN}}{v_{GN}} - 1\right)} \quad (10)$$

Substituting eqn. (10) in eqn. (8):

$$v_{PK} = \frac{V_{PK}}{1 + \frac{K_{PK}}{K_{GN}} \cdot \left(\frac{V_{GN}}{v_{GN}} - 1\right)} \quad (11)$$

Substituting eqn. (11) in eqn. (6):

$$v_{GN} = v_{PC} \cdot F - \left\{ V_{PK} / \left[1 + \frac{K_{PK}}{K_{GN}} \cdot \left(\frac{V_{GN}}{v_{GN}} - 1 \right) \right] \right\} \quad (12)$$

Multiplying through by

$$\left[1 + \frac{K_{PK}}{K_{GN}} \cdot \left(\frac{V_{GN}}{v_{GN}} - 1 \right) \right]$$

and making $v_{PC} \cdot F = v'_{PC}$:

$$v_{GN} + \frac{V_{GN}K_{PK}}{K_{GN}} - \frac{v_{GN}K_{PK}}{K_{GN}} = v'_{PC} + \frac{K_{PK}V_{GN}v'_{PC}}{K_{GN}v_{GN}} - \frac{v'_{PC}K_{PK}}{K_{GN}} - V_{PK}$$

Fig. 1. Simulation of the effects of α -cyano-4-hydroxycinnamate on gluconeogenesis from L-lactate under various conditions

Rates of gluconeogenesis were calculated by using eqns. (5) and (13). Values of the various parameters involved were varied as shown in Table 1. The major significance of the graphs is as follows. In (a) Dixon plots are shown for cases where (i) $V_{PT} \ll V_{PC}$, V_{PK} and V_{GN} and (ii) $V_{PC} \ll V_{PT}$, V_{PK} and V_{GN} . In (b) $V_{GN} \ll V_{PT}$, V_{PC} and V_{PK} and pyruvate kinase recycling becomes very much greater than the rate of gluconeogenesis. The control situation is given in (i) and the effects of stimulating gluconeogenesis by increasing V_{PC} (ii), V_{GN} (iii), K_{PK} (iv) and V_{PT} (v) are shown. In (c) conditions are thought to represent most closely the physiological situation. The control situation is shown in (i) and the effects of increasing V_{PK} (ii), V_{PC} , V_{PT} and K_{PK} (iii), K_{PK} (iv) and V_{PT} (v). In (vi) the effects of monochloroacetate are simulated assuming this compound stimulates pyruvate dehydrogenase and pyruvate transport and inhibits pyruvate carboxylase. The plots of (d, i) and (d, ii) show the data from (c, i) and (c, v) before subtracting the inhibitor-insensitive rate of gluconeogenesis and without reciprocation. Data from an actual experiment are superimposed, (●) representing control rates and (■) those of glucagon stimulated cells. Each point is the mean of results from three separate incubations on the same batch of cells. In (a) and (c) short-dashed lines (---) represent extrapolated linear portions of the plot.

Multiplying through by v_{GN} and re-arranging:

$$\left(1 - \frac{K_{PK}}{K_{GN}}\right)(v_{GN})^2 + \left[\frac{V_{GN}K_{PK}}{K_{GN}} - v'_{PC}\left(\frac{1 - K_{PK}}{K_{GN}}\right) + V_{PK}\right]v_{GN} - \frac{K_{PK}V_{GN}v'_{PC}}{K_{GN}} = 0 \quad (13)$$

This quadratic may now be solved for v_{GN} which will be calculated in nmol of pyruvate utilized for glucose synthesis/min per mg of cell protein. By dividing by 2 the value may be converted to the more usual units, nmol of glucose formed/min per mg of cell protein. For Dixon plots the calculated value of v_{GN} at 1M-inhibitor is used to represent the inhibitor-insensitive rate of gluconeogenesis (v'_{GN}) and this value is subtracted from v_{GN} .

Conclusions

The stimulation shown in Fig. 1(a,i) demonstrates that if pyruvate entry into the mitochondria is the slowest step in gluconeogenesis, inhibition of gluconeogenesis by α -cyano-4-hydroxycinnamate gives linear Dixon plots. This is not the case if pyruvate carboxylase is considerably slower than pyruvate transport [Fig. 1(a,ii)]. Under these conditions, to obtain a sensitivity of gluconeogenesis to the inhibitor similar to that observed in practice requires the use of a very much smaller K_i for inhibition of pyruvate transport. In Fig. 1(b) the effect of making a step beyond phosphoenolpyruvate become the slowest in the pathway is studied. Linear Dixon plots may still be observed and gluconeogenesis stimulated by increasing pyruvate transport, or pyruvate carboxylase as well as by stimulating the slowest step beyond phosphoenolpyruvate. However, under these conditions pyruvate kinase recycling is some 15-fold greater than the net flow of pyruvate to glucose, whereas the observed recycling is much less than this (Rognstad, 1975, 1976). As might be expected, an increase in the K_m of pyruvate kinase for phosphoenolpyruvate causes stimulation of gluconeogenesis.

The conditions which are thought to match most closely those found in isolated cells are shown in Figs. 1(c) and 1(d). Here the calculated rates of gluconeogenesis and pyruvate kinase recycling, as well as the concentrations of pyruvate and phosphoenolpyruvate, are in reasonable agreement with those observed (Rognstad, 1976; Siess *et al.*, 1977). Under these conditions a change in pyruvate kinase

K_m , pyruvate transport V_{max} , or pyruvate carboxylase V_{max} , all give changes in the rate of gluconeogenesis. Stimulation of all three together or just pyruvate carboxylase and pyruvate transport (results not shown) most closely represent the observed results. A relatively modest (20–30%) increase in each of these parameters produces a 40–50% increase in gluconeogenic rate without a change in the apparent K_i for α -cyano-4-hydroxycinnamate. Fig. 1(d) shows real data plotted on the simulated graphs and the fit is extremely good. Stimulation of gluconeogenesis by phenylephrine is unlikely to involve a significant change in pyruvate kinase K_m since no change in recycling is observed (Rognstad, 1976) and the activity of the enzyme in extracts of the cell is unchanged (see Table 2 of the main paper and related discussion). Under these circumstances increases in pyruvate transport and pyruvate carboxylase activities can account for the observed stimulation of gluconeogenesis. Increase in the V_{max} of pyruvate carboxylase alone would have to be considerable (3-fold or so) to give the observed increase in gluconeogenic rate and a drop in apparent K_i for α -cyano-4-hydroxycinnamate would be expected [Fig. 1(b,ii)]. This was not routinely observed (see Table 1 of the main paper).

References

- Denton, R. M. & Halestrap, A. P. (1979) *Essays Biochem.* **15**, 37–77
- Engström, L. (1980) in *Recently Discovered Systems of Enzyme Regulation by Reversible Phosphorylation* (Cohen, P., ed.), pp. 11–31, Elsevier/North-Holland, Amsterdam
- Halestrap, A. P., Scott, R. D. & Thomas, A. P. (1980) *Int. J. Biochem.* **11**, 97–105
- Rognstad, R. (1975) *Biochem. Biophys. Res. Commun.* **63**, 900–905
- Rognstad, R. (1976) *Int. J. Biochem.* **7**, 403–408
- Rognstad, R. (1980) *Biochim. Biophys. Acta* **628**, 116–118
- Siess, E. A., Brocks, D. G., Latte, H. K. & Wieland, O. H. (1977) *Biochem. J.* **166**, 225–235