Rate-limiting Steps in Metabolic Pathways*

Robert Rognstad

From the Cedars-Sinai Medical Research Institute, Los Angeles, California 90048

A method is proposed to detect whether a given enzyme catalyzes a rate-limiting step in a metabolic pathway. With the use of a range of concentrations of specific inhibitors of an enzyme, the finding of a biphasic response with an initial null effect indicates the non-rate-limiting nature of the enzyme. With this method, phosphoenolpyruvate carboxykinase is indicated to catalyze a rate-limiting step in lactate gluconeogenesis in hepatocytes from fasted rats.

There are a number of approaches for the localization of rate-limiting steps in metabolic pathways. However, in spite of rather intensive investigation, the site or sites of control of the gluconeogenic pathway are not completely known. We describe here a technique which may possibly be of some use in outlining limiting steps in metabolic pathways. The method involves determination of the effect, on the overall flux through a pathway, of a wide range of concentrations of a specific inhibitor of a given enzymic step in the pathway.

METHODS

Isolated hepatocytes were prepared from male Wistar rats essentially according to Berry and Friend (1) with the substitution of Krebs-Henseleit (2) buffer and the omission of hyaluronidase. Glucose (5 mM) and pyruvate (5 mM) were also present in the perfusion medium. Cells were washed several times in substrate-free Krebs-Henseleit buffer. The cells were incubated in 25-ml Erlenmeyer flasks at 38°C in Krebs-Henseleit buffer with added substrate and inhibitors with 5% CO2, 95% O2 in the gas phase. The incubations were terminated by addition of 0.5 ml of 20% HClO4; the medium was washed out, made to 10 ml in conical tubes, and centrifuged. Glucose was determined enzymically (3), with the substitution of NADP* for NADP+ and the use of glucose-6-P dehydrogenase from Leuconostoc mesenteroides. In experiments with hepatocytes from rats fasted 2 days and refed a high carbohydrate diet, lipids were extracted and saponified as described elsewhere (4).

Aminooxyacetate was obtained from Eastman Chemical Co. (Rochester, N. Y.); L-cycloserine was from Calbiochem (La Jolla, Calif.; no longer available from this source); hydroxycitrate was a generous gift from Dr. A. Sullivan, Hoffmann-La Roche (Nutley, N. J.); mercaptopropionylcarnitine was a generous gift from Dr. H. Saunders, Smith, Kline and French (Philadelphia, Pa.).

THEORETICAL

A given metabolic pathway may be composed of some reactions in which the reactants and products of a step are maintained close to equilibrium, and other steps in which reactants and products are far removed from thermodynamic equilibrium. In addition, there may be steps which do not exactly fall into these categories (e.g. some steps might be better characterized as “fairly close to equilibrium” more accurately than “near equilibrium”); however, we consider only the two distinct types here.

If a given enzymic step is rate-limiting, increasing (or decreasing) the concentration of the enzyme catalyzing this step should increase (or decrease) the overall flux through the pathway. Thus, if one could inject enzymes into cells (and into the proper location in the cell), one could readily test which enzyme or enzymes are rate-limiting. In general, it is not feasible to inject enzymes into (most) cells, and the question of whether even cytosolic enzymes might be spatially organized (5) offers other potential limitations. However, in principle, one can lower the effective activity of any of the enzymes with the use of specific inhibitors. Inhibitors generally have been used to test whether a given pathway involves the enzyme which catalyzes a rate-limiting step in the intact cell, even the lowest concentration of inhibitor which measurably decreases the activity of the given enzyme will correspondingly decrease the flux in the pathway.

In order to illustrate such effects, we will assume that simple kinetics of competitive inhibition of an isolated enzyme operates in the intact cell. It is realized that the actual situation in the intact cell will usually be much more complex and may only rarely approach the simplified kinetics of initial rates of an isolated enzyme system. The rate equation for a competitive inhibitor may be written as:

\[
\frac{1}{V} = \frac{K_m}{V_{max}K_i(s)} + \frac{K_m + K_i(s)}{V_{max}(s)}
\]

If the substrate concentration (S) remains constant, a plot of 1/V versus I would be linear (where V is the overall flux in the pathway). Fig. 1 illustrates the effects of two different inhibitors on two different enzymes in a metabolic pathway. In Fig. 1A, the inhibitor acts on a rate-limiting enzyme, while in Fig. 1B, the inhibitor acts on a near equilibrium enzyme. In the first case (Fig. 1A), the plot of 1/V versus I is linear down to the lowest inhibitor concentration used; whereas in Fig. 1B, there is an initial null effect, a region in which the inhibitor has no effect on the overall flux. Assuming that the simplified kinetics holds, one could extrapolate the inhibitory phase of Fig. 1B back to zero inhibitor concentration to obtain the maximal activity of the enzyme, under conditions prevailing in the intact cell (this would generally be less than the capacity of an enzyme as assayed under optimum conditions in a tissue homogenate). Thus, in the arbitrary scale of Fig. 1B, the actual rate at zero inhibitor concentration is 1.0, while the extrapolated rate is 2.5. Thus, the excess capacity of the enzyme over the actual metabolic flux would be 150% in this case, and until the enzyme is inhibited by more than 60% (2.5 - 1.0/2.5), no effect of the inhibitor on the overall flux will be seen.

Fig. 2, based again on the assumption of simple competitive kinetics, shows the expected effect of an inhibitor on overall flux; in this case, the excess capacity of the enzyme over the net flux, is varied stepwise from 0 to 200%, giving the different plots. As the excess capacity is increased, naturally an increased amount of inhibitor is required before any effect on the overall rate is noted. Also, the apparent Km, the concentration of inhibitor necessary for 50% inhibi-

* This work was supported by United States Public Health Service Grants AM 20417 and AM 21437. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accord ance with 18 U.S.C. Section 1734 solely to indicate this fact.

(Received for publication, June 19, 1978)
Rate-limiting Steps in Metabolic Pathways

FIG. 1. Expected effects of inhibitors on overall flux in metabolic pathways. A, effect of an inhibitor on a rate-limiting enzyme. B, effect of an inhibitor on a non-rate-limiting enzyme.

FIG. 2. Effect of varying excess capacity of an enzyme on the apparent $K_{i0}$ values of an inhibitor in intact cell studies.

The method should also be applicable (again to the degree that kinetics of isolated enzymes may be approximated in the intact cell) to the use of noncompetitive inhibitors of enzymes, where a linear relationship between $1/V$ and $I$ is found:

$$\frac{1}{V} = \frac{1}{K} \left( \frac{1}{V_{\text{max}}} + \frac{K_m}{V_{\text{max}}S} \right) + \frac{1}{V_{\text{max}}} + \frac{K_m}{V_{\text{max}}S}$$

The approach described here is an extension of studies on the effects of pyrazole on ethanol metabolism in rat liver hepatocytes, originally by Grunnet et al. (6) and later by Rognstad and Grunnet (7).

RESULTS AND DISCUSSION

Gluconeogenesis—We have applied this approach to the study of two enzymes in the pathway of gluconeogenesis from L-lactate, P-enolpyruvate carboxykinase and glutamate oxalacetate transaminase. Reasonably potent and specific inhibitors of these enzymes are available. P-enolpyruvate carboxykinase has been widely held to be a likely candidate for a rate-limiting enzyme in gluconeogenesis (8-10), while the glutamate oxalacetate transaminases generally have been considered to maintain near equilibrium in the liver, both in the cytosol and in the mitochondria (11, 12).

Mercaptopicolinate Inhibition of P-enolpyruvate Carboxykinase—For many years, P-enolpyruvate carboxykinase has been implicated in the acute control of gluconeogenesis by a large amount of indirect evidence (6-10). (The longer term control of gluconeogenesis by changes in the rate of synthesis of P-enolpyruvate carboxykinase has also been described (10), but is not considered here.) On the other hand, no one has yet shown a stable, acute increase in the assayable activity of P-enolpyruvate carboxykinase by hormonal treatments which do cause marked immediate increases in the rate of gluconeogenesis. For this reason, perhaps some of the relative certainty that this is a major control site has lessened; yet, it certainly remains a possible rate-limiting step. Mercaptopicolinate has been found to be a potent inhibitor of P-enolpyruvate carboxykinase (13, 14). It has a lower $K_i$ (14) than the formerly widely used inhibitor, quinolinate (15, 16), and also more readily enters the liver cell. As of yet, inhibitory effects of mercaptopicolinate on other gluconeogenic enzymes have not been described, and it is likely to be quite specific for P-enolpyruvate carboxykinase at the low concentrations required. However, Jomain-Baum et al. (14) have shown that 100 nM mercaptopicolinate inhibits gluconeogenesis from glycerol strongly in guinea pig liver and also partially in rat liver. These effects are not easy to relate to an inhibition of P-enolpyruvate carboxykinase; on the other hand, it is still possible that an accumulation of a metabolite (e.g. oxalacetate or malate) caused by P-enolpyruvate carboxykinase inhibition could be the cause of the inhibition of glucose formation from glycerol (although no mechanism for this effect can yet be postulated). Since mercaptopicolinate causes no inhibition of gluconeogenesis from fructose in rat liver (17), and since the enzymes catalyzing the reactions between P-enolpyruvate and the triose phosphates are present in high capacity in liver, it is also difficult to readily postulate another inhibitory site for mercaptopicolinate.

Fig. 3 shows the effect of a range of concentrations of mercaptopicolinate on the rate of gluconeogenesis ($V$) from L-lactate in isolated hepatocytes from fasted rats. The plot of $1/V$ versus $I$ is reasonably linear throughout the concentration range used, and no initial null effect at the low levels of inhibitor is found. To the extent that the approach has validity, and within the sensitivity of the experimental data, this...
result at least reaffirms the potential rate-limiting nature of P-enolpyruvate carboxykinase.

Aminoxyacetate and L-Cycloserine Inhibition of Glutamate-Oxalacetate Transaminase—By virtue of its rather high assayable activity (27 units/g in the cytosol and 42 units/g in the mitochondria of rat liver (18)), this transaminase has been assumed to maintain near equilibrium between its substrates (11, 12). Recent experiments using techniques to rapidly separate mitochondrial and cytosolic metabolites have demonstrated that at least the cytosolic glutamate-oxalacetate transaminase is near (or fairly near) equilibrium (19, 20). The status of the mitochondrial enzyme is less clear, possibly because of experimental difficulties in estimating mitochondrial oxalacetate (21).

Aminoxyacetate is a potent inhibitor of glutamate-oxalacetate transaminase and of transaminases in general, and it has been shown to inhibit both the cytosolic and mitochondrial isoenzyme (22). Fig. 4A shows the effect of aminoxyacetate on the rate of gluconeogenesis from L-lactate. There is an initial region, at very low inhibitor concentrations, of very little (but not zero) inhibition, followed by a region of rapidly increasing inhibition. Extrapolation of this reasonably linear second phase gives impossibly high capacities of the enzyme ($1/V < 0$). Obviously, the simple kinetics described in the theoretical section does not rigorously hold. One might rationalize the steepness of the inhibition by remembering that two consecutive transaminase enzymes are involved in the pathway of gluconeogenesis from L-lactate. The cytosolic enzyme will be inhibited by virtue of a direct effect of aminoxyacetate on this enzyme and also by virtue of the slower production of its substrate, aspartate, caused by aminoxyacetate inhibition of the mitochondrial isoenzyme. While the extrapolation obviously is meaningless in this case, there are definitely two distinct phases of inhibitor action, typical of a non-rate-limiting enzyme. Again, one should mention possible complications as to the specificity of action of aminoxyacetate. At higher concentrations (2 mM), some of the inhibitory effects were found to decrease in experiments with reduced substrates in kidney cortex slices (24). Aminoxyacetate, as a substituted hydroxylamine, can react chemically with keto-compounds. If exogenous keto compounds (especially aldehydes) are added at high concentrations, this complex formation may be significant (25). However, with lactate as the added substrate and with aminoxyacetate added at the low concentrations required (Fig. 4A), complex formation with endogenous keto compounds would not be expected to occur significantly.

L-Cycloserine is a less potent inhibitor of glutamate-oxalacetate transaminase, but has the advantage that it reportedly does not inhibit the mitochondrial enzyme, because of restricted permeability (26). Fig. 4B shows the effect of L-cycloserine on gluconeogenesis from L-lactate. The inhibitory effect appears in three fairly distinct phases. At low inhibitor concentrations, there is only a slight inhibitory effect; this is followed by a linear inhibitory region, at higher inhibitor concentrations, a more strongly increasing inhibitory effect is then found. Extrapolation of the linear phase gives values for the capacity of glutamate-oxalacetate transaminase about 4-fold in excess of the net flux in Fig. 4B. In other experiments, the extrapolated values vary, but have always been found to be in over 2-fold excess. The cause of the onset of nonlinearity at higher L-cycloserine concentrations is not known, but possibly some inhibition of the mitochondrial enzyme might be involved.

Lipogenesis: Hydroxycitrate Inhibition of Citrate Cleavage Enzyme—The method has also been used to a limited degree on other metabolic pathways. The citrate cleavage enzyme has some characteristics of potential rate-limiting step. The substrates and products are far from equilibrium in the intact cell, and the enzyme activity is markedly changed with different dietary regimes (27).

Fig. 5 shows the effect of hydroxycitrate on fatty acid synthesis from L-[U-14C]lactate in hepatocytes from fasted-refed rats. Rates of lipogenesis are very high under these conditions (28). A null effect region is found, followed by a linear region over a fairly wide inhibitor concentration range. Extrapolation of the linear region to zero inhibitor concentration yields a capacity of citrate cleavage enzyme about 70% in excess of the actual rate of fatty acid synthesis. This suggests that the citrate cleavage enzyme is not a rate-limiting step under the dietary and hormonal conditions used in this experiment.

The reason for the departure from linearity at the higher concentrations of hydroxycitrate is not known. It could possibly be related to the known activating effect of hydroxycitrate on acetyl-CoA carboxylase (29, 30). Triscari and Sullivan (31) have shown that while hydroxycitrate inhibits liver lipogenesis in vivo in fed rats, it actually increases the low rate of lipogenesis in the liver of fasted rats, probably as a result of activation of acetyl-CoA carboxylase.
Rate-limiting Steps in Metabolic Pathways

General Discussion—We have presented a technique to aid in the localization of rate-limiting steps in metabolic pathways. The approach is rather simplistic and its usefulness in general remains to be seen. It is offered because the detection of rate-limiting steps in complex pathways is apparently much more difficult than might be thought. Various techniques have been proposed and applied, e.g., the crossover method. This remains a valuable tool to show a possible site of action of a given agent, but it has been shown that a “crossover” can also occur at near equilibrium steps which are not rate-limiting (32, 33). Steps which are far from equilibrium in the intact cell are likely candidates for control. However, there may be a number of such steps in a given pathway, and not all of these may be of equal capacity. Control steps have been found at the first unique and committed step of various pathways. Probably this should remain the best guide, but corroboration is always necessary. Here, we have provided at least supportive evidence for the rate-limiting nature of the P-enolpyruvate carboxykinase reaction in gluconeogenesis, under the conditions used (lactate as substrate in hepatocytes from fasted rats).

It is difficult to predict the general usefulness of the inhibitor approach. When, in the future, one can actually write a mathematical equation for the rate of a very complex metabolic pathway, it is possible that the rate may be a sensitive function of the activity of several enzymes, and also possible that the rate may be to a lesser degree affected by changes in the activity of enzymes which normally catalyze near equilibrium or fairly near equilibrium reactions. Thus, our suggested interpretation that P-enolpyruvate carboxykinase catalyzes a rate-limiting step in gluconeogenesis from lactate does not necessarily rule out the possibility that other steps (e.g., those involving mitochondrial reactions (34)) may be essentially equally rate-limiting and that a hormonal stimulation of gluconeogenesis may involve a coordinated action at several sites.

REFERENCES

1. Berry, M. N. & Friend, D. S. (1969) J. Cell Biol. 43, 506-520
2. Krebs, H. A. & Henseleit, K. (1932) Hoppe-Seyler's Z. Physiol. Chem. 210, 33-66
3. Bergmeyer, H. U., ed (1974) Methods of Enzymatic Analysis, Vol. 3, Academic Press, New York
4. Katz, J., Landau, B. R. & Bartsch, G. E. (1966) J. Biol. Chem. 241, 727-740
5. Stere, P. A. & Mosbach, K. (1974) Annu. Rev. Microbiol. 28, 61-83
6. Grunnet, N., Quistorff, B. & Theiden, H. I. D. (1973) Eur. J. Biochem. 40, 275-282
7. Rognstad, R. & Grunnet, N. (1979) in Biochemistry and Pharmacology of Ethanol (Maichrowicz, E. & Noble, E. P., eds) Vol. 1, pp. 65-85, Plenum Press, New York
8. Exton, J. H., Mallette, L. E., Jefferson, L. S., Wong, E. H. A., Friedmann, N., Miller, T. D. & Park, C. R. (1970) Recent Prog. Horm. Res. 26, 411-461
9. Ui, M., Claus, T. H., Exton, J. H. & Park, C. R. (1973) J. Biol. Chem. 248, 3534-3549
10. Tilghman, S. M., Hanson, R. W. & Ballard, F. J. (1976) in Gluconeogenesis: Its Regulation in Mammalian Species (Hanson, R. W. & Mehlman, M. A., eds) pp. 41-91, John Wiley & Sons, Inc., New York
11. Veech, R. L., Eggleston, L. V. & Krebs, H. A. (1969) Biochem. J. 118, 609-619
12. Greenbaum, A. L., Gumaa, K. A. & McLean, P. (1971) Arch. Biochem. Biophys. 143, 617-663
13. Kostos, V., DiTullio, N. W., Rush, J., Cieslinski, L. & Saunders, H. L. (1975) Arch. Biochem. Biophys. 171, 459-465
14. Jomain-Baum, M., Schramm, V. L. & Hanson, R. W. (1976) J. Biol. Chem. 251, 37-44
15. Snoke, R. E., Johnston, J. B. & Lardy, H. A. (1971) Eur. J. Biochem. 24, 342-346
16. McDaniel, M. G., Reddy, W. J. & Boshell, B. R. (1972) Biochim. Biophys. Acta 276, 543-550
17. DiTullio, N. W., Berhoff, C. E., Blank, B., Kostos, V., Stack, E. J. & Saunders, H. L. (1974) Biochem. J. 138, 387-394
18. Marco, R., Pestana, A., Sebastian, J. & Sols, A. (1974) Mol. Cell. Biochem. 3, 53-70
19. Zaurendonk, P. F., Akerboom, T. P. M. & Tager, J. M. (1976) in Use of Isolated Liver Cells and Kidney Tubules in Metabolic Studies (Tager, J. M., Soling, H. D. & Williamson, J. R., eds) pp. 17-27, North-Holland, Amsterdam
20. Tischler, M. E., Friedrichs, D., Coll, K. & Williamson, J. R. (1977) Arch. Biochem. Biophys. 184, 222-236
21. Krebs, H. A. (1976) in Use of Isolated Liver Cells and Kidney Tubules in Metabolic Studies (Tager, J. M., Soling, H. D. & Williamson, J. R., eds) pp. 299-308, North Holland, Amsterdam
22. Soling, H. D., Kleineke, J., Willms, B., Janson, G. & Kuhn, A. (1973) Eur. J. Biochem. 37, 233-243
23. Cornell, N. W., Loolu, P. & Krebs, H. A. (1974) Biochem. J. 142, 327-337
24. Rognstad, R. & Clark, D. G. (1974) Arch. Biochem. Biophys. 161, 636-649
25. Smith, S. B., Briggs, S., Triebwasser, K. C. & Freedland, R. A. (1987) Biochem. J. 162, 453-455
26. Metzger, A. J., Gimpel, J. A., Deleeuw, G. A., Tager, J. M. & Williamson, J. R. (1975) J. Biol. Chem. 250, 7728-7738
27. Kornacker, M. S. & Lowenstein, J. M. (1965) Biochem. J. 94, 209-219
28. Clark, D. G., Rognstad, R. & Katz, J. (1974) J. Biol. Chem. 249, 2028-2036
29. Hackenschmidt, J., Barth, C. & Decker, K. (1972) FEBS Lett. 27, 131-133
30. Cheema-Dhadii, S., Halperin, M. L. & Leznoff, C. C. (1973) Eur. J. Biochem. 48, 98-102
31. Triscari, J. & Sullivan, A. C. (1977) Lipids 12, 357-363
32. Mallette, L. E., Exton, J. H. & Park, C. R. (1969) J. Biol. Chem. 244, 5713-5723
33. Williamson, J. R., Anderson, J. & Browning, E. T. (1970) J. Biol. Chem. 245, 1717-1726
34. Garrison, J. C. & Haynes, R. (1973) J. Biol. Chem. 248, 2679-2777
Rate-limiting steps in metabolic pathways.
R Rognstad

J. Biol. Chem. 1979, 254:1875-1878.

Access the most updated version of this article at http://www.jbc.org/content/254/6/1875

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/254/6/1875.full.html#ref-list-1