The Control of the Synthesis of Pyruvate Carboxylase in 
Pseudomonas citronellolis

EVIDENCE FROM DOUBLE LABELING STUDIES*

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The level of pyruvate carboxylase in Pseudomonas citronellolis is controlled by the carbon source of the growth medium. The activity of the enzyme is highest in cells grown on lactate or glucose and virtually absent in cells grown on malate or aspartate. Double labeling studies with 3H- and 14C-labeled leucine confirm that pyruvate carboxylase is synthesized in the presence of lactate but not in the presence of aspartate. The studies also show that coordinated regulation occurs at the level of the synthesis of the two polypeptides which make up pyruvate carboxylase in P. citronellolis, rather than at the stages of their assembly into protomers or the biotinylation of the apoenzyme. There is no evidence for control of the catalytic activity of the holoenzyme via effectors. In all other varieties of pyruvate carboxylase examined thus far, the enzyme appears to be constitutive with regulation accomplished either through effector modulation of holoenzyme activity (pyruvate carboxylase from animal sources, yeast, several species of bacteria) or through control of the biotinylation of the apoenzyme by holocarboxylase synthetase (Bacillus stearothermophilus, yeast).

Pyruvate carboxylase (pyruvate:CO2 ligase (ADP), EC 6.4.1.1) is important in regulating the entry of pyruvate into gluconeogenic and anaplerotic pathways (1-5). The enzyme from chicken liver and other animal sources appears to be dependent for activity on the presence of acetyl-CoA or a closely related analog (4). In addition the enzyme from chicken liver may be inhibited by a variety of other acyl-CoA compounds such as methylmalonyl-CoA and acetoacetyl-CoA (4, 5). Thus, in animal species effective control of pyruvate carboxylase may be provided by a balance of effectors that reflects the physiological state (6). Pyruvate carboxylase from yeast (7) and from a number of bacterial species such as Bacillus stearothermophilus (8) and Arthrobacter globiformis (9) are stimulated by acyl-CoA compounds and inhibited by aspartate which may be considered to be an end product of the anaplerotic function of pyruvate carboxylase (3, 4). In these microbial species pyruvate carboxylase may also be regulated by a balance between positive and negative stimuli. In addition, in some microbial species, an additional form of control is provided by the influence of activators (acyl-CoA) and inhibitors (aspartate) on the biotinylation of the apoenzyme. This unique type of control has been demonstrated thus far in B. stearothermophilus (10, 11) and yeast (12) but does not appear to occur in liver (13).

In contrast to the apparent regulation of many varieties of pyruvate carboxylase by effectors, an extensive search for similar activators or inhibitors of the enzyme from Pseudomonas citronellolis has not disclosed any regulating metabolites. The bacterial enzyme is not activated by acetyl-CoA (14) or by palmityl-CoA (15, 16) which is the most effective activator of the enzyme from yeast (3, 17) and is not inhibited by aspartate or by more than 20 other metabolites that have been tested (15, 16). On the other hand, the present studies show that the level of pyruvate carboxylase in P. citronellolis is dependent on the carbon source of the growth medium and hence control of the oxalacetate concentration level in this organism may be vested at least in part in this sort of “coarse” control. The present communication is concerned with the nature of this process.

MATERIALS AND METHODS

Studies of Changes in Enzyme Activities

Growth of Bacteria—Pseudomonas citronellolis was obtained from Dr. W. Seubert (18) University of Göttingen and grown in a minimal medium (pH 7.2) which contained per liter: KH2PO4, 9.45 g; KH2PO4, 2.7 g; NH4Cl, 1.0 g; MgSO4, 0.2 g; CaCl2, 10 mg; (NH4)2MoO4, 5 mg; MnSO4, 1 mg; and a carbon source. Except where stated otherwise, the concentration of the various carbon sources per liter of medium were: acetate, 5 g; L-aspartate, 10 g; citrate, 10 g; D-glucose, 10 g; L-glutamate, 10 g; L-malate, 6 g; L-malate, 10 g; pyruvate, 10 g; succinate, 10 g. The cells were maintained on a medium containing lactate.

 Cultures (610 ml in a 2.8-liter Fernbach flask) were agitated at 30° on a rotary shaker. After centrifugation (2°) the cells were suspended in cold extracting buffer (potassium phosphate, pH 7.2, 100 mM; EDTA, 5 mM; MgCl2, 1 mM; dithioerythritol, 0.1 mM). No loss of enzyme activity...
was detected when cells were stored at -15°C for several weeks.

**Measurement of Enzyme Activities**—Cells were normally disrupted in a French pressure cell at 8,000 p.s.i. for assay of pyruvate carboxylase, the crude extract was centrifuged (25') at 80,000 x g for 60 min and the activity was measured spectrophotometrically in the supernatant fraction by the procedure described previously (15) in which the reaction was coupled with malate dehydrogenase. The reaction (25') was started by the addition of ATP and the observed activity was corrected for the rate of NADH oxidation before ATP was added. Protein concentrations were determined by the method of Lowry as described by Layne (19).

**Double Labeling Studies**

**Growth of Cells**—Cells were grown as described above except that the medium in the Fennbach flask was supplemented with L-[4,5-3H]leucine (1.66 µCi per ml) or L-[1-14C]leucine (0.42 µCi per ml) and with nonradioactive L-leucine (5 µg per ml). Unless otherwise noted in the simultaneous double labeling studies the cells in which pyruvate carboxylase was induced were grown in lactate medium in the presence of [14C]leucine and cells without pyruvate carboxylase were grown in aspartate medium in the presence of [3H]leucine. Growth was terminated in late exponential phase (275 to 278 Klett units). The cultures (610 ml) were rapidly cooled to 5°C, combined with thorough mixing and harvested at 2°C by centrifugation.

**Partial Purification of Pyruvate Carboxylase**—Cells were washed twice in 0.1 M Tris-HCl, pH 7.6, and resuspended in extracting buffer (final volume, 8 ml). After addition of approximately 100 µg of DNase I, the cells were lysed in a French pressure cell. The lysate was heated to 50°C for 6 min, cooled rapidly to 8°C, and centrifuged (35,000 x g for 40 min). To the supernatant fraction was added 1.5 volumes of 2% lysozyme in distilled water. The extensive precipitate of nucleic acids and acidic proteins (20) was removed by centrifugation (27,000 x g for 20 min). Approximately 5 ml (50 to 70 mg of protein) from the supernatant solution from the lysozyme precipitation was applied to a small DEAE-Sephadex A-50 column (1.4 x 9.4 cm) and eluted at 23°C using a 100-ml linear gradient from 2 to 5% ammonium sulfate in column buffer (25 mm Tris-HCl, pH 8.0, at 0°C). 0.8 mm EDTA, 0.1 mm dithiothreitol). The two effluent fractions (volume, 1 ml) with the highest activity of pyruvate carboxylase were combined and stored at 25°C overnight. The specific activity of the partially purified enzyme was 0.46 to 0.69 unit per mg compared with a specific activity of 14 to 18 units per mg for the highly purified enzyme (15, 16).

**Polyacrylamide Gel Electrophoresis**—The pH 5.5/0.75 M acrylamide gel procedure of Williams and Reisfeld (21) was modified to include 0.1 M sucrose in the 5% acrylamide gels. The sample was dialyzed for 30 min against 50 mm Tris-HCl, pH 7.6, and from 6 to 120 µg of protein applied to each gel (0.6 x 7.0 cm). Gels were stained for 2 hours in Coomassie blue and destained by transverse electrophoresis in 2,000 spectrophotometer. Electrophoresis of the sodium dodecyl sulfate. The molecular weights of the dissociated peptides were estimated from a calibration curve that used phosphorylase A (molecular weight, 94,000), bovine serum albumin (68,000), glutamate dehydrogenase (53,000), and ovalbumin (43,000) as standards.

**Fractionation of Polyacrylamide Gels**—Best results were obtained when a new razor blade was used to slice each gel into 2-mm sections, care being taken to separate the bands cleanly. For the double labeling experiments, gel sections were placed in test tubes (1.0 x 7.5 cm), covered with 1.0 ml of NCS tissue solubilizer (Amersham-Searle) and incubated for 4 hours at 65°C with occasional shaking (23). The gel and solubilizer were then transferred to scintillation vials with the aid of 10 ml of toluene scintillation fluid (16) and the vials stored in the dark at 5°C overnight before counting.

To determine the distribution of pyruvate carboxylase activity in the gel (Fig. 4B) the Autogel divider (Savant Instrument Co.) was used with elution of crushed fragments by 0.1 M Tris-HCl, pH 7.6. Selected fractions were added to cuvettes containing 0.5 ml of double strength pyruvate carboxylase assay mixture. For comparison of the distribution of the enzymatic activity with the [14C]leucine labeling in this experiment (Fig. 4B), the contents of each cuvette were transferred to an unsealed scintillation vial and incubated with 1 ml of 15 M NH4OH overnight (23') before Bray's solution (25) (10 ml) was added to the vials.

**Counting of Radioactivity**—Radioactivity was counted in a Packard Tri-Carb model 3290 Liquid Scintillation spectrometer. The 3H radioactivities were corrected for crossover from the 14C channel and were multiplied by the average 14C:3H ratio in the fractions not significantly enriched with 14C or 3H (26). The 14C:3H ratio in each fraction was normalized by dividing by the average 14C:3H ratio.

**Materials**

[3H]Biotin (115 µCi per mg), L-[4,5-3H]leucine (5 µCi per mmol) and L-[1-14C]leucine (28.7 µCi per ml) were obtained from New England Nuclear. Acrylamide was recrystallized from chloroform.

**RESULTS**

**Changes in Activity of Pyruvate Carboxylase and Lactate Dehydrogenase**

The specific activity of pyruvate carboxylase in *Pseudomonas citronellolis* depends on the carbon source supplied in the growth medium. When the organism is grown on glucose or lactate, a relatively high activity of pyruvate carboxylase is found in the cells whereas cells grown on dicarboxylic acids such as aspartate or malate have almost no detectable activity (Table I). Certain other compounds result in intermediate levels of enzyme activity. For cells grown in lactate medium the specific activity of pyruvate carboxylase varies during the growth cycle, reaching a peak about the middle of the exponential phase of growth and then declining to a value about half that of the peak by the time the stationary phase is reached (Fig. 1). However, the variations in enzyme activity that are experienced within the growth cycle are much smaller than the differences in specific activity that are observed in Table I with different carbon sources. To confirm this, cells were grown in aspartate medium and enzyme activities were measured. The specific activity of pyruvate carboxylase during the entire period of growth on aspartate (not shown) did not vary significantly from a mean value of 0.003 unit per mg (16). This level of activity is 1 order of magnitude lower than the lowest specific activity (0.042 unit/mg) observed during growth on lactate (Fig. 1).

1 This procedure is a modification of the method of Inouye and Guthrie (24) by Dr. A. Csordas (personal communication).
stearothermophilus is inhibited by aspartate. The low level of was exerted at the biotinylation step. Sundaram et al. (27) whether the control of carboxylase synthesis in P. citronellolis suggest that protein synthesis is required for the formation of failed to grow and the specific activity of the pyruvate carboxylase increased from 0.006 to 0.11 unit per mg. In another experiment, cells grown on lactate medium were transferred to new media containing aspartate medium were added biotin,2 suggesting that the formation of pyruvate carboxylase of Bacillus stearothermophilus is inhibited by aspartate. The low level of holoenzyme in these cells was apparently due to a biotin deficiency. To test for the possibility of a similar situation in P. citronellolis, cells were grown on a biotin-enriched medium containing aspartate. The specific activity of the pyruvate carboxylase was not increased over that of cells grown without added biotin, suggesting that the formation of pyruvate holocarboxylase in aspartate cells is not limited by the availability of biotin. In another experiment, cells grown on aspartate medium were transferred to new media containing (a) lactate, and (b) lactate and chloramphenicol (100 µg per ml), respectively. The cells in the lactate medium grew exponentially after a 1 hour lag and the pyruvate carboxylase specific activity increased from 0.006 to 0.11 unit per mg. In contrast, the cells in the medium containing chloramphenicol failed to grow and the specific activity of the pyruvate carboxylase remained constant at a low level. These results suggest that protein synthesis is required for the formation of pyruvate carboxylase and that control of this process is not exerted at the biotinylaton step as in the case of Bacillus subtilis (27) where conversion of the apoenzyme to the holoenzyme occurs in the presence of inhibitors of protein synthesis.

**Double Labeling Studies Using [14C]Leucine and [3H]Leucine**

The inhibition by chloramphenicol of the formation of pyruvate carboxylase in the previous experiment was not sufficient evidence to establish the control of synthesis of pyruvate carboxylase as the mechanism regulating the level of this enzyme in P. citronellolis. It was possible, for example, that the protein synthesized in the absence of chloramphenicol was not pyruvate carboxylase but another protein that was required for the assembly of the polypeptides into the apoenzyme or for the biotinylation of pre-existing apoenzyme. To identify the protein whose synthesis is controlled, we employed the double labeling technique of Kolber and Stein (28).

Partially purified pyruvate carboxylase was obtained from cells grown on aspartate-[14C]leucine and lactate-[3H]leucine as described under “Materials and Methods” and subjected to disc gel electrophoresis. The results are shown in Fig. 2 where the upper half of the figure shows the various protein peaks and the lower half of the figure gives the radioactivity in the various peaks. Peak II (lower part of Fig. 2) is greatly enriched in 14C relative to its 3H content and therefore is synthesized on lactate but not aspartate. This is in contrast to the other seven peaks where the 14C:3H ratio is constant. The results suggest that Peak II might be pyruvate carboxylase synthesized only in lactate cells.

**Identification of Pyruvate Carboxylase—Pyruvate carboxylase is a biotin-containing protein and advantage was taken of this fact to identify Peak II more definitively. The partially purified fraction from the double labeling experiment was treated with avidin prior to fractionation by disc gel electrophoresis with results as shown in Fig. 3. Two new protein peaks have appeared (designated A1 and A2) corresponding to the avidin-biotin protein complexes. Avidin itself has a net positive charge (29) and does not enter the gel unless it forms a complex with a negative-charged biotin-protein such as pyruvate carboxylase. In the bottom half of the same figure it will be seen that most of Peak II has disappeared and that there is no excess 14C in the residual Peak II. Rather, the excess 14C is now found in A1 and A2 confirming that Peak II is a biotin-containing protein that is synthesized only in the

2B. L. Taylor, S. Routman, and M. F. Utter, unpublished observation.

3Avidin has four binding sites for biotin (28) and Pseudomonas pyruvate carboxylase is a dimer with two biotin moieties (15). The most likely complex to be formed in the presence of an excess of avidin would consist of one pyruvate carboxylase and two avidins but other complexes are possible and no doubt account for the presence of more than one avidin complex in Fig. 3.
IV also contained a small amount of radioactivity but this may be derived from acetyl-CoA carboxylase or geranyl-CoA carboxylase (14) which are also present in P. citronellolis. Highly purified pyruvate carboxylase from P. citronellolis showed an $R_f$ essentially identical with that of Peak II under similar conditions. Finally, direct measurement of pyruvate carboxylase activity after elution of the protein from the gel also indicated that the activity was located in Peak II (Fig. 2).

The sum of this evidence strongly supports the view that Peak II is pyruvate carboxylase.

Identification of Band II as pyruvate carboxylase. A comparison of the distribution of $[^{3}H]$biotin (A-A) and cellular proteins (- - - -) in a partially purified preparation from cells grown in lactate media with the distribution of $[^{14}C]$radioactivity (O-O) in a gel similar to the one used for Fig. 2 but fractionated with the Autogel-divider. The specific activity of pyruvate carboxylase before electrophoresis was 0.41 unit per mg. The details of the experimental procedures are described under "Materials and Methods."

In another experiment, cells were grown in lactate medium containing $[^{3}H]$biotin (but not labeled leucine) in which case pyruvate carboxylase is labeled with $[^{3}H]$biotin (12). The cells grown on labeled biotin were fractionated in a similar fashion to that described above. The partially purified material gave a protein profile (Fig. 4A) that is generally similar to that shown in Fig. 2. Peak II was highly labeled with tritiated biotin as indicated by a spectrophotometric scan of the fractionated proteins; B, comparison of the distribution of radioactivity in proteins from cells grown in lactate (O---O, $[^{14}C]$leucine) and aspartate (O-O, $[^{3}H]$leucine) media.

The presence of lactate. These results suggest very strongly that the protein in question is pyruvate carboxylase.

The results of such an experiment are shown in Fig. 5. There are nine major bands in the upper portion of the figure with molecular weights ranging between 91,000 and 32,000. The radioactivity measurements of the various peaks are shown in the middle of Fig. 5 and the normalized $[^{14}C]/[^{3}H]$ in the bottom graph. The results show that Peaks 3 and 6 contain excess $[^{14}C]$ for the appropriate polypeptide should be one or perhaps even lower (31). This would be true, of course, only if the unassembled reacting form of the bacterial enzyme (15) would thus contain 2 molecules of each of these peptides (i.e., $\alpha_{1}\beta_2$).

To obtain information about the synthesis of individual peptides, the partially purified material from a double labeling experiment was fractionated by the sodium dodecyl sulfate gel electrophoretic procedure of Weber and Osborn (22). If the component polypeptides of pyruvate carboxylase are synthesized only in lactate cells, the $[^{14}C]/[^{3}H]$ ratio of the polypeptides derived from the enzyme should be similar to that observed for the holoenzyme in Fig. 2. However, if either or both of the polypeptides are also synthesized in the aspartate medium but not assembled into the holoenzyme, the ratio of $[^{14}C]/[^{3}H]$ for the appropriate polypeptide should be one or perhaps even lower (31). This would be true, of course, only if the unassembled polypeptides are retained by the partial fractionation procedures employed in these experiments.

The results of such an experiment are shown in Fig. 5. There are nine major bands in the upper portion of the figure with molecular weights ranging between 91,000 and 32,000. The radioactivity measurements of the various peaks are shown in the middle of Fig. 5 and the normalized $[^{14}C]/[^{3}H]$ in the bottom graph. The results show that Peaks 3 and 6 contain excess $[^{14}C]$ in ratios of 7 and 2, respectively. The estimated molecular weights of the two polypeptides represented in Peaks 3 and 6 are 67,000 and 55,000, respectively, which are in excellent agreement with the previously determined molecular weights (16, 32) of the two polypeptides which make up the bacterial pyruvate carboxylase. In Fig. 5 the ratio of $[^{14}C]/[^{3}H]$ in Peak 3 is
Fig. 5. The composition of the sodium dodecyl sulfate-dissociated soluble proteins from doubly labeled cells. A, spectrophotometric scan of the separated polypeptides. The molecular weights of the peptides were Band 1, 91,000; Band 2, 79,000; Band 3, 67,000; Band 4, 64,000; Band 5, 59,000; Band 6, 55,000; Band 7, 50,000; and Band 8, 46,000; and Band 9, 32,000. B, the distribution of the radioactivity in the polypeptides from lactate (---O, [14C]leucine) and aspartate (O——O, [3H]leucine) cells. C, normalized ratios for the 14C and 3H radioactivities in B. The details of the procedures and calculations are described in the text.

about 7 which is in good agreement with the ratio of 8 observed for the holoenzyme, but the ratio is considerably lower in Peak 6. An examination of the middle part of Fig. 5 would suggest that Peak 6 is poorly resolved with the 14C spreading over two fractions, thereby lowering the apparent ratio of 14C:3H. A less likely explanation is that the smaller polypeptide is synthesized in the aspartate cells. Further evidence to be discussed in the next section does not support this hypothesis.

Sequential Labeling of Cells with [14C]Leucine and [3H]Leucine

The above experiment (Fig. 5) supports the thesis that in P. citronellolis the regulation of pyruvate carboxylase synthesis by the carbon source occurs at the level of polypeptide synthesis rather than at the stage of assembly of these chains or their biotinylation. Further, the experiments suggest that the synthesis of the two polypeptides must be subject to some coordinated type of control. To test this hypothesis cells were grown in aspartate medium with [14C]leucine (turbidity = 1.35) and then transferred to lactate medium containing [3H]leucine (turbidity = 0.8). Growth was continued for 8.4 hours (final turbidity = 1.4). Any pyruvate carboxylase components which were synthesized in the aspartate medium would be assembled in the lactate medium and would be detected as increased 14C in Peak II (cf. Fig. 2). In addition, since the radioactive labels added to the aspartate and lactate media in this experiment were reversed with respect to those used previously, it would be possible to detect any unusual quenching of tritium that might have occurred in the other experiments, particularly in Peak II. The results are shown in Fig. 6. By comparison with Fig. 2, Peak II in the upper half of the figure was increased relative to Peak I and other proteins. This was due to the higher specific pyruvate carboxylase activity in the partially purified material used in this experiment. In the lower half of Fig. 6, Peak II had a normalized 3H:14C ratio of about 9.5 which was actually a little higher than the converse experiments and is consistent with the synthesis of pyruvate carboxylase in lactate medium only. The distribution of radioactivity in the sodium dodecyl sulfate-dissociated sample (not shown here) from the sequential labeling study was very similar to that of Fig. 5B. Tritium enrichment was noted only in Peaks 3 and 6 with ratios of 3H:14C of 5.8 and 2.3 in the two peaks, respectively. These ratios are lower than that of the holoenzyme (Fig. 6). The discrepancy probably reflects a failure to separate Peak 3 and 6 cleanly from their neighbors, particularly in the case of Peak 6. The general results are very similar to those of Fig. 5B in this regard. However, in this second experiment the cells were grown sequentially on 14C medium and then 3H and any polypeptide synthesis during growth on aspartate in the 14C should have been reflected in the enrichment ratio of the holoenzyme as well as in the polypeptides. This was not the case, supporting the thesis that the individual polypeptides are synthesized only in the lactate medium.

Other experiments not shown here indicate that the turnover
of pyruvate carboxylase was not a significant factor in reducing the ¹⁴C in Peak II. In those experiments, cells grown in lactate medium were transferred to aspartate medium containing chloramphenicol (100 μg per ml) and the specific activity of pyruvate carboxylase was measured at intervals. There was no significant loss of pyruvate carboxylase activity over 7 hours indicating that pyruvate carboxylase apparently turns over at a relatively slow rate. Considered in toto, the double labeling studies indicate that aspartate cells contain little or no pyruvate apo-carboxylase or unassembled polypeptides.

Induction of Pyruvate Carboxylase by Pyruvate or Closely Related Metabolite

To distinguish between induction (derepression) of pyruvate carboxylase by a product formed from glucose and lactate (perhaps pyruvate) and repression by a product formed from aspartate and malate (cf. Table I), cells grown on aspartate were used to inoculate a medium containing both aspartate and lactate. It was anticipated that if control was achieved by repression, pyruvate carboxylase would not be synthesized in the medium with aspartate and lactate present. Alternatively, if the enzyme was induced in the presence of lactate, pyruvate carboxylase levels should increase sharply when aspartate cells were transferred from aspartate medium to one with both carbon sources. Growth studies of the type shown in Fig. 1 showed that pyruvate carboxylase and lactate dehydrogenase were synthesized at normal levels in medium which contained both aspartate and lactate. This suggested that the synthesis of both enzymes is controlled by the presence of lactate or a product derived from this compound rather than by the presence of aspartate.

On the other hand, when lactate cells were washed and transferred to aspartate medium there was little growth in the first 6 hours, presumably because the aspartate transport system is not constitutive and was synthesized only slowly or was inhibited in the lactate medium. The possibility had to be considered that aspartate was not transported into the cells in the presence of lactate and was therefore unable to repress the synthesis of pyruvate carboxylase in lactate plus aspartate medium. Other experiments made this appear unlikely. When the concentration of aspartate in the lactate plus aspartate medium was increased from 2.5 to 15 g/liter there was no significant decrease in pyruvate carboxylase levels as might be expected if active transport of aspartate was impaired. Furthermore, in cells grown on medium containing succinate, glutamate, malate, or citrate, pyruvate carboxylase was invariably induced if lactate was also present in the medium (Table II) but was not induced in the absence of lactate (Table I). It is possible but unlikely that the transport mechanisms for each of these possible repressors and for aspartate is affected in an identical fashion by lactate.

It has not yet been possible to identify the metabolite responsible for pyruvate carboxylase synthesis. However, activity (0.048 unit/mg, cf. Table II) was induced in cells grown on pyruvate, suggesting that pyruvate or a closely related metabolite such as acetyl-CoA may be responsible. Attempts to induce pyruvate carboxylase synthesis by including 5 mM cyclic adenosine 3':5'-monophosphate in aspartate medium were not successful.

DISCUSSION

The regulation of pyruvate carboxylase by allosteric effectors has been suggested for all varieties of the enzyme that have been investigated, with the exception of that from P. citronellolis (see the introduction), but there has been considerable discussion as to whether synthesis of pyruvate carboxylase is also regulated. It has been claimed that the level of this enzyme in liver is increased in diabetes, after glucocorticoid administration and during fasting (32-34). These claims have been disputed by other investigators (35-37) and recent studies in this laboratory have shown that when pyruvate carboxylase is assayed under optimal conditions that no significant difference in the amount of total enzyme present in the liver can be detected in the enzyme activity in diabetic and normal rats. At present there is no compelling evidence from any vertebrate tissue that the total amount of pyruvate carboxylase is increased in diabetes or fasting.

Similarly, until the present study the induction or repression of pyruvate carboxylase had not been shown in a microbial species. Where changes in the levels of pyruvate carboxylase had been investigated in B. stearothermophilus (10, 11, 27) and yeast (12) as noted earlier, it would appear that the biotinylation step catalyzed by pyruvate holocarboxylase synthetase is regulated rather than protein synthesis (10, 11). Regulation of the formation of pyruvate carboxylase at the holoenzyme synthetase reaction by a limiting supply of biotin has also been shown in rat liver (38, 39) and chicken liver (13, 40).

It is clear from the results that have been presented here that the formation of pyruvate carboxylase in P. citronellolis is controlled at the level of the synthesis of the polypeptides and not by other mechanisms that have been mentioned. The presence of two ¹⁴C-enriched peaks in the sodium dodecyl sulfate-dissociated fractions (Fig. 5) is additional evidence that the two bands previously observed (16, 30) after sodium dodecyl sulfate gel electrophoresis of highly purified Pseudomonas enzyme are both derived from pyruvate carboxylase and that the protomer of the Pseudomonas enzyme consists of two different polypeptides.

In P. citronellolis the action of pyruvate carboxylase is opposed by oxaloacetate decarboxylase, that is, pyruvate is converted to oxalacetate by pyruvate carboxylase and oxalacetate is converted to pyruvate by oxalacetate decarboxylase. Together, these two reactions constitute a potential futile cycle with ATPase activity (41). The induction of pyruvate carboxylase in this organism provides one mechanism for regulating the futile cycle. From the results obtained it would seem likely that the inducing compound is pyruvate or a closely related metabolite such as acetyl-CoA (Tables I and II). Thus, in the

\*R. Yonasaki and M. F. Utter, unpublished experiments.

| Table II |
| Effect of second carbon source on synthesis of pyruvate carboxylase during growth in lactate medium |
| Additional carbon source | Specific activity (μmoi/min/mg) |
|--------------------------|-----------------------------|
| None                     | 0.084-0.117                 |
| Malate                   | 0.139                       |
| Succinate                 | 0.077                       |
| L-Glutamate               | 0.056                       |
| Citrate                  | 0.089                       |
| L-Aspartate              | 0.056                       |

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presence of excess pyruvate, oxalacetate formation may be stimulated by the induction of pyruvate carboxylase, thereby facilitating the oxidation of pyruvate. Also, oxalacetate decarboxylase is inhibited by acetyl CoA (41, 42). By these two mechanisms acetyl-CoA may be controlling the level of pyruvate in P. citronellolis as in most other species that have been studied but in an indirect fashion.

At present the properties of pyruvate carboxylase from P. citronellolis are unique among the varieties of this enzyme that have been studied extensively. The bacterial enzyme is a dimer Pseudomonas pyruvate carboxylase probably reflects the lim-

citronellolis are unique among the varieties of this enzyme that

ited investigation of this enzyme in microorganisms and it is likely that similar properties will be found in some other varieties of the enzyme. Scruton has recently investigated pyruvate carboxylase from Azotobacter vinlandii. The results suggest that this variety of the enzyme is also without activators or inhibitors, that the reacting form of this enzyme is a dimer and that the enzyme is not constitutive (43). Large fluctuations in the levels of pyruvate carboxylase accompany changes in the growth conditions for Rhizopus nigricans (44) and Aspergillus niger (45). It is possible that further studies will reveal that pyruvate carboxylase is not constitutive in these organisms also.

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