Proc. Nat. Acad. Sci. USA
Vol. 72, No. 11, pp. 4308–4312, November 1975
Biochemistry

Structural properties of pyruvate carboxylases from chicken liver and other sources

(biotin enzyme/subunits/avidin-Sepharose/protomer/enzymatic structure)

ROLAND E. BARDEN*, BARRY L. TAYLOR†, FUMIHIDE ISOHASHI‡, WILLIAM H. FREY, II§, GARY ZANDER, JAMES C. LEE¶, AND MERTON F. UTTER‖

Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio 44106

Contributed by Merton F. Utter, August 18, 1975

ABSTRACT Varieties of pyruvate carboxylase [pyruvate: CO_2 ligase (ADP-forming), EC 6.4.1.1] obtained from the livers of several species of vertebrates, including humans, all show the same basic structure. They are composed of large polypeptide chains of molecular weights ranging from 1.2 to 1.3×10^5 for the different varieties of the enzyme. The native form of the enzyme appears to be a tetramer with a molecular weight of about 5×10^5. In the case of pyruvate carboxylase from chicken liver each polypeptide chain contains a biotin moiety, thus supporting the thesis that the tetramer contains four identical polypeptide chains. Pyruvate carboxylase from yeast appears to be basically similar to those from the vertebrate species and has a tetrameric structure.

Each protomer contains a single polypeptide chain with a molecular weight of 1.25×10^5. In contrast, pyruvate carboxylase from two bacterial species, *Pseudomonas citronellolis* and *Azotobacter vinelandii*, appears to be a dimer with a molecular weight (2.5×10^5) about half that of the animal and yeast species. As a further difference, each of the protomers of the bacterial enzymes contain two polypeptides of 6.5 and 5.4×10^5 molecular weight in the case of the *Pseudomonas* enzyme. The larger of the two polypeptides contains the biotin moiety. The functional units of the bacterial enzyme thus appear to contain two polypeptides while that of the liver and yeast enzymes is made up of a single chain. Neither of these arrangements corresponds with those of other biotin enzymes whose structure has been extensively studied (acetyl-CoA carboxylases from liver or *Escherichia coli*, and transcarboxylase from *Propionibacterium*).

Pyruvate carboxylase [pyruvate:CO_2 ligase (ADP-forming) EC 6.4.1.1] is a biotin-containing enzyme which forms oxalacetate from pyruvate by CO_2 fixation (1, 2). The overall reaction mechanism is similar to that of other biotin enzymes and consists of two partial reactions. In the first partial reaction biotin is carboxylated; in the second the carboxyl group is transferred to an acceptor (cf. 3). For transcarboxylase (4, 5) and for pyruvate carboxylase from the liver of chicken (6) and rat (7, 8), kinetic studies indicate that the active site is composed of two separate catalytic sites, one for each partial reaction.

Structural studies with transcarboxylase from *Propionibacterium shermanii* (9) and acetyl-CoA carboxylase from *Escherichia coli* (3, 10) have revealed a close correlation between function and structure. These biotin enzymes exhibit a tripartite structure–function relationship in that each of three different polypeptides contributes an essential part to a given active site.

For acetyl-CoA carboxylase from *E. coli*, one polypeptide carries the biotin, a second polypeptide participates in catalysis of the biotin carboxylation partial reaction, and a third polypeptide carries out the carboxyl-group transfer partial reaction. For transcarboxylase, one polypeptide carries the biotin while each of the other two polypeptides carries out a carboxyl-group transfer reaction between the biotin and the α-keto acid or acyl-CoA substrates, respectively. Acetyl-CoA carboxylase from chicken liver contains four different polypeptides, but the function of each has not been established (11).

In order to determine whether the similarity in overall reaction mechanisms among biotin enzymes is also reflected in structural similarities, the structure–function relationship for pyruvate carboxylase has been investigated. This paper reports on the structure of pyruvate carboxylases from a number of vertebrate and two microbial sources. The results clearly establish that the structure of pyruvate carboxylase is not similar to that reported for other biotin enzymes. Furthermore, at least two different structural types exist among the pyruvate carboxylases. A preliminary report of some of the data presented here has been given (12).

EXPERIMENTAL PROCEDURE

Enzyme Purification. Highly purified pyruvate carboxylase was isolated from chicken liver (13), *Saccharomyces cerevisiae* (14), and *Pseudomonas citronellolis* (15) essentially as previously described. The isolation of pyruvate carboxylase from the livers of rat, pig, turkey and calf followed procedures similar to those used with chicken liver. A partially purified preparation was also obtained from an autopsy sample of human liver.

Pyruvate carboxylase was routinely assayed at 25° by coupling the reaction with malate dehydrogenase and observing the decrease in absorbance at 340 nm (13, 14, 16).

Electrophoresis Techniques. Electrophoretic analysis of dissociated enzyme on sodium dodecyl sulfate-polyacrylamide gels was performed by the method of Weber and Osborn (17), except that the gels contained 5 or 8% acrylamide. Immediately after mixing the enzyme with sodium dodecyl sulfate and 2-mercapotoethanol, the sample was placed in a boiling-water bath for 5 min to ensure denaturation of any proteolytic enzymes that might be present (18). Protein in the gels was fixed and stained with Coomassie blue (17). Destaining was accomplished by transverse electrophoresis in 7% acetic acid, or by leaching in a solution containing 75

* Present address: Department of Chemistry and Division of Biochemistry, University of Wyoming, Laramie, Wyo. 82071.
† Present address: Department of Biochemistry, University of California, Berkeley, Calif. 94720.
‡ Present address: Department of Biochemistry, Cancer Institute of Osaka, University Medical School, Osaka, Japan.
§ Present address: Department of Pharmacology, University of Minnesota, Minneapolis, Minn. 55455.
¶ Present address: Graduate School of Biochemistry, Brandeis University, Waltham, Mass. 02154.
‖ To whom correspondence should be addressed.

Table 1. Weight of sodium dodecyl sulfate polypeptides, molecular weights, and number of polypeptides per molecule for pyruvate carboxylase from various species

Species	Weight of sodium dodecyl sulfate polypeptide	Molecular weight	Poly-peptides per molecule
Chicken liver	125,000	500,000*	4
Turkey liver	110,000	500,000*	4
Calf liver	120,000	500,000*	4
Rat liver	130,000	500,000	4
Pig liver	130,000	—	—
Human liver	130,000	—	—
Yeast	125,000	475,000	4
P. citronellolis	65,000	256,000	2
	54,000†		2

* Wallace and Utter, unpublished data (see *text*).
† Does not contain biotin.

ml of acetic acid, 425 ml of methanol, and 500 ml of H_2O. Spectrophotometric scans (at 570 nm) of the destained gels were prepared with the linear transport accessory for Gilford spectrophotometers. The radioactivity of labeled polypeptides ([^{3}H]biotin) in polyacrylamide gels was determined as previously described (19). In a few cases, the protein was dissociated in 8 M urea either with or without sodium dodecyl sulfate and subjected to electrophoresis on 5% polyacrylamide gel containing urea (20).

Ultracentrifugation Techniques. Experiments were performed in a Beckman-Spinco model E analytical ultracentrifuge as previously outlined (16). Where required, pertinent procedures are given in the figure legends.

For pyruvate carboxylase from chicken liver, the apparent partial specific volume for the native enzyme was determined by density measurements with a Precision Density Meter, DIKA-02, following the procedure of Lee and Timasheff (21). A value of 0.765 ± 0.004 ml/g was obtained in 0.1 M K phosphate, pH 7.0.

Protein Determinations. The protein concentration of pyruvate carboxylase solutions was routinely determined spectrophotometrically using the equation of Warburg and Christian (22). Frey and Utter (unpublished data) have shown that the values obtained by this method are too low by a factor of 2 for pyruvate carboxylase from chicken liver on the basis or protein values determined by dry weight and the biuret procedure of Layne (23). Conversion factors of 1.4 and 1.0 were obtained for the enzymes from yeast and *P. citronellolis*, respectively. These factors were used in the calculations of protein concentrations.

Preparation of Avidin-Sepharose. The covalent attachment of avidin to Sepharose 4B was accomplished by the procedure of Bodanszky and Bodanszky (24).

RESULTS AND DISCUSSION

Polypeptide Composition of Pyruvate Carboxylases. When pyruvate carboxylases from the livers of chicken, rat, pig, turkey, calf, and human, and from yeast were analyzed on sodium dodecyl sulfate-polyacrylamide gels, one prominent polypeptide band was observed in each case. Examples of the spectrophotometric scans of the electrophoretic patterns of pyruvate carboxylase from rat, chicken, and pig liver are shown in Fig. 1. The molecular weight is in the range of 1.3×10^5 in all three cases. However, the polypeptide chain from the chicken liver is slightly smaller than that

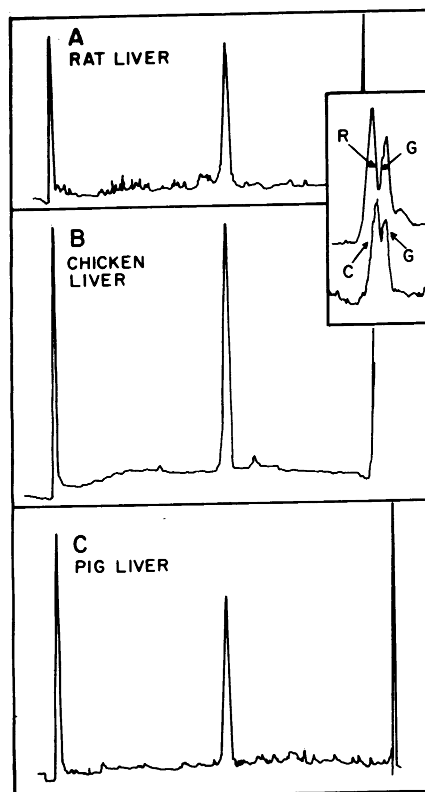

FIG. 1. Spectrophotometric scans of pyruvate carboxylase after electrophoresis in sodium dodecyl sulfate-polyacrylamide (5%) gel. Migration was from left to right. (A) A 5 μg sample of rat liver pyruvate carboxylase, specific activity = 15 μmol of oxalacetate per min per mg of protein; (B) a 7 μg sample of chicken liver pyruvate carboxylase, specific activity = 18; (C) a 5 μg sample of pig liver pyruvate carboxylase, specific activity = 23. The insert shows scans of gels containing 5 μg each of rat liver pyruvate carboxylase (R) and β-galactosidase (G) in the top panel and 5 μg each of chicken liver pyruvate carboxylase (C) and β-galactosidase (G) in the lower panel.

from rat liver, as shown in the insert of Fig. 1, where β-galactosidase has been used as an internal marker. Here, the protein from chicken liver is found closer to the standard than that from rat liver. The polypeptides from rat and pig liver appear to be virtually identical in size when tested in this same manner.

The molecular weight of the constituent polypeptides from all species of liver tested thus far has been in the range of 1.1 to 1.3×10^5 (Table 1) when proper precautions are taken to prevent proteolytic activity. After reduction and carboxymethylation of pyruvate carboxylase from rat liver, McClure *et al.* observed an apparent dissociation of a polypeptide of the 1.3×10^5 dalton polypeptide into a mixture of smaller polypeptides (25). Similar results were obtained in this work in preliminary studies with the enzymes from chicken liver and yeast. However, when traces of proteolytic activity were destroyed by placing the untreated enzyme samples in a boiling-water bath immediately after the addition of sodium dodecyl sulfate and mercaptoethanol (cf. 18), subsequent analysis on polyacrylamide gels revealed a single polypeptide band. With this treatment, the enzyme from rat liver also dissociated to give only the 1.3×10^5 polypeptide (Fig. 1A). Warren and Tipton (26) have reported that pyruvate carboxylase from pig liver has four subunits of 130,000 each but that each subunit is composed of three polypeptide chains of 47,000 molecular weight. These authors reported

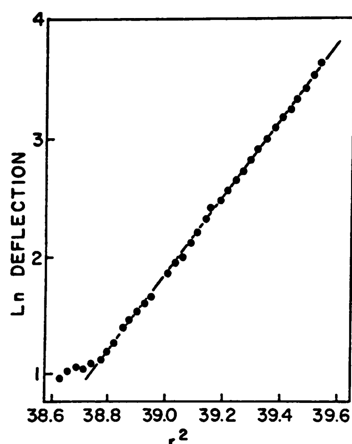

FIG. 2. The molecular weight of pyruvate carboxylase from chicken liver after dissociation in guanidine·HCl. The enzyme was dissociated in 6 M guanidine·HCl, 1 mM dithiothreitol for 24 hr. Prior to analysis, the guanidine·HCl concentration was reduced to 2 M by dialysis. Speed: 24,000 rpm; temperature: 5°; protein concentration: 0.4 mg/ml. The molecular weight was determined by the meniscus depletion method of sedimentation equilibrium using the interference optical system.

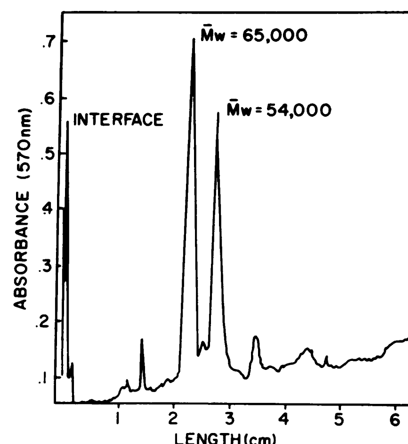

FIG. 3. Spectrophotometric scan of pyruvate carboxylase from *Pseudomonas citronellolis* in 8% polyacrylamide gel containing sodium dodecyl sulfate. A 7 μg sample of specific activity 17 was used.

that electrophoresis with sodium dodecyl sulfate showed a major band at 47,000 and a minor band at 125,000. As shown in Fig. 1C, there is no evidence for more than one band in our preparation. The basis of this discrepancy is not clear although Warren and Tipton (26) did not mention any procedure that would obviate proteolytic activity and their preparation had a specific activity of 3.2 compared with that of 23 in the present case.

Dissociation of the chicken and rat liver enzymes in 8 M urea or urea and sodium dodecyl sulfate followed by electrophoresis on polyacrylamide gel containing urea also showed only a single large polypeptide for each of the two enzymes. It is concluded, therefore, that the about 1.3×10^5 polypeptide (Table 1) represents the fundamental structural unit of the vertebrate varieties of pyruvate carboxylase; smaller peptides that have been observed presumably are the result of proteolysis or impurities.

Further evidence for the presence of a single large polypeptide for the enzyme from chicken liver was obtained by equilibrium sedimentation analysis of the dissociated enzyme in the presence of guanidine hydrochloride (Fig. 2). The molecular weight calculated from these data was 1.2×10^5, using the determined $\bar{v}$ value of 0.765 ml/g. These data strongly support the view that pyruvate carboxylase from chicken liver, and from other vertebrate sources, is composed entirely of polypeptides of large molecular weights and that for any given variety of the enzyme, the polypeptides are similar or identical in size. As will be noted below, these varieties of the enzyme appear to be tetrameric in their native form.

The polypeptide composition of pyruvate carboxylase from *P. citronellolis* is quite different from that of the enzymes described above. Even when precautions are taken to prevent proteolysis, two different polypeptides with molecular weights of 6.5×10^4 and 5.4×10^4 are observed in polyacrylamide gels (Fig. 3, Table 1). The two polypeptides are present in equimolar amounts, and the ratio between them is unchanged for preparations of the enzyme with various degrees of purity.

Determination of Biotin Distribution among the Polypeptide Chains of Pyruvate Carboxylase. The chicken liver enzyme contains 1 mole of biotin per polypeptide chain (G. Zander, J. Swack, and M. Utter, unpublished data). It has now been possible to show that each polypeptide chain contains a biotin. This was achieved by: (*i*) dissociating the enzyme in cold 0.4 M urea into the individual polypeptide chains (cf. 27), (*ii*) trapping all biotin-containing polypeptides by passage through an avidin-Sepharose column, (*iii*) analyzing by sodium dodecyl sulfate polyacrylamide electrophoresis the material which passes through the column (non-biotin-containing), and (*iv*) similarly analyzing that material which is trapped by the column (biotin-containing). About 90% of the applied material was trapped by the avidin-Sepharose column, while in a control experiment in which the column had been pre-treated with a tenfold excess of biotin almost no protein was bound.

This experiment demonstrates that almost all of the material in the dissociated enzyme is associated with biotin and therefore that each of the polypeptide chains probably contains biotin. For example, if the four biotins had been distributed among only two of the four chains only 50% of the protein should have been bound to the Sepharose-avidin column.

Further evidence to support this view is shown in Fig. 4. Spectrophotometric scans of the polyacrylamide gels in which the protein has been stained by Coomassie blue are shown for the material which was applied to the avidin-Sepharose column (Fig. 4A); the material which did not adhere to the column (Fig. 4B); and that which did adhere to the column (Fig. 4C). In the latter case the polypeptides were removed from the column by treatment with 1% sodium dodecyl sulfate. These results show clearly that the large polypeptide which is characteristic of pyruvate carboxylase (Fig. 1) has been almost completely removed by passage through the avidin-containing column (Fig. 4B) and that the peptide can be recovered from the column. The final panel in Fig. 4 shows that prior treatment of the avidin-Sepharose column with biotin prevents binding of the pyruvate carboxylase polypeptide. Thus, these results show that each large polypeptide chain contains covalently bound biotin and, since the stoichiometric relationship is 1:1, each chain must contain a single biotin. The results strengthen the view that the polypeptide chains of the avian enzyme are identical.

Pyruvate carboxylases from yeast and *P citronellolis* have been isolated from cells grown in the presence of [^{3}H]biotin. The tritiated biotin was incorporated into the enzyme in

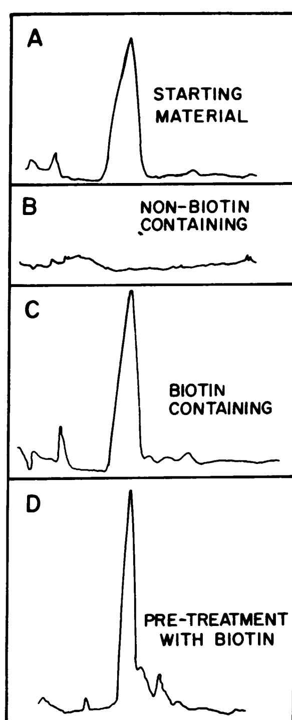

FIG. 4. Treatment of dissociated pyruvate carboxylase from chicken liver with avidin-Sepharose. (A) Spectrophotometric scan of a sodium dodecyl sulfate-polyacrylamide gel showing the starting material without treatment. (B) After 1 hr in cold 0.4 M urea, the inactivated and dissociated enzyme was treated with avidin-Sepharose. The material which did not bind to the column is shown in this scan. (C) After washing with 0.4 M urea, the avidin-Sepharose was extracted with 1% sodium dodecyl sulfate and the scan shows the pattern of the extracted material. (D) The avidin-Sepharose was pre-treated with an excess of biotin prior to its use and the scan shows the polypeptide pattern of the enzyme solution after treatment with avidin-Sepharose.

both cases. When the labeled yeast enzyme was subjected to electrophoresis in polyacrylamide gels, electrophoretic analysis indicated a single polypeptide with a molecular weight of 1.3×10^5. The [^{3}H]biotin was entirely associated with this polypeptide. The ratio of the moles biotin per mole polypeptide is 1:1 (cf. 14). Thus, it seems likely that each polypeptide in the yeast enzyme contains one biotin. On the other hand, analysis of the labeled *P. citronellolis* enzyme on sodium dodecyl sulfate gels revealed that biotin was associated with only the larger polypeptide (6.5×10^4 daltons); no radioactivity was associated with the smaller polypeptide (5.4×10^4 daltons).

Molecular Organization of Pyruvate Carboxylases. As a guide for establishing the number of polypeptides per molecule of pyruvate carboxylase, molecular weights for several species of the enzyme were determined by ultracentrifugation techniques. The enzyme from yeast was analyzed by the meniscus depletion sedimentation equilibrium method of Yphantis (28). The plot of ln deflection versus the square of the radium was linear (Fig. 5). A molecular weight of 4.75×10^5 was calculated from these data, assuming a partial specific volume of 0.765 ml/g (Table 1). Pyruvate carboxylases from the livers of chicken, calf, and turkey have been studied by the approach to equilibrium procedure of Trautman and Crampton (29), and in each case a mean molecular

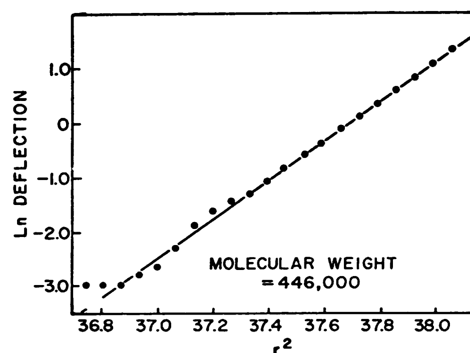

FIG. 5. The molecular weight of pyruvate carboxylase from yeast as determined by the meniscus depletion method of sedimentation equilibrium. Speed: 12,000 rpm; temperature: 6.1°; protein concentration: 0.17 mg/ml; solvent: 0.5 M Tris·HCl, pH 7.2, 1 mM EDTA, 0.1 mM dithiothreitol, and 0.2 M KCl; optics: absorption with a photoelectric scanner. Final scans were made after 20 hr. The base line was determined by overspeeding at 52,000 rpm for 2 hr, and scanning the cell after relaxing the speed to 12,000 rpm.

weight of about 5×10^5 was obtained (J. C. Wallace and F. Utter, unpublished data). Further support for a tetrameric structure as the native form of pyruvate carboxylases of animal origin comes from experiments that show the predominant reacting forms of the enzymes have sedimentation coefficients in the 15–16S range (16). This is entirely consistent with a tetrameric structure. Also, in electron micrographic studies of several species of the enzyme from animal liver and from yeast, tetrameric figures were observed with negative staining (1, 30, 31).

A molecular weight of 2.6×10^5 was recently reported for pyruvate carboxylase from *P. citronellolis* (16). If the two different polypeptides of this enzyme are added, the size (1.2×10^5 daltons) and biotin content are essentially the same as for the protomer of the tetrameric enzymes. Thus, these data suggest that a protomer of the *P. citronellolis* enzyme consists of two different polypeptides. Since the proposed protomer has a molecular weight which is one-half that of the native enzyme, pyruvate carboxylase from *P. citronellolis* is apparently a dimer. However, the exact arrangement of polypeptides in this enzyme is not known. Preliminary studies of pyruvate carboxylase from *Azotobacter* indicate that this enzyme is quite similar to the *Pseudomonas* enzyme (32).

Concluding Discussion. The available evidence suggests that the different biotin-containing enzymes vary significantly in their structural and functional relationships. For transcarboxylase (33) and acetyl-CoA carboxylase from *E. coli* (3, 10), each of the three different polypeptides found in these two enzymes contributes to a given catalytic site. There is one polypeptide for catalysis of each partial reaction and one peptide which carries the biotin. In contrast, pyruvate carboxylases from *P. citronellolis* and *A. vinelandii* have only two different polypeptides and thus one of these polypeptides must carry out at least two of the three functional requirements. A third possibility is demonstrated by pyruvate carboxylase from yeast and vertebrate sources, where each polypeptide appears to have a 3-fold functional role and must serve as biotin carrier as well as the catalyst for the two partial reactions. Acetyl-CoA carboxylase from chicken liver (11) may constitute still a fourth variety, since it has been reported that the protomeric unit of about 500,000 molecular weight contains four different polypeptide chains ranging in size from 117,000 to 139,000. Only

one of these contained biotin and the functional role of the four polypeptides is not understood.

The authors are grateful to Judy Swack and Maxwell Adams for assistance with some of the experiments reported here and to Dr. S. N. Timasheff of Brandeis University for the use of a density meter. This investigation was supported by Grant AM 11712 from the National Institutes of Health and by Atomic Energy Commission Contract AT-(11-1)-1242.

1. Utter, M. F., Barden, R. E. & Taylor, B. L. (1975) *Adv. Enzymol.* **42**, 1–72.
2. Scrutton, M. C. & Young, M. R. (1972) in *The Enzymes*, ed. Boyer, P. D. (Academic Press, New York), Vol. 6, pp. 1–35.
3. Moss, J. & Lane, M. D. (1971) *Adv. Enzymol.* **35**, 321–442.
4. Northrop, D. B. (1969) *J. Biol. Chem.* **244**, 5808–5819.
5. Northrop, D. B. & Wood, H. G. (1969) *J. Biol. Chem.* **244**, 5820–5827.
6. Barden, R. E., Fung, C.-H., Utter, M. F. & Scrutton, M. C. (1972) *J. Biol. Chem.* **247**, 132–1333.
7. McClure, W. R., Lardy, H. A., Wagner, M. & Cleland, W. W. (1971) *J. Biol. Chem.* **246**, 3579–3583.
8. McClure, W. R., Lardy, H. A. & Cleland, W. W. (1971) *J. Biol. Chem.* **246**, 3584–3590.
9. Wood, H. G. (1972) in *The Enzymes*, ed. Boyer, P. D. (Academic Press, New York), Vol. 6, pp. 83–115.
10. Alberts, A. W. & Vagelos, P. R. (1972) in *The Enzymes*, ed. Boyer, P. D. (Academic Press, New York), Vol. 6, pp. 37–82.
11. Guchhait, R. B., Zwergel, E. E. & Lane, M. D. (1974) *J. Biol. Chem.* **249**, 4776–4780.
12. Barden, R. E. & Taylor, B. L. (1973) *Fed. Proc.* **32**, 510.
13. Scrutton, M. C. & Fung, C.-H. (1972) *Arch. Biochem. Biophys.* **150**, 636–647.
14. Scrutton, M. C., Young, M. R. & Utter, M. F. (1970) *J. Biol. Chem.* **245**, 6220–6227.
15. Taylor, B. L. (1973) Ph.D. Dissertation, Case Western Reserve University.
16. Taylor, B. L., Barden, R. E. & Utter, M. F. (1972) *J. Biol. Chem.* **247**, 7383–7390.
17. Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412.
18. Pringle, J. R. (1970) *Biochem. Biophys. Res. Commun.* **39**, 46–52.
19. Taylor, B. L., Routman, S. & Utter, M. F. (1975) *J. Biol. Chem.*, **250**, 2376–2382.
20. Lee, J. C., Frigon, R. P. & Timasheff, S. N. (1973) *J. Biol. Chem.* **248**, 7253–7262.
21. Lee, J. C. & Timasheff, S. N. (1974) *Biochemistry* **13**, 257–265.
22. Warburg, O. & Christian, W. (1941) *Biochem. Z.* **310**, 384–421.
23. Layne, E. (1957) in *Methods in Enzymology*, eds. Colowick, S. P. & Kaplan, N. O. (Academic Press, New York), Vol. 3, pp. 450–454.
24. Bodanszky, A. & Bodanszky, M. (1970) *Experientia* **26**, 327.
25. McClure, W. R., Lardy, H. A. & Kneifel, H. P. (1971) *J. Biol. Chem.* **246**, 3569–3578.
26. Warren, G. B. & Tipton, K. F. (1974) *Biochem. J.* **139**, 297–310.
27. Irias, J. J., Olmstead, M. R. & Utter, M. F. (1969) *Biochemistry* **8**, 5136–5148.
28. Yphantis, D. A. (1964) *Biochemistry* **3**, 297–317.
29. Trautman, R. & Crampton, C. F. (1959) *J. Am. Chem. Soc.* **81**, 4036–4040.
30. Valentine, R. C., Wrigley, N. G., Scrutton, M. C., Irias, J. J. & Utter, M. F. (1966) *Biochemistry* **5**, 3111–3116.
31. Nakashima, K., Rudolph, F. B., Wakabayashi, T. & Lardy, H. A. (1975) *J. Biol. Chem.* **250**, 331–336.
32. Scrutton, M. C. & Taylor, B. L. (1974) *Arch. Biochem. Biophys.* **164**, 641–654.
33. Wood, H. G., Ahmad, F., Jacobson, B., Chuang, M. & Brattin, W. (1975) *J. Biol. Chem.* **250**, 918–926.