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Research Article

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ABSTRACT Pyruvate dehydrogenase complex (PDC) activity in human skin fibroblasts appears to be regulated by a phosphorylation-dephosphorylation mechanism, as is the case with other animal cells. The enzyme can be activated by pretreating the cells with dichloroacetate (DCA), an inhibitor of pyruvate dehydrogenase kinase, before they are disrupted for measurement of PDC activity. With such treatment, the activity reaches 5–6 nmol/min per mg of protein at 37°C with fibroblasts from infants. Such values represent an activation of about 5–20-fold over those observed with untreated cells. That this assay, based on [1-^{14}C]pyruvate decarboxylation, represents a valid measurement of the overall PDC reaction is shown by the dependence of $^{14}CO_2$ production on the presence of thiamin-PP, coenzyme A (CoA), Mg^{++}, and NAD^+. Also, it has been shown that acetyl-CoA and $^{14}CO_2$ are formed in a 1:1 ratio. A similar degree of activation of PDC can also be achieved by adding purified pyruvate dehydrogenase phosphatase and high concentrations of Mg^{++} and Ca^{++}, or in some cases by adding the metal ions alone to the cell homogenate after disruption. These results strongly suggest that activation is due to dephosphorylation. Addition of NaF, which inhibits dephosphorylation, leads to almost complete loss of PDC activity.

Assays of completely activated PDC were performed on two cell lines originating from patients reported to be deficient in this enzyme (Blass, J. P., J. Avigan, and B. W. Ublendorf. 1970. *J. Clin. Invest.* **49**: 423–432; Blass, J. P., J. D. Schuman, D. S. Young, and E. Ham. 1972. *J. Clin. Invest.* **51**: 1545–1551). Even after activation with DCA, fibroblasts from the patients showed values of only 0.1 and 0.3 nmol/min per mg of protein. A familial study of one of these patients showed that both parents exhibited activity in fully activated cells about half that of normal values, whereas cells from a sibling appeared normal. These results demonstrate the inheritance nature of PDC deficiency, and that the present assay is sufficient to detect the heterozygous carriers of the deficiency. Application of the same procedures to fibroblasts obtained from 16 individuals who were believed to have normal PDC activities showed a range from about 2–2.5 nmol/min per mg protein for adults to 5–6 nmol/min per mg protein for cells from infants.

INTRODUCTION

Pyruvate dehydrogenase complex (PDC)[1] catalyzes the oxidative decarboxylation of pyruvate:

$$\text{Pyruvate} + \text{coenzyme A (CoA)} + \text{NAD}^+ \xrightarrow{\text{Mg}^{++},\ \text{thiamin pyrophosphate (TPP)}} \text{Acetyl-CoA} + \text{NADH} + \text{CO}_2.$$

The mammalian multienzyme complex can be resolved into three catalytic components and two regulatory enzymes (1, 2). The three catalytic components are pyruvate dehydrogenase (PDH) (EC 1.2.4.1), dihydrolipoyl transacetylase (EC 2.3.1.12), and dihydrolipoyl dehydrogenase (EC 1.6.4.3), which act sequentially in that order. The two regulatory enzymes are pyruvate dehydrogenase kinase (PDH_a kinase) (EC 2.7.1.99), which catalyzes the Mg-ATP-dependent phosphorylation of PDH with concomitant inactivation, and pyruvate dehydrogenase phosphate phosphatase (PDH_b phosphatase) (EC 3.1.3.43) which dephosphorylates PDH_b with concomitant activation of the enzyme (3).

A preliminary account of this work was presented in 1980. *In* Enzyme Therapy in Genetic Disease. R. J. Desnick, editor. Alan R. Liss, Inc., New York. 289–304.

Dr. Sheu and Ms. Hu wish to dedicate this work to the late Dr. Merton F. Utter.

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[1] *Abbreviations used in this paper*: CoA, coenzyme A; DCA, dichloroacetate; PBS, phosphate-buffered saline, Dulbecco's "A" solution; PDC, pyruvate dehydrogenase complex; PDH, pyruvate dehydrogenase; PDH_a, active, or dephosphorylated form of PDH; PDH_b, inactive or phosphorylated form of PDH; TPP, thiamin pyrophosphate.

PDC activity is acutely regulated at two levels. Acetyl-CoA and NADH, the reaction products, exert product inhibition by competing with the substrates, CoA and NAD^+, respectively (4–6). Additionally, PDC activity is determined by the degree of phosphorylation of PDH. The phosphorylation in turn depends on the relative activities of PDH_a kinase and PDH_b phosphatase which are subject to control by various metabolites (1, 2, 7, 8). PDH_a kinase is activated by acetyl-CoA and NADH (9, 10) and inhibited by pyruvate, CoA, NAD^+, TPP, and ADP (9, 11–17). The kinase is also inhibited by dichloroacetate (DCA), a hypoglycemic agent (17, 18), which probably acts as an analog of pyruvate. The PDH_b phosphatase is activated by Mg^{++} and Ca^{++} (12, 19, 20) and inhibited by F^- (12). The phosphorylation-dephosphorylation process furnishes a major regulatory mechanism whereby the rate of pyruvate oxidation can be controlled by availability of pyruvate and metal ions and changes in the NAD^+/NADH, CoA/acetyl-CoA, and ADP/ATP ratios.

Numerous cases have been reported in which a defect in the pyruvate oxidation system has been proposed or tested (21–23). The clinical disorders involved in these cases include lactic acidemia, motor and mental retardation, and other neurological defects. Blass et al. (21) have correlated the severity of clinical symptoms and the age of onset with the residual PDC activity found in cultured fibroblasts. Various attempts have also been made to measure the activities of the component enzymes of PDC, and the deficiencies in overall pyruvate oxidation have been attributed to defects in specific enzymes, including abnormalities in kinetic parameters (21, 23–38).

The PDC activity measurements in the previous studies, particularly those conducted with disrupted cell preparations, have the disadvantage that they do not take into account the phosphorylation state of PDC. Since the activation state of this enzyme can vary widely, depending on the metabolic situation, it is important that this parameter be controlled. The fully activated enzyme probably furnishes the most reliable estimates of enzymatic activity for studies of PDC deficiencies.

In the present paper, we present evidence to show that PDC, in preparations from untreated, cultured human skin fibroblasts, appears to be 90–95% inactivated. Measurements of PDC activity are thus subject to considerable error and can conceivably give misleading results. The full activity of PDC can be expressed by pretreatment of the fibroblasts with the activator DCA or by treating the disrupted fibroblasts with PDH_b phosphatase or metal ions or both. These procedures have been used to measure PDC activity in fibroblasts with a series of normal controls, patients with unexplained lactic acidosis and two cases of PDC deficiency. A preliminary account of some of these studies has been presented previously.

METHODS

Chemicals, enzymes. [1-^{14}C]Pyruvic acid and [2-^{14}C]pyruvic acid were obtained from New England Nuclear, Boston, Mass. [2-^{14}C]Pyruvic acid was further purified before use on cellulose thin-layer chromatogram (Eastman Kodak Co., Rochester, N. Y.) using *n*-butanol:formic acid:H_2O (95:5:10) as the mobile phase. [1-^{14}C]Pyruvic acid was routinely dissolved in 30 mM HCl with added carrier pyruvic acid (Sigma Chemical Co., St. Louis, Mo.) to give a 50 mM solution which contained 0.5 μCi/μmol. This solution was stored in small aliquots at −20°C. Prior to use, contaminating [1-$^{14}CO_2$] was removed from the solution by incubating in an assay vial (40) for at least 1 h with hyamine hydroxide (New England Nuclear).

Purified PDC from bovine heart (41) was a gift from Dr. L. J. Reed, University of Texas, Austin, Tex. PDH_b phosphatase was purified from bovine heart according to Siess and Wieland (42) through step 5. The phosphatase was further purified by centrifuging twice in a Beckman L2-65 ultracentrifuge (Beckman Instruments, Fullerton, Calif.) with a type 42.1 rotor at 70,000 *g* for 90 min. This preparation reactivated bovine heart PDH_b which had been rendered 95% inactive by treatment with 0.02 mM ATP (41). The phosphatase had a specific activity of 12.8 mU when expressed as PDC activated per minute per milligram of phosphatase protein at 30°C. The purified phosphatase contained a slight amount of contaminating PDC activity (0.26 nmol/min per mg). All the PDC assays using this phosphatase preparation as activation agent have been corrected for the contaminating PDC activity.

All other chemicals and enzymes were obtained from commercial suppliers at the highest grade available and used without further purification.

Radioactive measurements. Radioactivity was measured on a Packard Model 3320 Tri-Carb Scintillation Spectrometer (Packard Instrument Co., Downers Grove, Ill.) using scintillant Econofluor (New England Nuclear) for $^{14}CO_2$ measurements and Formula 963 (New England Nuclear) for determination of radioactivity in aqueous samples.

Fibroblast cultures. Human skin fibroblasts were grown in Eagle's minimum essential medium with 4× concentration of vitamins and amino acids (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.), supplemented with 15% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were fed twice a week, and harvested 5–7 d past confluency. To harvest the cells, the tissue culture flasks were rinsed twice with 0.5 mM EGTA in Ca^{++}, Mg^{++}-free phosphate-buffered saline (Dulbecco's "A" solution) (PBS), followed by treatment with 0.5% trypsin (trypsin 1:250) (Difco Laboratories, Detroit, Mich.), in National Institutes of Health medium 307 at 37°C for 5–10 min. One-half volume of the growth medium was then added, and the cells were collected by low-speed centrifugation. The cells were further washed twice with PBS. Only cells of early passage (5–15) were used in the experiments. The cell cultures were spot checked for *Mycoplasma* contamination either by Microbiological Associates (Bethesda, Md.) or in a few cases as a courtesy of Dr. J. A. Barranger of National Institutes of Health, Bethesda, Md. The cells used in these experiments were free from *Mycoplasma* contamination on the basis of the negative results of such examinations. As we will report elsewhere,[2] we have found that

[2] Miller Paulson, S., K-F. R. Sheu, and M. F. Utter. Unpublished data.

fibroblasts contaminated with *Mycoplasma* behave very differently from normal fibroblasts during the activation and inactivation of PDC. This property appears to provide a useful additional method for detection of the presence of *Mycoplasma*. Fibroblast lines were obtained as follows: IMR90 and GM3093 from the Human Genetic Mutant Cell Repository, Camden, N. J.; TC78761 and TC78766 from Dr. A. M. Glosgow, Children's Hospital, National Medical Center, Washington, D. C.; SK8177, SK5616, and SK8167 from Dr. J. C. Haworth, Health Science Center, Children's Hospital, Winnipeg, Manitoba; B.J.R., D.R., L.R., and B.R. from Dr. J. P. Blass, Cornell University Medical Center, New York, N. Y.; M.L. from Dr. D. Vine, Mt. Sinai Hospital, New York, N. Y.; B.J. from Dr. K. N. Rosenbaum, Children's Hospital, National Medical Center, Washington, D. C.; G-1 and G-2 from Dr. I. A. Schaefer, Cleveland Metropolitan General Hospital, Cleveland, Ohio; TC346 and TC349 from Dr. D. Kerr, University Hospitals, Cleveland, Ohio; TC194, TC313, and TC318 from Cytogenetics Laboratory, Case Western Reserve University, Cleveland, Ohio, and W.P. from Dr. D. C. Chuang, Veterans Administration Hospital, Cleveland, Ohio.

Cell treatment and PDC assay. Immediately after harvesting, the cells were suspended at ~5 mg protein/ml in PBS, which contained either 5 mM DCA or 15 mM NaF. These suspensions were incubated at 37°C for 15 min in a water-bath shaker with constant gentle gyratory shaking. The pretreatment process was stopped by adding 1/3 vol of ice-cold "stopping mixture" (43) which contained 40% vol/vol ethanol, 25 mM NaF, 25 mM EDTA, and 4 mM dithiothreitol at pH 7.4. The suspension was then frozen rapidly in a dry ice-ethanol mixture and stored at −76°C. The enzymatic assays were normally performed within 48 h after freezing.

For assay, the fibroblast suspension was further frozen and thawed twice. PDC activity was determined by measurement of $^{14}CO_2$ production from [1-^{14}C]pyruvate, by modifying the method of Leiter et al. (40). The assay mix with a final volume of 0.2 ml and pH of 8.0 contained K phosphate, 50 mM; K oxalate, 15 mM; MgCl, 2 mM; CoA, 0.3 mM; dithiothreitol, 1 mM; NAD^+, 2 mM; TPP, 0.1 mM; [1-^{14}C]pyruvate, 0.5 mM; phosphotransacetylase, 1 U/ml (Boehringer Mannheim Biochemicals, Indianapolis, Ind.); and the fibroblast suspension, 20 or 30 μl, containing 0.05–0.15 mg protein. The assay mix (except the fibroblast extract and [1-^{14}C]pyruvate) was pipetted into assay vials made by shortening test tubes (10 mm) and cutting out vents. These tubes were suspended from rubber serum stoppers which were then used to cover snap-cap scintillation vials containing 0.2 ml hyamine hydroxide. These vials were put in a 37°C water bath shaker; and the reaction was started by injecting the fibroblast extract and [1-^{14}C]pyruvate into the reaction mix through the septum of the rubber stopper with Hamilton syringes fitted with PB-600 repeating dispensers. After 4 min incubation, the reaction was stopped by injecting 50 μl of 10% TCA, and the incubation was continued for another 1 h. The serum stoppers and reaction vials were then removed, and the radioactivity associated with hyamine hydroxide was measured. CoA was omitted to serve as a blank except where otherwise indicated. Three to five replicates were performed to obtain each data point. For a complete assay which includes determination of PDC activity in untreated cells and after treatment with DCA or NaF, cells from 300 cm^2 were required.

Protein was determined according to Lowry et al. (44), using bovine serum albumin as standard.

Activation of PDC in cell-free homogenates. The cell suspension prepared as described above was centrifuged at top speed in an Eppendorf model 3200 centrifuge for 30 s and the resulting cell pellets were quickly frozen in a dry ice-ethanol bath. The pellets were thawed in a solution containing K phosphate, 10 mM, pH 7.4; EDTA, 1 mM; $MgCl_2$, 2 mM; fatty acid-poor bovine serum albumin, 0.5 mg/ml (Sigma Chemical Co.); and fibroblasts, 3 mg protein/ml, homogenized in a ground glass homogenizer for 6–8 passes, followed by two cycles of freezing and thawing. To the homogenate were added (as final concentrations) $MgCl_2$, 15 mM; $CaCl_2$, 0.5 mM; TPP, 40 μM; DCA, 2 mM; and PDH_b phosphatase, 2 mg/ml. Either PDH_b phosphatase or PDH_b phosphatase plus the other components listed above were omitted from control incubations. Aliquots of 20 μl were withdrawn at different time intervals for measurement of PDC activity by the procedures described above, except that 1 mM EDTA was also present in the assay medium.

Identification of acetyl-CoA as a reaction product. The PDC reaction product, acetyl-CoA, was converted to citrate by adding citrate synthase and oxalacetate and the citrate isolated chromatographically. For these experiments, the reaction mixture of 1 ml contained the components described above at the same concentrations except that [1-^{14}C]pyruvate was replaced by [2-^{14}C]pyruvate, and phosphotransacetylase was replaced by 1 μmol of freshly dissolved oxalacetate and 7 μg of citrate synthase (Boehringer Mannheim Biochemicals). After 4 min incubation, the reaction was stopped by adding 50 μmol of carrier citrate and heating in a boiling water bath for 2 min. The heated suspension was chromatographed on a DEAE-Sephadex A-25 ion exchange column (Pharmacia Fine Chemicals, Piscataway, N. J.). Citrate was quantitated spectrophotometrically by measuring the oxidation of NADH in the presence of citrate lyase (Boehringer-Mannheim Biochemicals) and malate dehydrogenase (Sigma Chemical Co.). The amount of acetyl-CoA formed was calculated by dividing the total radioactivity associated with citrate by the known specific activity of the [2-^{14}C]pyruvate.

RESULTS

Activation of PDC in fibroblasts. When intact fibroblasts obtained from infants were incubated with 5 mM DCA and then disrupted and assayed for PDC activity, the specific activity reached a value of 5.5–6 nmol $^{14}CO_2$ produced/min per mg of fibroblasts protein after 5 min activation and remained at that level at least through a 17 min activation period (Fig. 1). Lower concentrations of DCA such as 0.5 mM were insufficient to activate the PDC completely, even in 17 min. Preincubation with NaF caused a drop of ~50% in the control level over a 10-min period. The control values were relatively steady over the same incubation period. Although the DCA-incubated values were relatively consistent (5–6 nmol/min per mg protein), the values for untreated normal cells varied considerably (from 0.3 to 1.4 nmol/min per mg). That shown in Fig. 1 was the highest of any line yet tested. Likewise, the values for NaF-treated cells varied considerably although they were always low. As shown in Fig. 2, the activity of PDC was linear for only ~4 min during incubation of the DCA-treated cells, and it was necessary to restrict the assay to this period. As is also shown in Fig. 2, the activity of the PDC from untreated cells appeared to be linear with time for a longer period. The production of

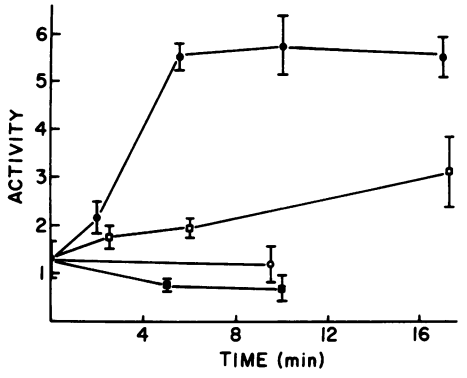

FIGURE 1 Time-course of activation and inactivation of PDC in fibroblasts by dichloroacetate and NaF. The values are mean±SD (n = 3–4). ○, control ●, 5 mM DCA; □, 0.5 mM DCA; ■, 15 mM NaF. Assay conditions were described in Methods. Activity is expressed as nanomoles $^{14}CO_2$ produced per minute per milligram protein.

$^{14}CO_2$ from [1-^{14}C]pyruvate was linear with protein concentration up to 1 mg/ml of the assay mix, and assays were conducted with 0.15–0.75 mg protein/ml.

The effects of the various cofactors and substrates on the rate of pyruvate oxidation by PDC are shown in Table I. The results are presented as counts present at 0 and 4 min. The former values show the actual extent of blank value. The reaction is almost completely dependent on the presence of TPP, CoA, NAD^+, and Mg^{++}. Routinely, CoA was omitted to serve as a blank. In an early stage of this study, the blanks were unacceptably high, but this problem has been largely obviated by proper storage of the pyruvate, by preincubation of the pyruvate with hyamine hydroxide and by addition of the dithiothreitol and CoA to the assay medium just prior to addition of enzyme. Even so, the blank formation of $^{14}CO_2$ could not be completely eliminated, as shown in Table I.

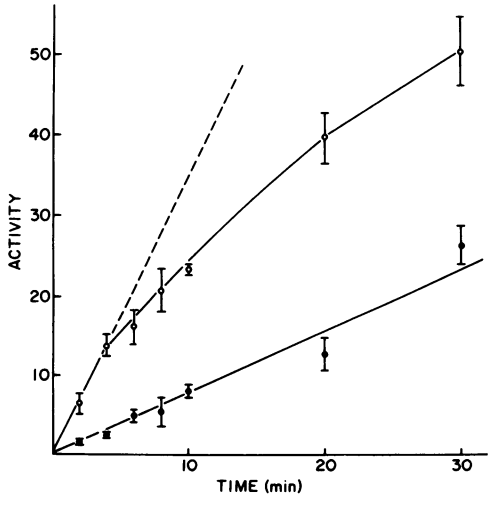

FIGURE 2 The activity of PDC in fibroblasts as a function of the incubation time. The values are mean±SD (n = 3–4). ○, DCA pre-treated cells; ●, untreated cells. Activity is expressed as nanomoles $^{14}CO_2$ produced per milligram protein.

TABLE I
Cofactor Requirement of DCA-activated PDC Assay

Assay condition	$^{14}CO_2$ 0 min	$^{14}CO_2$ 4 min	$^{14}CO_2$ Net*
		cpm	
Complete	131±11	2177±125	2,046
-TPP	116±4	228±19	112
-CoA	122±11	240±24	118
-NAD^+	300±18	351±47	51
-Mg^{++}	175±19	223±16	48
-CoA, -NAD^+, -Mg^{++}	163±23	204±9	81

The assays were performed with 90.5 μg protein in each tube. The specific radioactivity of [1-^{14}C]pyruvate was 1,220 cpm/nmol. Values are mean±SD (n = 3–4).
* Net is the difference of counts at 0 min subtracted from that at 4 min.

Relative rates of production of CO_2 and acetyl-CoA as products. To show that measurement of $^{14}CO_2$ produced from [1-^{14}C]pyruvate constitutes a valid assay for the complete reaction catalyzed by PDC, the rate of production of another major product, acetyl-CoA, was compared with that of $^{14}CO_2$ production. The assay reaction was initiated with [2-^{14}C]pyruvate and the radioactive product [1-^{14}C]acetyl-CoA was trapped as [^{14}C]citrate in the presence of oxalacetate and citrate synthase. Citrate was isolated by chromatography on a DEAE-Sephadex A-25 column as depicted in Fig. 3. A radioactive peak, well separated from the initial peak of [2-^{14}C]pyruvate, co-migrated with authentic citrate added as carrier. The latter was recovered in 96% yield. As shown in Fig. 3, the second radioactive peak was absent in the parallel experiment from which CoA was omitted. The specific radioactivity of the citrate was constant over most of the peak, indicating that the radioactive product was citrate. The enzymatic activity, calculated from the amount of citrate formation, was 3.86 nmol/min per mg protein. In a similar experiment where the [2-^{14}C]pyruvate was replaced by [1-^{14}C]-pyruvate, and enzymatic activity calculated from $^{14}CO_2$, production was 3.67±0.18 nmol/min per mg protein. Thus, the stoichiometry of product formation, acetyl-CoA/CO_2 = 1.02, was in excellent agreement with the expected value of 1.0.

Activation of PDC in cell-free homogenates. To establish that the mechanism of DCA activation involves an alteration of the dephosphorylation/phosphorylation ratio in favor of the former process and further to establish that DCA activates the enzymatic complex completely, dephosphorylation by other pro-

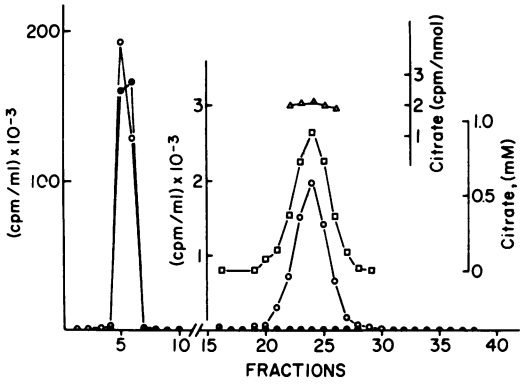

FIGURE 3 Formation of [^{14}C]citrate of PDC reaction from [2-^{14}C]pyruvate when coupled with citrate synthase reaction. The heat-inactivated reaction mixture (Methods) was chromatographed on a DEAE-Sephadex A-25 column (1.0 × 5.5 cm). After washing the column with 40 ml 0.4 M NH_4OAc, pH 5.9, 36 ml of 0.4–1.5 M linear gradient of the same buffer was applied starting from fraction 15. Fractions of 1.5 ml were collected. ○, radioactivity, complete reaction mixture; ●, radioactivity, CoA omitted; □, citrate concentration, △, specific radioactivity of citrate.

cedures was attempted. While PDH_a kinase is believed to be bound tightly to the PDC complex, PDH_b phosphatase is probably associated only loosely with the complex after disruption of the cells and their mitochondria (20, 41). Further, high concentrations of Mg^{++} and Ca^{++} are required for binding of the phosphatase to the complex (20). Therefore, the endogenous phosphatase may not be sufficient to provide rapid or perhaps maximal activation in broken cell preparations. Fig. 4 shows that the addition of Mg^{++} and Ca^{++} to stimulate the phosphatase, along with DCA and TPP to inhibit the kinase, provided reasonably good activation in a disrupted cell experiment. However, the addition of external PDH_b phosphatase gave a more rapid rate of activation, although the maximal value reached was probably not significantly higher. Addition of ATP resulted in a lowering of the initial control value, demonstrating the presence of an active kinase during these conditions. In an accompanying experiment, in which the cells were preincubated with 5 mM DCA for 15 min and homogenized with 2 mM DCA and 40 μM TPP, the PDC activities were 4.5±0.46 and 4.0±0.91 nmol/min per mg protein after 4 and 7.5 min, respectively. In a later part of the investigation, DCA and TPP were omitted from the medium. No significant difference was found (data not shown). These results indicate that PDC can be activated to about the same extent by any of the three types of treatment, although the addition of PDH_b phosphatase may be the most effective procedure.

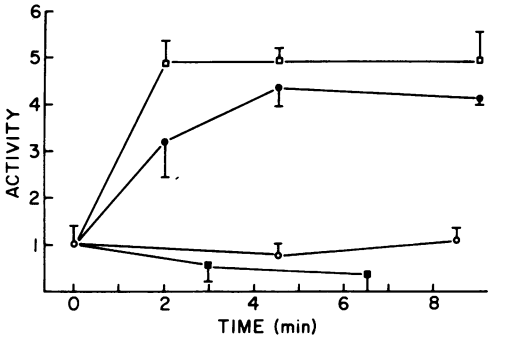

FIGURE 4 Phosphatase activation and ATP inactivation of PDC in fibroblast homogenate. Aliquots of 20 μl were withdrawn at times indicated from the cell-free homogenate (Methods) for enzyme assay. Values are mean±SD (n = 3–4). ○, control; ●, 15 mM $MgCl_2$, 2mM DCA, 0.5 mM $CaCl_2$ and 0.4 mM TPP added; □, 2 mg/ml phosphatase also added in the latter incubation; ■, 3 mM ATP added into the control incubation. Activity is expressed as nanomoles per minute per milligram protein.

Application of activation procedures to normal cell lines. Table II lists the PDC activities of cell cultures that we have assayed and consider to be within the normal range. Infant skin fibroblasts (except for IMR90, which is a lung culture) consistently exhibited maximal PDC activities up to 4–6 nmol/min per mg protein after activation with DCA, while cells obtained from older individuals showed somewhat lower activities. The decrease appears to take place in early childhood. Table II also shows that untreated values are always much lower than those obtained after activation and that these values exhibit a wider range. NaF-treated cells have still lower activities and also vary considerably between different cell lines. These results demonstrate the values from untreated cells and especially from those treated with NaF provide much less reliable estimates of the activity of PDC.

Assays on PDC-deficient cell lines. These methods have also been applied to cells from two PDC-deficient patients, who have been previously studied by Blass et al. (patient B.J.R. [26, 45]; patient E.G. [46]). The results shown in Table III indicate that both of these patients are indeed PDC deficient even when DCA activation is used; indeed, the defects now appear to be more severe than previously realized because of the higher normal values. After DCA activation, fibroblasts from B.J.R. had an activity of only 0.1 nmol/min per mg, while those from E.G. were only 0.28 nmol/min per mg protein. Fibroblasts from the parents and a sibling of B.J.R. were also tested. The results (Table III) suggest that the sibling has activity in the normal range for his age group, but that the parents have activities half or less than those of any other individuals tested thus far (cf. Table II), except the two patients.

DISCUSSION

The studies presented here demonstrate that PDC in fibroblasts, as in all other mammalian systems tested

TABLE II

PDC Activities in Fibroblasts That Contain Normal Amount of Total Enzyme

Cell line	Sex	Age	Remarks	PDC*		
				DCA-activated	Untreated	NaF-inactivated
				nmol $^{14}CO_2$/min/mg protein		
PW	M	Newborn	Normal control	5.8±0.66 (5)	1.23±0.067 (2)	0.45±0.066 (5)
TC318	F	Newborn	Possible renal agnesis	4.9±0.21 (4)	0.65±0.094 (4)	0.52±0.142 (4)
TC313	F	3 d	Potter's Syndrome	5.1±0.20 (4)	0.34±0.002 (3)	0.30±0.35 (3)
IMR90	F	16 wk	Normal control, fibroblast lung culture	3.2±0.092 (3)	0.57±0.117 (3)	—
TC349	M	4 mo	Lactic acidosis	5.5±0.38 (4)	1.36±0.153 (3)	0.62±0.254 (4)
TC78766	M	10 mo	Lactic acidosis, fructose intolerance	5.8±0.12 (4)	0.33±0.050 (3)	0.005±0.070 (4)
TC78761		infant	Normal control	4.7±0.21 (4)	0.41±0.134 (4)	0.059±0.23 (4)
SK8167 (GB)	M	4 mo	Lactic acidosis, pyruvate carboxylase deficiency	3.5±0.24 (4)	0.71±0.122 (8)	0.054±0.156 (3)
SK5616 (DM)	M	23 mo	Lactic acidosis, pyruvate carboxylase deficiency	3.6±0.68 (4)	0.62±0.069 (3)	0±0.075 (4)
SK8177 (RS)	M		Lactic acidosis, pyruvate carboxylase deficiency	4.9±0.20 (4)	1.20±0.293 (4)	0.17±0.080 (4)
ML	F	20 mo	Lactic acidosis, mitochondrial myopathy	2.7±0.49 (4)	0.53±0.064 (4)	0.104±0.082 (4)
BJ	M	3 yr	Lactic acidosis	4.6±0.62 (4)	0.50±0.194 (4)	0.042±0.040 (4)
TC346	M	5 yr	Lactic acidosis, fasting hypoglycemia, galactose intolerance	2.7±0.25 (4)	0.44±0.066 (4)	0.091±0.080 (4)
TC194	F	Adult	Turner Syndrome	2.3±0.68 (3)	0.60±0.278 (3)	—
G-1		Adult	Normal control	2.5±0.26 (4)	0.30±0.088 (4)	0.18±0.108 (4)
G-2		Adult	Normal control	2.0±0.11 (4)	0.106±0.189 (3)	0.154±0.130 (4)

* Values are mean±SD. Numbers of determinations are in parentheses.

thus far, is subject to regulation by phosphorylation and dephosphorylation. Thus, these cells may be subject to wide variations in the catalytic activity of this enzyme, depending on the metabolic situation, hormonal influences, and other factors. Assay of PDC activity without proper regard for the phosphorylation state may give a very misleading picture of the total activity of this enzymatic complex. Mammalian tissues have been shown to vary widely in their proportion of active and inactive forms, e.g., active PDC: 20–30% in rat liver (51, 52); about 60% in rat heart and adipose tissue (51, 53–57).

Examination of the literature shows that previous measurements of PDC activity in disrupted fibroblasts range from 0.14 to 0.6 nmol/min per mg protein (24, 29–31, 33, 35, 38, 46, 48–50). It is surprising to note that the highest of these values could be no higher than 20% of the levels of activity observed here after activation, and some of the previous values may be as low as 3% of the expected maximal activity. These observations suggest that the PDC in isolated fibroblasts exists largely in the inactivated phosphorylated state. However, such low values may be an artifact of isolation and handling of the cells, and it seems

TABLE III

PDC in Fibroblasts of Enzyme-deficient Patients, BJR and EG, and BJR's Family

Cell line	Sex	Age	Remarks	PDC activities*	
				DCA-activated	Untreated
		yr		*nmol $^{14}CO_2$/min/mg protein*	
BJR	M	8	Patient	0.10±0.054 (3)	0.083±0.065 (3)
DR	M	5	Sibling	2.3±0.18 (3)	0.34±0.022 (2)
LR	F	31	Mother	0.74±0.042 (2)	0.051±0.0044 (3)
BR	M	43	Father	1.05±0.080 (3)	0.32±0.058 (3)
EG (GM3093)	F	4-1/2	Patient	0.28±0.513 (3)	0.03±0.124 (3)

* Values are mean±SD. Numbers in parentheses are numbers of determinations.

likely that the rates of pyruvate oxidation in fibroblasts under physiological conditions are considerably higher.

It is important to note (Table III) that in the two cases of previously reported PDC deficiency included in the present study, the values of PDC activity were so low that they could be distinguished from the normal untreated fibroblast preparations. With the activation procedures, the deficiencies appear to be much more severe because of the much higher control values. The problem of detection of PDC deficiencies in untreated fibroblasts may become much more difficult in cases where the deficiency is less severe, e.g., in Friedreich's ataxia where PDC activities of 40–50% of normal have been reported (34, 49, 58, 59) by some investigators and normal activities by others (60). Results obtained with the familial study of patient B.J.R. (Table III), in which both parents exhibit approximately half the total PDC activity compared with normal control, and that of the sibling appears to be normal, indicate the inheritance nature of the PDC deficiency. Both parents are probably heterozygous carriers. It is interesting to note that Blass et al. (26), in their original studies on this parent, found that rates of pyruvate oxidation in both intact fibroblasts and white cells from the parents were about half the levels of those obtained with control cells. This relationship, however, could not be demonstrated in assays involving disrupted cells. The present method appears to be sensitive enough to detect certain genetic heterozygous states. Efforts are underway to apply this method to different cell types, e.g., leukocytes and amniotic cells, to provide biochemical basis for prenatal diagnosis.

The DCA activation procedure appears to provide a relatively simple and reproducible method for measuring maximal PDC activity in fibroblasts, but it has the disadvantage that relatively large numbers of cells are required. This is due mainly to the short period (4 min) when the activity is linear with time. The reason for the decrease in activity after 4 min is not clear. Lynen et al. (61) have suggested that the dihydrolipoyl transacetylase component of PDC may be subject to proteolysis, and it is possible that such an effect is being seen here. However, the preparation from untreated cells (Fig. 2) does not seem to be subject to the same decrease in activity, as would be expected if proteolysis were involved. As an alternative explanation, preliminary results from experiments in which PDH_b phosphatase was added suggest that the length of the linear response with time can be greatly extended by the presence of the phsophatase. This result suggests that a shift toward increased phosphorylation is responsible for the decrease in activity with time. If these results are confirmed, the use of PDH_b phosphatase may be the preferred method of activation.

The present method of PDC measurement is aimed only at detecting defects in total activity of the complex, although the procedure could be modified to test for alterations in the K_m for the various substrates. The DCA method is probably not well adapted to detect changes in the regulatory components of the complex, the kinase and phosphatase, unless these are severe.

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