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Absence of Cross-Reacting Material in Isolated Propionyl CoA Carboxylase Deficiency: Nature of Residual Carboxylating Activity

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SUMMARY

Fibroblast extracts and fetal liver homogenates from patients with propionic acidemia due to inherited deficiency of propionyl CoA carboxylase (PCC) were analyzed for the presence of immunologically cross-reactive PCC protein. Using several rabbit antisera raised against homogeneous human liver PCC, homogeneous pig heart PCC, or the individual nonidentical subunits of the human liver enzyme, we found no detectable cross-reacting material by direct or competitive immunotitration in several cell lines from patients in either major complementation group (*pcc A*; *pcc C*) with isolated PCC deficiency. In contrast, cells of a patient from the *bio* complementation group contained normal amounts of immunoreactive PCC. Further analysis of the *pcc A* and *pcc C* mutants revealed that their residual propionyl CoA carboxylating activity varied greatly depending on the concentration of extract or homogenate protein used in the PCC assay. When propionyl CoA carboxylation was assayed at high protein concentration in a fetal liver homogenate from a *pcc C* patient, the apparent PCC activity was comparable to that found in normal human fetal liver. Significantly, the specific activity in the mutant, but not in the control, extract declined steeply as protein concentration was lowered, and this loss could not be prevented by adding PCC substrates, bovine serum albumin, glycerol, or 2-mercaptoethanol. Moreover, detailed analyses of immunotitration curves of control fibroblast extracts showed that fresh extracts contained an amount of nonimmunotitratable carboxylating activity corresponding to the residual activity present in fresh extracts of mutant cell lines. We conclude that the residual propionyl CoA carboxylating activity found in isolated PCC deficiency represents another carboxylase that can utilize propionyl CoA as a substrate rather

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than a mutant form of PCC with markedly different immunochemical and physicochemical properties.

INTRODUCTION

Human liver propionyl CoA carboxylase (PCC; E.C.6.4.1.3), a biotin-dependent mitochondrial enzyme, has a native mol. wt. of ~540,000 daltons with an $\alpha_4\beta_4$ tertiary structure. The larger (α) subunit contains biotin and has a mol. wt. of ~72,000 daltons; the smaller (β) has a mol. wt. of ~56,000 [1]. PCC deficiency is observed in a group of recessively inherited disorders of organic acid metabolism characterized by both clinical and biochemical heterogeneity [2]. Some patients with defective PCC activity are susceptible to episodic ketoacidosis [3, 4], while others are not [5]. However, all patients described retain some residual propionyl CoA carboxylating activity in leukocytes [6], fibroblasts [2], and liver [7]. Three major genetic complementation groups, *pcc A*, *pcc C*, and *bio*, have been defined from studies with fibroblast heterokaryons [8]. Biochemical analysis has suggested that the *pcc A* and *pcc C* classes, in which isolated PCC deficiency is observed [9], result from structural gene mutations in the loci coding for the α and β subunits, respectively. The primary defect in the *bio* class, in which the activities of several biotin enzymes are affected pleotropically [10–13], involves a reduction in the activity of a holocarboxylase synthetase required for the covalent attachment of biotin to the apocarboxylases [14–15].

McKeon et al. [16], using antiserum prepared against homogeneous pig heart PCC, reported that the amount of cross-reacting material (CRM) in liver and cultured fibroblasts of PCC-deficient patients from all three major complementation groups is comparable to that found in normal tissues. We examined in more detail the immunotitration of PCC activity in normal and mutant human fibroblasts and liver. Our results differ sharply from those of McKeon et al. [16] and indicate that neither *pcc A* nor *pcc C* mutant cells have any detectable CRM.

MATERIALS AND METHODS

Tissue Extracts

Cultured skin fibroblasts were propagated and harvested as described [9]. Seven PCC-deficient (three *pcc A*, three *pcc C*, one *bio*) and three control cell lines were used. One of the *pcc A* mutant lines (Yale accession number 49) and one of the *pcc C* mutant lines (Yale accession number 269) that we have used were also examined by McKeon et al. [16]. Biotin depletion was achieved by the addition of 1.5 μg/ml of avidin to the medium [17]. Except when stated otherwise, cell pellets were used immediately after harvesting. In most cases, they were suspended in 3 vol of either distilled water or 50 mM Tris-HCl buffer, pH 7.5, and sonicated on ice 10 times for 2 seconds each time. Cell debris was then removed by centrifugation at 10,000 *g* for 10 min. Liver samples were kept at −70°C before use. Liver homogenates were made in 50 mM Tris-HCl buffer, pH 7.5.

Immunotitrations

PCC activity was assayed and calculated as described [1]. Protein content was estimated by the method of Lowry et al. [18]. Homogeneous PCC from normal human liver and pig

heart were prepared either by the method of Kalousek et al. [1] or by the use of an avidin-Sepharose column [19, 20]. The α and β subunits of PCC were prepared as described [1]. Antisera were raised by injecting 150 μg of antigen (either native holoenzyme or individual subunits) in complete Freund's adjuvant into the hind foot pads and abdominal dermis of white male New Zealand rabbit. After 6 weeks, a booster of the same amount of antigen in incomplete Freund's adjuvant was injected twice during a 2-week interval into the hind thigh and back. Intracardiac bleeding followed 2 weeks after the last immunization. The antisera were used directly or after partial purification according to Lau et al. [21]. In a typical immunotitration experiment, 40 μl of the fibroblast extract or liver homogenate was incubated with 5 μl of varying concentrations of antiserum prepared by dilution with normal rabbit serum. After 1 hr at 4°C, 5 μl of goat antirabbit γ-globulin antibody was added, and the mixture was allowed to stand at 4°C for 1 hr. PCC activity was assayed in the supernatant recovered after centrifugation at 7,000 *g* for 5 min at 4°C. Using this method, as little as 1% CRM was detectable.

RESULTS

Direct Immunotitration of PCC-deficient Fibroblast Extracts and Fetal Liver Homogenate

When PCC activity in extracts of control cell lines was titrated with increasing amounts of antihuman PCC antiserum, typical curvilinearly decreasing plots of activity were obtained (fig. 1). When increasing amounts of antiserum were added to cell extracts from *pcc A* or *pcc C* mutants, however, no fall in apparent PCC activity was seen when either the initial amount of activity (fig. 1*A*) or the amount of protein (fig. 1*B*) were comparable to that in the control extract. Identical results were observed when human fetal liver homogenates were used as the enzyme source (fig. 2). Immunotitration curves of apparent PCC activity from *pcc C* mutant fetal liver homogenate failed to reveal any detectable CRM (fig.

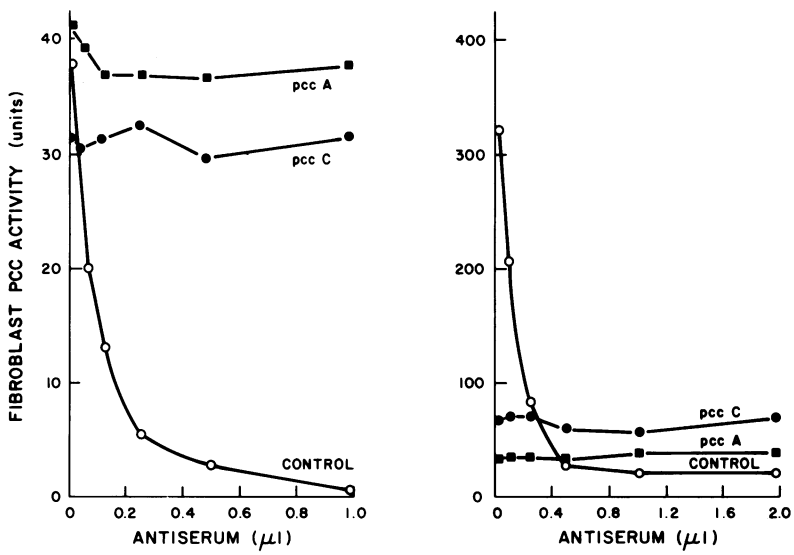

FIG. 1.—Immunotitration of apparent PCC activity in fibroblast extracts from control (○), *pcc A* (■), and *pcc C* (●) cells. Titrations were carried out using equivalent amounts of carboxylating activity (*left frame*) or cell protein (*right frame*).

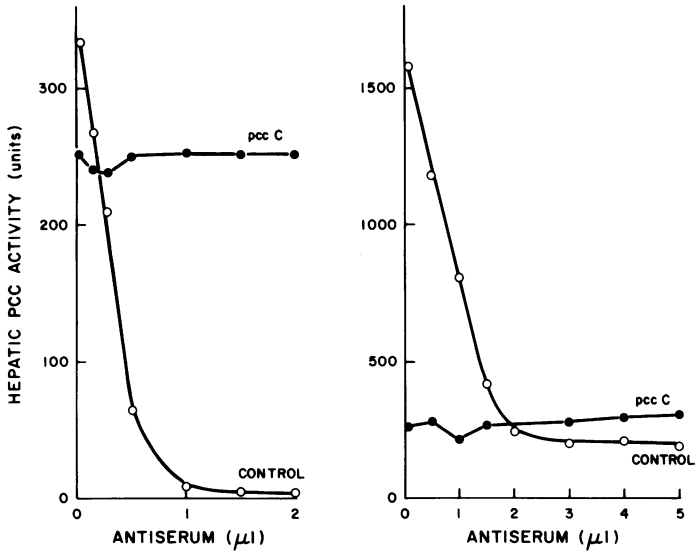

FIG. 2.—Immunotitration of apparent PCC activity in control (○) and *pcc C* (●) fetal liver homogenates. See legend to figure 1 for other details.

2), while normal fetal liver homogenate contained an amount of CRM comparable to that found in normal adult liver homogenate (data not shown).

PCC activity and immunotitration behavior were also examined in extracts of fibroblasts from a *bio* mutant. The specific activity of PCC in this biotin-responsive strain was 14 times lower when the growth medium was depleted of biotin by the addition of avidin. Titration curves of PCC activity in extracts of control and *bio* fibroblasts grown in the presence or absence of biotin had a very similar profile (fig. 3). The amount of antiserum required to decrease initial activity by 50% is between 0.2 and 0.25 μl in both extracts under both conditions, suggesting that they contain comparable amounts of immunocross-reactive PCC.

Our results, with the exception of those with the *bio* mutant, are substantially different from those of McKeon et al. [16]. The latter used partially purified rabbit antiserum raised against PCC prepared from pig heart. They also used titration methods different from ours with respect to time of antigen-antibody interaction, temperature, volume, and, in some cases, protein concentration of the extract. In an attempt to resolve the disparate findings, we first used our antihuman PCC antiserum under the precise conditions described by them [16], but the results did not differ from those reported above. To eliminate the possibility that, for some reason, antihuman PCC antiserum cannot recognize the mutant enzyme in fibroblasts and liver, we purified pig heart PCC to homogeneity and raised antiserum against it in rabbits. Once again, neither crude nor purified antibody titrated the apparent PCC activity in *pcc A* and *pcc C* mutant extracts regardless of methodology used (data not shown). Finally, similarly negative results were also obtained with antiserum raised against the human enzyme denatured by sodium dodecyl sulfate prior to the injection into rabbits and with antisera raised against the individual human α or β subunits.

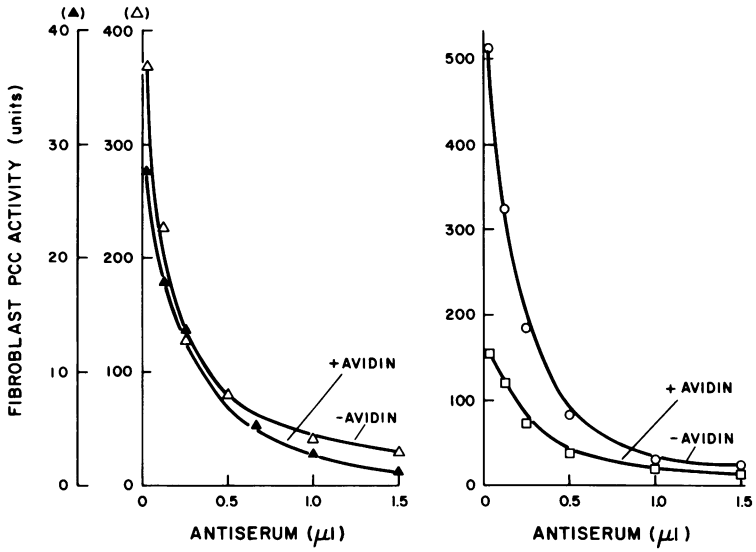

FIG. 3.—Immunotitration of PCC activity in fibroblast extracts of *bio* (*left*) and control (*right*) cells. Cells were grown in presence of biotin (△; ○) or in avidin-supplemented media (▲; □).

Competitive Immunotitration

To further exclude the possibility that the *pcc A* and *pcc C* extracts contained immunologically reactive but catalytically inactive PCC, we mixed extracts from normal and PCC-deficient fibroblasts at a protein ratio of 1:4, and a PCC activity ratio of 3:1. Figure 4 shows that only 75% of the PCC activity in one such mixture (*pcc C*: control) was titrated by antiserum, corresponding exactly to the activity present in the control extract. Moreover, the amount of antiserum required to inactivate 50% of the PCC activity in the mixed extract (0.85 μl) was not different from that needed for the control extract only (0.80 μl). Similar results were obtained with mixtures of extracts of *pcc A* and control cells.

An analogous experiment was performed using fetal liver homogenates (table 1). In this experiment, the protein and activity ratios were as described above, but the mutant homogenate was allowed to interact with the antiserum for 20 hrs before the addition of normal homogenate. This extended interaction served a twofold purpose: it ensured complete interaction of mutant enzyme protein (active or inactive) with antibody; and it eliminated the interference in the assay of normal PCC activity caused by the large amount of carboxylating activity in the mutant homogenate (after 20 hrs, more than 90% of the apparent PCC activity in the mutant extract had disappeared; see below). In a parallel experiment, pure human liver PCC was first denatured by treatment with glycine-NaOH buffer, pH 9.0, and an amount of denatured PCC corresponding to that in the mutant homogenate was then added to the control fetal liver homogenate. While the pure, denatured PCC competed fully for the antibody, the *pcc C* liver homogenate did not compete at all. When the experiment was repeated using similarly denatured control homogenate instead of pure enzyme, the result was identical (table 1).

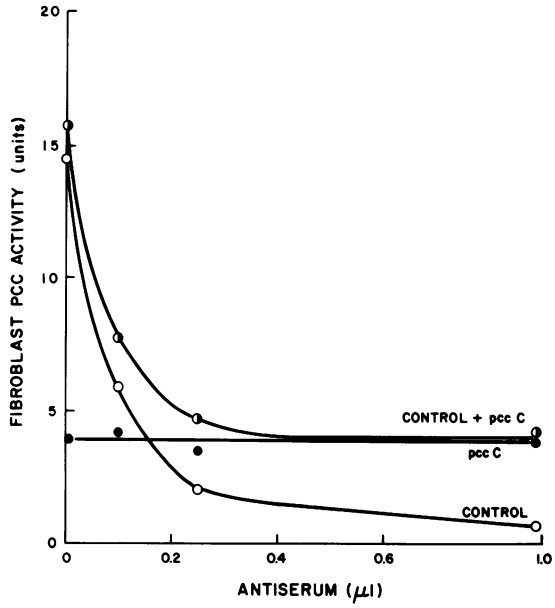

FIG. 4.—Competitive immunotitration of PCC activity in fibroblast extracts: 16 U of control activity (○); 12 U of control activity mixed with 4 U of carboxylating activity from *pcc C* cells (◑); and 4 U of mutant activity alone (●).

Effect of Immunoglobulins and Temperature on PCC Activity

Because our method of disrupting fibroblasts was also different from that used by McKeon et al. [16], we compared the amount of propionyl CoA carboxylating activity in control and mutant extracts using three different methods for releasing the enzyme from fibroblasts: 0.5% Triton X-100, which was used by McKeon et al. [16]; freezing at −70°C followed by addition of 0.5% Triton X-100; and sonication. Sonication released 10–20 times more carboxylating activity from both mutant and control cell lines (table 2). In no case, however, was mutant carboxylating activity titratable with anti-PCC antiserum (data not shown).

The small amount of propionyl CoA carboxylating activity released from mutant cells by Triton X-100 or by freezing followed by Triton X-100 was also more sensitive to temperature and to nonspecific inactivation by the immunoglobulins present in control rabbit serum. When mutant extracts were incubated at 37°C for 30 min under the conditions used by McKeon et al. [16], about 50% of the original activity was lost, whereas in control extracts, a slight increase was noted (table 2). The addition of immunoglobulins purified from control rabbit serum resulted in the loss of 75% or more of the original activity in the mutants (table 2).

Residual Propionyl CoA Carboxylating Activity in Mutant Extracts

No *pcc* mutant fibroblast line reported previously has been completely devoid of PCC activity, the specific activity in fibroblast extracts from both *pcc A* and *pcc C* cell lines ranging between 0.3% and 8% of that found in control cell lines

TABLE 1

COMPETITIVE IMMUNOTITRATION OF PROPIONYL CoA CARBOXYLATING ACTIVITY IN FETAL LIVER

ENZYME SOURCE	PCC ACTIVITY (U)	
	− Anti PCC antiserum	+ Anti PCC antiserum
Control fetal liver	1,708	654
Control fetal liver homogenate:		
+ *pcc C* fetal liver homogenate	1,774	771
+ Denatured pure PCC	1,698	1,523
+ Denatured control fetal liver homogenate	1,803	1,743

NOTE: Immunotitration was performed as described in MATERIALS AND METHODS using 5 μl of normal rabbit serum (left column) or 1.5 μl of anti-PCC antiserum diluted to 5 μl with normal rabbit serum (right column).

[9]. However, during our competitive immunotitration studies in which mutant extracts were assayed at high protein concentrations, we found that the apparent specific activities for PCC were often higher than reported previously. Therefore, we conducted a systematic study of PCC activity over a wide range of protein concentration in mutant and control extracts. Using β-methylcrotonyl CoA carboxylase (MCC) as a control enzyme, we found that the specific activity of propionyl CoA carboxylation in fibroblast extracts from both *pcc A* and *pcc C* complementation groups reached values higher than 25% of those in control extracts when an extract protein concentration of 12 mg/ml was used; activity decreased rapidly as the extract was diluted, however (table 3). With mutant fetal liver homogenate, we observed as much as 100% of the activity found in control fetal liver homogenate at protein concentrations of 16 mg/ml or higher (table 3). Again, dilution of the mutant homogenate led to a sharp fall in specific activity. With both tissue sources, however, specific activity of PCC in control fibroblasts and liver homogenates remained unchanged over a wide range of protein con-

TABLE 2

EFFECT OF TEMPERATURE AND IMMUNOGLOBULINS (IgG) ON PROPIONYL CoA CARBOXYLATING ACTIVITY RELEASED FROM CONTROL AND MUTANT FIBROBLASTS

METHOD OF CELL DISRUPTION	INCUBATION CONDITIONS	UNITS OF PCC ACTIVITY IN EXTRACTS		
		Control	*pcc A*	*pcc C*
Triton X-100	0°C	20.1	1.25	1.70
	37°C	24.4	0.62	1.05
	37°C + IgG	21.8	0.34	0.49
	37°C + Anti-PCC	0.6	0.22	0.39
Freezing and Triton X-100	0°C	47.0	3.30	3.48
	37°C	54.5	1.16	1.34
	37°C + IgG	43.8	0.57	0.31
	37°C + Anti-PCC	1.9	0.51	0.36
Sonication	0°C	406	63.4	29.5
	37°C	472	22.3	25.1
	37°C + IgG	393	24.6	17.1
	37°C + Anti-PCC	34	23.5	17.6

TABLE 3

EFFECT OF DILUTION ON THE SPECIFIC ACTIVITY OF PROPIONYL CoA CARBOXYLATING ACTIVITY IN CONTROL AND MUTANT FIBROBLAST EXTRACTS AND FETAL LIVER HOMOGENATES

FIBROBLAST EXTRACT				FETAL LIVER HOMOGENATE*		
	SPECIFIC ACTIVITY (U/mg)				SPECIFIC ACTIVITY (U/mg)	
Protein concentration† (mg/ml)	Control	*pcc A*	*pcc C*	Protein concentration† (mg/ml)	Control	*pcc C*
12	904	248	227	24	9,562	11,741
6	881	154	138	16	10,074	10,607
3	892	88	74	8	9,830	5,204
1.5	902	53	49	4	10,318	2,696
0.75	920	30	28	2	10,232	718
0.38	940	17	18	1	10,108	148

* Incubation time for PCC assay was a 1-min rather than the 15-min interval employed for fibroblast extracts.
† Protein concentration was first adjusted to 12 or 24 mg/ml, respectively, and then diluted with 50 mM Tris-HCl, pH 7.5.

centrations. Because the dramatic loss of carboxylating activity in diluted mutant extracts might have been caused by dilution of "stabilizing factors" required by the mutant enzyme, we added a variety of possible candidates to the reaction mix. Neither the addition of PCC substrates (used individually or in combination) nor that of bovine serum albumin, control fibroblast extract (in which normal PCC was inactivated by treatment at pH 9.0), different buffers, 2-mercaptoethanol, or glycerol had any demonstrable effect.

Further Characterization of the Propionyl CoA Carboxylating Activity in Mutant Extracts

To determine whether control fibroblast lines also contain some propionyl CoA carboxylating activity that does not interact with anti-PCC antisera, we compared PCC immunotitration curves in fresh control extracts with those in control extracts kept at 4°C for 20 hrs prior to titration. We found that about 10% of PCC activity in fresh extracts did not react with antiserum, whereas in extracts stored at 4°C for 20 hrs, all PCC activity could be titrated (fig. 5). It is significant that, at similar protein concentrations, the fraction of nontitratable PCC activity in freshly prepared and immediately titrated control extracts corresponded to the residual activity found in the mutant cell lines. This residual carboxylating activity is not produced by MCC, which can also utilize propionyl CoA [22], because 50% of

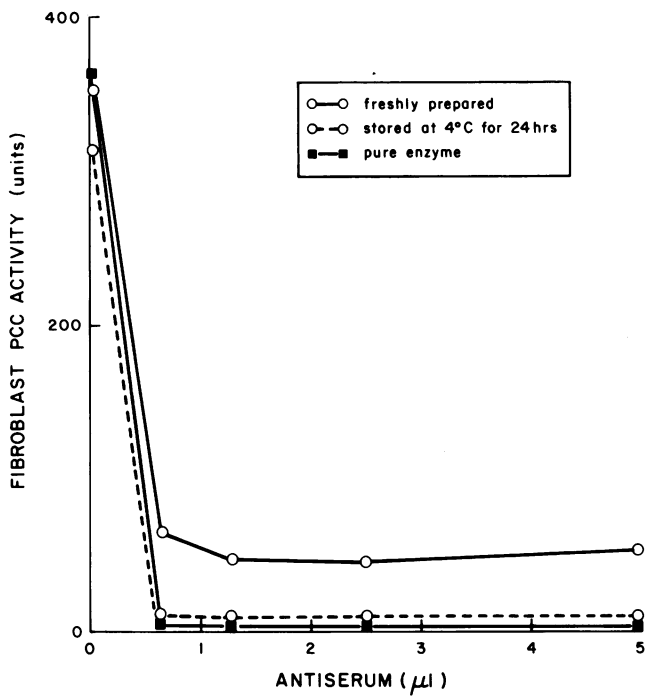

FIG. 5.—Immunotitration of PCC activity in control fibroblast extract immediately after sonication (o—o) and after 20 hrs at 4°C (o- - -o). Identical amounts of pure human hepatic PCC (■—■) were used in parallel to ensure that all PCC activity was neutralized by the antiserum.

MCC activity was retained after 20 hrs of storage at 4°C. We tried to purify the enzyme responsible for the residual activity in mutant extracts using a method of monomeric avidin affinity chromatography; more than 90% of the residual activity was lost, thereby making any further characterization impractical.

DISCUSSION

Both we [9] and others [8] have proposed that the two major genetic complementation groups identified in patients with isolated deficiency of PCC activity (*pcc A* and *pcc C*) result from mutations in the structural genes coding for the nonidentical α and β subunits of PCC, respectively (reviewed in [23]). This thesis, however, in no way accounts for the observation that all PCC-deficient patients manifest in their tissues some residual propionyl CoA carboxylating activity [9]. To explore this matter further, we raised antisera in rabbits against native PCC purified from human liver and pig heart and against its respective subunits and estimated the amount of CRM in mutant cells from patients in the *pcc A* and *pcc C* groups and in the distinct group (*bio*) in which PCC deficiency occurs secondary to impaired holocarboxylase synthetase activity.

Not unexpectedly, extracts prepared from fibroblasts of the *bio* mutant contained an amount of CRM comparable to that noted in control extracts. None of three *pcc A* or three *pcc C* cell lines, however, contained any detectable CRM, despite residual apparent PCC activity in the range of that in the *bio* mutant. Moreover, a fetal liver homogenate from a *pcc C* patient also lacked detectable CRM, thereby excluding the possibility that our results with fibroblast extracts reflected some artifact of cell propagation or disruption. Because our results were in complete disagreement with those of McKeon et al. [16], who reported that *pcc A* and *pcc C* mutant cells have normal amounts of CRM, we explored as many explanations for the disparity as possible. Hence, we used some of the same cell lines employed by them (one *pcc A* mutant and one *pcc C* mutant), and attempted to reproduce exactly their conditions of cell disruption and immunotitration, and we used antisera raised against pig heart PCC as they did. In no instance did we find detectable CRM in *pcc A* and *pcc C* mutants. Further, neither modifying the method of fibroblast disruption, nor using antisera raised against individual PCC subunits, nor carrying out competitive (rather than direct) immunotitrations yielded any measurable CRM in extracts from *pcc A* and *pcc C* cells. Significantly, Lam Hom Wah et al. [24] obtained results consistent with ours. They labeled intact fibroblasts with [^{35}S]methionine and then attempted to immunoprecipitate PCC subunits from partially purified cell extracts using antisera raised against native PCC. In neither *pcc A* nor *pcc C* extracts were their findings compatible with the existence of assembled PCC.

In the course of these experiments, we found that the residual propionyl CoA carboxylating activity in *pcc A* and *pcc C* mutants was sensitive to dilution, to storage (even at room temperature), and to the addition of normal immunoglobulins that we used to adjust protein concentrations in our immunotitrations. We suggest that McKeon et al. [16] overlooked these phenomena, thereby obtaining immunotitration curves that they interpreted as indicating the existence of CRM in their mutant extracts.

How then do we account for the residual propionyl CoA carboxylating activity found reproducibly in *pcc A* and *pcc C* mutant cells? Conceivably, in each mutant class a modified PCC enzyme exists that has lost all of the antigenic determinants present on normal PCC and, hence, does not react in our immunotitration experiments. This seems highly unlikely given the range of antisera we employed and the results of Lam Hom Wah et al. [24]. A more likely possibility is that the residual activity found in mutant cells reflects another carboxylase capable of using propionyl CoA as substrate. This explanation is supported by previous findings that biotin-dependent carboxylases have overlapping substrate specificities [22], and by our current observations that the residual activity in mutant extracts has stability characteristics, such as its marked dependence on protein concentration (table 3) and its lability to storage even in the cold unlike those of "true" PCC. Our past [25] and present findings make it highly unlikely that this putative carboxylase is either acetyl CoA carboxylase or β-methylcrotonyl CoA carboxylase. Its nature and physiologic role remain obscure at this time. Given the heteropolymeric (αβ) nature of many biotin-dependent carboxylases, however, it is possible that the residual activity in mutant cells could reflect association of carboxylase subunits designed for one purpose (e.g., propionyl CoA carboxylation) with those for another (e.g., β-methylcrotonyl CoA carboxylation). This thesis can be tested only when methods of in vitro reconstitution of carboxylase activity from isolated subunits are perfected.

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