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Genetics

Analysis of genetic complementation by whole-cell microtechniques in fibroblast heterokaryons

[cell fusion/propionate incorporation/propionicacidemia/propionyl-CoA carboxylase (ATP-hydrolyzing)]

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ABSTRACT **A whole-cell microtechnique for the determination of complementation of human metabolic disorders is presented. This procedure permits the isolation of individual multinucleate cells produced by cell fusion for the quantitative evaluation of complementation. Mutant fibroblasts with a deficiency of propionyl-CoA carboxylase activity (EC 6.4.1.3) that had been mapped to complementation groups *pcc* and *bio* were used to evaluate the microtechnique. Complementation was monitored by the determination of [^{14}C]propionate incorporation into cellular macromolecules. Single cells or a small number of cells were isolated from plastic film dishes after radioactive incubation by cutting out the portion of the plastic film holding the desired cells. Isotope incorporation was linear in 10–50 unfused cells and in 10–50 fused normal cells containing five or more nuclei. There was also a direct correlation between the nuclear content of cells and the amount of isotope incorporated. Three *pcc* and two *bio* mutants were fused in pairwise combinations by means of polyethylene glycol and complementation was determined by isotope incorporation in sets of 50 multinucleate cells, each cell isolated individually. The results agreed with autoradiographic data for both complementing and noncomplementing strains. The method is quantitative and gives severalfold higher sensitivity than current procedures. The method can be applied to the complementation analysis of a wide variety of inherited disorders of intermediary metabolism.**

Human metabolic disorders are often characterized by a range of clinical expression. It is unknown if this variation results from the effects of different allelic mutations, or if more than one gene may be involved in the same disorder. In some cases, what is thought to be the same clinical disease may even be a combination of genetically distinct disorders. One approach to this question involves the use of complementation analysis (1). Complementation can be studied by fusing mutant fibroblasts in the presence of polyethylene glycol (PEG) or inactivated Sendai virus to produce heterokaryons (multinucleate cells containing nuclei of both mutant strains) and determining if the abnormal phenotypes associated with the mutation are corrected. With current procedures, true heterokaryons generally make up 10–30% of a fused cell population. The remainder is composed of unfused parental cells and homokaryons containing nuclei of only one parent.

Several metabolic disorders have been examined by complementation analysis. These include maple syrup urine disease (2), GM_1 (3–5) and GM_2 (6–8) gangliosidosis, xeroderma pigmentosum (9), propionicacidemia (10–12), and methylmalonicacidemia (13–14). Complementation has usually been determined by assaying extracts of the complete fusion mixture (batch assay method) (2, 5–9, 12, 14) and comparing these results to those obtained by self-fusion or in unfused mixtures of cells. The method is considered valid when complementation is clearly demonstrated. The difficulty in using the batch method as a general test for complementation lies in attempting to interpret negative results. Because only true heterokaryons are competent to complement, it is uncertain whether mutants that fail to show complementation are truly defective in the same gene. There is the possibility that the enzyme assay is not sensitive enough to detect complementation in the proportion of cells that are truly heterokaryotic, given the background activity of the unfused parental or homokaryotic cells.

Autoradiographic and histochemical methods have been used in other studies to detect complementation. Here the results of individual multinucleate cells can be observed visually by light microscopy. In our own work on complementation we have used autoradiographic methods in the study of propionicacidemia (10, 11) and methylmalonicacidemia (13) and histochemical methods for analysis of GM_1 gangliosidosis, sialidosis (4), and I-cell disease (unpublished data). All of these methods represent unique approaches specific to the defect in question and are not generally available for most metabolic disorders.

We have turned to whole-cell microtechniques for the quantitative determination of complementation in isolated multinucleate cells. This is a simplification of the microtechnique developed by Hösli (15). In principle, a population of fused living cells is incubated in the presence of a radioactive precursor. At the end of incubation, multinucleate cells are isolated and assayed for the presence of radioactive product.

Because we had previously demonstrated complementation in propionicacidemia by using an autoradiographic method to monitor [^{14}C]propionate incorporation in fused cells (10), we have used known complementers from this system to establish the conditions for complementation analysis by whole-cell microtechniques. In this report we present a microtechnique involving the [^{14}C]propionate incorporation system in which individual heterokaryons are isolated and assayed directly for radioactivity. This method proves the validity of the autoradiographic procedure and is a highly sensitive assay method of detecting activity in as few as 10–50 cells.

MATERIALS AND METHODS

Source and Growth of Cells. The methods for the growth and maintenance of cultured fibroblasts have been described (10), except that antibiotic-free medium was used in the present experiments. The mutant strains were from individuals with propionicacidemia or biotin-responsive organicacidemia (10, 11). Propionicacidemia results from a deficiency of propionyl-CoA carboxylase activity (EC 6.4.1.3), and biotin-responsive organicacidemia is a disorder in which there is a deficiency of several carboxylase activities, including propionyl-CoA carboxylase, β-methylcrotonyl-CoA carboxylase (EC 6.4.1.4), and pyruvate carboxylase (EC 6.4.1.1). The latter defect is expressed

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Abbreviation: PEG, polyethylene glycol.

in cells grown in biotin-free medium under conditions that do not affect the activities of normal cells, but the enzyme activities are restored to normal levels with biotin supplementation. Complementation groups were previously determined by an autoradiographic method that detects [^{14}C]propionate incorporation in multinucleate cells (10, 11). The nomenclature used to describe the complementation groups is *pccA* and *pccBC* for propionicacidemia and *bio* for biotin-responsive organicacidemia.

The following mutant strains were kindly provided by L. Sweetman, University of California at La Jolla: strains 456 (patient initials J.R.), 500 (L.S.) 536 (K.F.), and 541 (B.B); and strains 447 (R.H.) and 450 (N.M.) were provided by L. E. Rosenberg, Yale University, New Haven, CT. One normal strain, 595, was included as a control. All of the strains reported in this study were certified mycoplasma free by broth or agar assay or determination of UDP phosphorylase activity after at least two passages in antibiotic-free medium (done by P. Quinn, Hospital for Sick Children).

Cell Fusion. The cells were fused in the presence of polyethylene glycol 1000 (PEG 1000, Merck), according to Davidson *et al.* (16), except that the PEG was made up as a 40% (vol/vol) solution in α medium (17) without fetal calf serum. PEG at a higher concentration, including a 50% solution (16), was often lethal to a large portion of the culture being fused. The proportion of cells demonstrating multiple nuclei after dilution plating was 20–40%.

For complementation studies involving biotin-responsive cells, all strains to be fused were grown in biotin-free medium for at least 2 weeks prior to fusion. The propionyl-CoA carboxylase activity of the mutant strains was reduced to background levels in this medium but remained normal in wild-type cells (11).

[^{14}C]Propionate Incorporation. The procedure for detecting [^{14}C]propionate incorporation in cells *in situ* by using an autoradiographic method was described by Hill and Goodman (18). It has also been adapted to the direct determination of radioactivity by scintillation counting (19, 20). Several modifications were instituted in order to apply it to the microtechnique. The composition of the incubation medium was modified to include 0.5 mM alanine and 10 mM glucose (see *Results*). The specific activity of [1-^{14}C]propionate was increased to the maximum available, 59 mCi/mmol (1 Ci = 3.7×10^{10} becquerels), and the acid was used at 50 μM.

The conditions for isotope incorporation were established on cells incubated at a density of 5×10^4 cells per cm^2 in Linbro wells (ICN). The day after plating, the cells were incubated at 37°C in the presence of the [^{14}C]propionate medium. After up to 16–20 hr, the reaction was stopped by washing twice with ice-cold phosphate-buffered saline (21), and once, for 10 min, with ice-cold 10% trichloroacetic acid. The cells were rinsed thoroughly with water and were removed by exposing them to 0.3 ml of a 1:10 dilution of concentrated ammonium hydroxide in water until they were completely lysed as observed by microscopy. This was followed by two more rinses of the same volume of water. All three washes were combined and radioactivity was determined by scintillation counting in Aquasol.

For microtechniques, cells were plated in plastic film dishes (22) at $1–3 \times 10^5$ cells per dish. The density of plating was varied depending on the number of cells to be cut out. The cells were incubated in the isotope medium and processed as they were in Linbro wells. After the trichloroacetic acid wash and water rinse, the plastic film dishes were drained thoroughly and air dried. The dishes were placed on a glass plate and observed under a stereomicroscope or an inverted transmission microscope. Small sections of the plastic film containing a desired number of cells, usually 5–50, were cut out with a scalpel or other microsurgery knife. The cutting was done in a "tic-tac-toe" pattern with the center rectangle containing the desired cells. The crosscuts were made with a convex blade (no. 15 surgical blade or eye surgery blade, Beaver, Belmont, MA) by pressing and rocking the blade so as not to pull against the plastic film. The leaflets of cells were removed by stabbing with the pointed end of a straight-edge scalpel and transferring directly to a scintillation vial containing 1 ml of water. The leaflets readily fell off the knife on contact with the water. Aquasol was added and the radioactivity was determined in a scintillation counter.

Complementation Tests. Cells were fused and after incubation for 3 days at 37°C were transferred to plastic film dishes. The cells were incubated overnight in the [^{14}C]propionate medium as described above. At the end of incubation 20 μl of 0.05% acridine orange in water was added directly to the incubation mix and incubation was continued for an additional 10 min. The cells were fixed, washed, and dried as described above. The dried dishes were examined for multinucleate cells. The nuclei could be readily identified by their fluorescence in UV light under a Leitz Diavert inverted microscope equipped for fluorescence illumination. Fifty fused cells containing five or more nuclei were cut out. Each multinucleate cell was placed on a moist piece of filter paper while being observed through a stereomicroscope to ensure successful transfer. After all 50 cells had been transferred, the filter paper was carefully placed in a scintillation vial containing 1 ml of water. Aquasol was added and radioactivity was determined. Fifty fused cells could usually be isolated from a single plastic film dish and the procedure for each set took 1–2 hr.

RESULTS

For the microtechniques, it was necessary to modify the [^{14}C]propionate incorporation method of Hill and Goodman (18) to maximize the absolute amount of radioactivity detected in cellular macromolecules. Table 1 shows that a $2\frac{1}{2}$-fold increase in radioactive incorporation was obtained by reducing the glucose concentration to 10 mM and adding 0.5 mM alanine to the incubation mix. A similar stimulation was observed for isotope incorporation by a mutant strain. Interestingly, pyruvate had an inhibitory effect on isotope incorporation when compared to the value obtained in 10 mM glucose alone. Lactate had no effect.

[^{14}C]Propionate is metabolized via succinyl-CoA and the Krebs cycle to produce ^{14}C-labeled trichloroacetic acid-precipitable material. The latter material has not been analyzed, but most of it must be radioactive protein produced by the transamination of [^{14}C]oxaloacetate to [^{14}C]aspartate. Therefore, the stimulation of isotope incorporation by alanine prob-

Table 1. Conditions of [^{14}C]propionate incorporation

Modification of incubation conditions*	^{14}C incorporated, cpm ± % SD	
	Normal, $n = 4$	450, *pccC*,† $n = 2$
None (50 mM glucose)	32,520 ± 7.1%	1425 ± 1.0%
10 mM glucose	53,156 ± 19.7%	1950 ± 3.9%
10 mM glucose + 0.5 mM alanine	81,674 ± 6.0%	2542 ± 4.7%
10 mM glucose + 0.5 mM pyruvate	27,403 ± 12.7%	1700 ± 43.9%

* Incubation conditions: 50 mM glucose, 15% dialyzed fetal calf serum, 50 μM sodium [^{14}C]propionate (10 mCi/mmol), in Puck's saline.

† A mutant with a defect of propionyl-CoA carboxylase assigned to complementation group *pccC*.

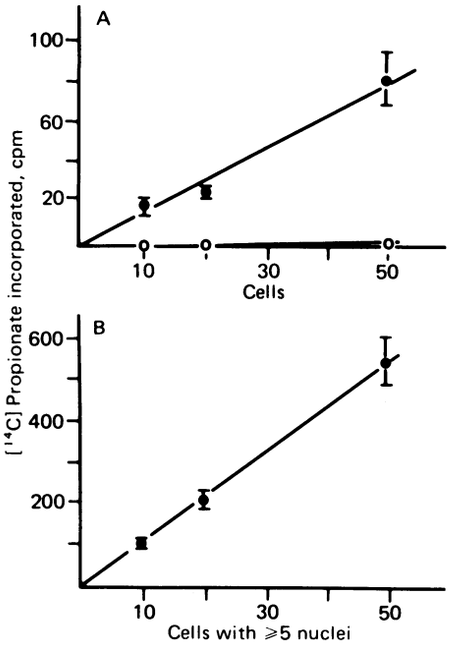

FIG. 1. [^{14}C]Propionate incorporation, showing linearity with cell number. Cells, unfused (*A*) or fused (*B*), were incubated in isotope medium in plastic film dishes overnight. They were processed and dried as described in text. (*A*) Samples of 10, 20, or 50 cells, $n = 7$ samples, were cut out and radioactivity was determined. ●, Strain 595, *pcc+*; ○, strain 500, *pccBC*. Blank values of 21 cpm were subtracted. They were obtained by determining radioactivity in plastic film leaflets, $n = 4$, of a size corresponding to 50 cells, taken from experimental plates. (*B*) Individual multinucleate cells of strain 595 containing five or more nuclei were cut out and combined to produce samples of four sets of 10, four sets of 20, or 3 sets of 50 cells. A blank of 22 cpm was subtracted as for *A*. Specific activity of [^{14}C]propionate, 59 mCi/mmol. Error bars represent 1 SD.

ably results from its utilization as nitrogen source for the transamination reaction. The inhibition by pyruvate can be explained by competition with oxaloacetate for endogenous nitrogen.

Although not shown here, isotope incorporation continues for 15–18 hr and remains unchanged for at least 24 hr. Presumably by this time the cells are depleted of endogenous amino acids. The incubation medium did not include amino acid supplementation because it results in a significant reduction in isotope incorporation. This reduction may be caused by isotope dilution, particularly in the Krebs cycle, because several amino acids can be converted to Krebs cycle intermediates.

The stimulation of isotope incorporation obtained by reducing the glucose concentration is more difficult to explain. Hill and Goodman (18) reported that little incorporation occurred at 5 mM glucose, the concentration in culture medium, and that maximal incorporation occurred at 50 mM glucose. We did not obtain similar results, although the possibility exists that such a high concentration of glucose was toxic to the cells for the period of incubation used in this study. All of the studies described below were done in the alanine-supplemented medium; high specific activity [^{14}C]propionate was used.

Fig. 1*A* shows the isotope incorporation obtained for samples of 10, 20, and 50 cells. The graph shows the [^{14}C]propionate incorporation in normal cells to be about 80 cpm per 50 cells. This is close to the expected value that can be calculated from the results in Table 1 after correcting for the higher specific activity of the isotope. Mutant cells produced negligible incorporation of isotope. The background of approximately 22 cpm was obtained from a blank piece of plastic film from each experimental plate of a size that could hold about 50 cells; however, the same result was obtained for scintillation fluid alone.

The isolation of a fused cell is shown in Fig. 2. The cells were stained with Giemsa so that clear pictures could be produced. Fifty such cells were combined for radioactive counting.

Fig. 3 shows the distribution of the number of nuclei per cell obtained after fusion with 40% PEG 1000. This is the pattern of nuclear fusion obtained after the dilution plating of the fused monolayer. It is important to make this distinction because PEG produces well over 50% fusion but most cells with more than 10–15 nuclei do not survive trypsinization. Cells containing at least five nuclei were used to test for complementation because the probability that such a cell is a homokaryon is low, $2 \times (\frac{1}{2})^5$. It was not possible to restrict the isolation of cells to those with a specific nuclear content because of the difficulty of finding as many as 50 such cells (only 1 in 70 contained five nuclei). However, by isolating all cells with at least five nuclei, a sufficient number of multinucleate cells could be isolated, and the nuclear distribution from sample to sample remained fairly constant. For example, the average nuclear content of the 398 cells containing at least five nuclei used to obtain the data in Fig. 3 was 6.1 nuclei.

The amount of [^{14}C]propionate incorporated as a function

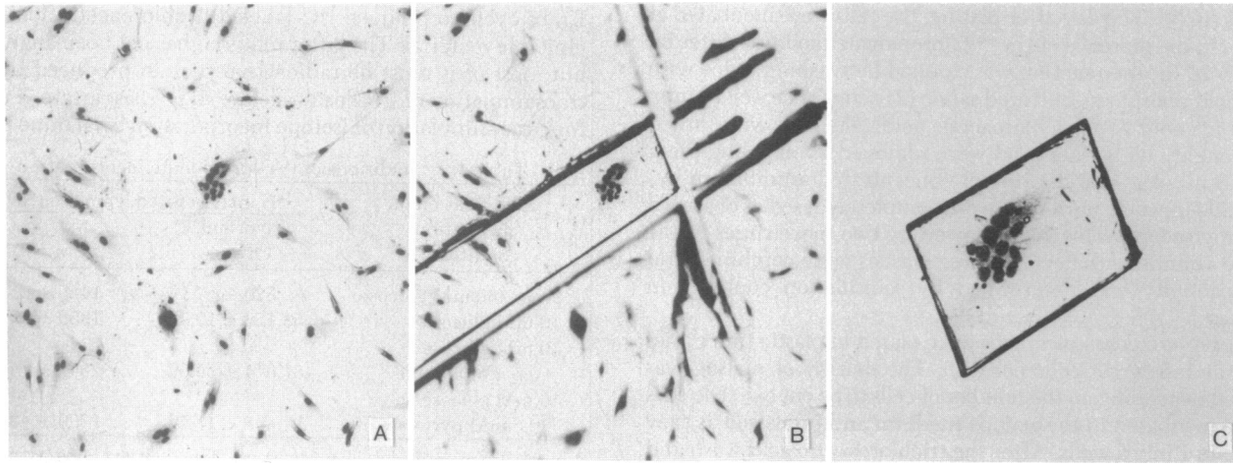

FIG. 2. (*A*) Giemsa-stained multinucleate cells in a dried plate after isotope incorporation and processing. (*B*) Multinucleate cell in a field after three of the four cuts required to isolate the cell have been made. (*C*) Enlargement of the cell after it was cut out. (*A* and *B*, ×64; *C*, ×128.)

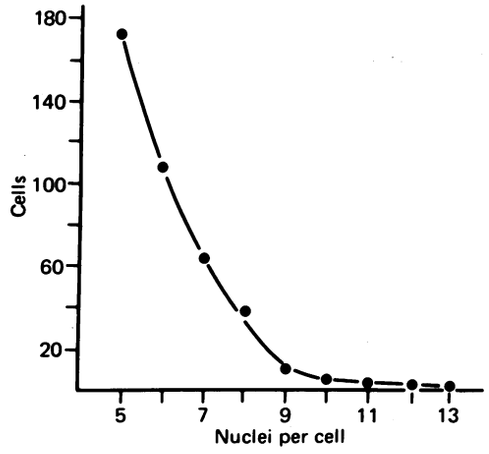

FIG. 3. Distribution of cells as a function of the number of nuclei per cell. Cells were fused in the presence of 40% PEG as described in the text. After three days, the cells were sparsely plated in plastic film dishes. The next day acridine orange was added and the cells were processed and dried as for the isotope incorporation procedures. Cells were counted and their nuclear contents were recorded. This fusion produced 40% multinucleate cells, of which 3% of the total contained five or more nuclei.

of the number of fused cells isolated is shown in Fig. 1*B*. The relationship is linear. These results confirmed the validity of doing complementation assays in sets of the larger multinucleate cells.

Mutant strains lacking propionyl-CoA carboxylase activity are unable to metabolize [^{14}C]propionate beyond the enzyme defect. First, one mutant, previously mapped by the autoradiographic method to complementation group *pccA*, and two others mapped to *pccBC* (10, 11), were examined for complementation by the whole-cell microtechnique. The results of fusions between the *pccA* and two *pccBC* mutants are given in Table 2, experiment 1. Self-fusion of each of the mutant strains gave values ranging from 24 to 35 cpm above background. When the *pccA* mutant was fused to either of the *pccBC* mutants, the isotope incorporation increased 10- to 12-fold above the values obtained for the average of the corresponding self-fusions. The radioactivity incorporated in these fusions represents about half the value obtained for the self-fusion of normal cells shown in Fig. 3. When the different *pccBC* mutants were fused to each other, no stimulation of [^{14}C]propionate incorporation was observed. These complementation results agree directly with those obtained by the autoradiographic method.

Table 2. Complementation tests between mutant strains

Strains	Genotype	[^{14}]Propionate incorporated,* cpm	Observed/ expected†
	Experiment 1		
447/447	*pccA/pccA*	24	
500/500	*pccBC/pccBC*	35	
536/536	*pccBC/pccBC*	24	
447/500	*pccA/pccBC*	359	12.2
447/536	*pccA/pccBC*	249	10.4
500/536	*pccBC/pccBC*	29	1.0
	Experiment 2		
456/456	*bio/bio*	34	
541/541	*bio/bio*	22	
500/500	*pccBC/pccBC*	30	
456/500	*bio/pccBC*	238	7.4
541/500	*bio/pccBC*	192	7.4
456/541	*bio/bio*	26	0.9

* Radioactivity was determined for samples of 50 fused cells each containing five or more nuclei. Blank values averaging about 25 cpm were subtracted. Specific activity of [^{14}C]propionate, 59 mCi/mmol.

† cpm incorporated for test fusions divided by average of cpm incorporated of the appropriate self-fusions.

Second, we examined two mutant strains, designated *bio*, that show a multiple carboxylase deficiency and in which enzyme activity can be restored to normal in medium containing excess biotin (11). Complementation of the propionyl-CoA carboxylase deficiency was examined in fusions between these strains and with a *pccBC* mutant by microtechniques. Table 2, experiment 2, shows the results of these fusions. When either of the *bio* mutants, strain 456 and 541, was fused with a *pccBC* mutant, strain 500, a 7- to 8-fold increase in isotope incorporation was obtained. Thus the *bio* mutants complement strain 500. However, when the *bio* mutants were fused to each other there was no stimulation of isotope incorporation. Similar results were obtained by using the standard autoradiographic procedure (11). Thus these results confirm that the *bio* mutants belong to a unique complementation group.

DISCUSSION

Hill and Goodman (18) pioneered the use of measures of [^{14}C]propionate incorporation to detect inborn errors of propionate metabolism, including propionicacidemia and methylmalonic acidemia. We (10, 11, 13) and others (12, 14) have employed this procedure to detect complementation in both of these disorders by autoradiographic or direct radioactive counting methods. Here we have described a method for the whole-cell determination of [^{14}C]propionate incorporation that can be applied to isolated multinucleate cells and studies of complementation.

The method we have used to isolate and analyze multinucleate cells is a simplification of the microtechnique of Hösli (15). In the full procedure, usually 5–50 cells are isolated from freeze-dried cultures in plastic film dishes for the assay of enzymes in lysates of less than 1 μl. For complementation analysis this would require isolating individual multinucleate cells and assaying them singly or in groups for enzyme activity. Complementation studies involving several lysosomal hydrolases have been done in this way (3, 22, 23). Unfortunately, to assay the enzymes of intermediary metabolism a large number of multinucleate cells would have to be isolated to produce sufficient enzyme activity. The time required to combine sets of as many as 50 cells for extract assay would make this procedure impractical. Therefore our approach was to do complementation by whole-cell assay in which the enzyme reaction could be completed in the plastic film dish before cells were isolated for product determination. In this way the later isolation of multinucleate cells could be done without the pressure of time or the problem of enzyme instability.

The results of our study show that amount of isotope incorporation per cell correlates directly with the number of nuclei per cell. Thus sets of cells with single nuclei incorporated about 80 cpm per 50 cells and fused cells containing an average of 6.1 nuclei incorporated 544 cpm per 50 cells, or 89 cpm per nuclear equivalent. Such a striking correlation suggests that the amount of enzyme per cell correlates directly with the gene dosage and that the nuclei are functionally equivalent. Because the fused cells appear to be large, it may be that the specific activity of the enzymes remain unchanged but what does change is cell volume in fused cells.

We have used acridine orange to stain nuclei so that mul-

tinucleate cells could be identified on dried plates by fluorescence microscopy. In our studies, the cells were fixed and washed with trichloroacetic acid to remove small molecules. Under these conditions, and with some practice, the nuclei of cells could be discerned by bright-field or phase-contrast microscopy without staining. This situation is unique to the requirement for the acid wash. If the product to be determined were a small molecule, the cells would have to be washed in cold, phosphate-buffered saline and air dried. In this situation, the acridine orange step would be essential if the nuclear content of the cells were to be determined. One possibility for greatly simplifying the procedure is to forego the examination of nuclear content. After a fair amount of practice, it becomes fairly easy to recognize multinucleate cells as large broad cells. Large elongated cells usually contain only one to three nuclei.

The most commonly used method to test for complementation is the batch method (2, 5–9, 12, 14). In this procedure the complete fused cell monolayer can be extracted and assayed for enzyme activity or it can be assayed by a whole cell method. The results obtained are generally compared to the average of self-fusions or mixtures of the strains in question. There are some difficulties inherent in this approach. In our study, cell fusion in the presence of PEG 1000 produced about 40% multinucleate cells, of which it can be calculated that about 60% of these were true heterokaryons. Therefore, about 25% of all of the cells would be competent to show complementation. Yet, all of the cells in mixtures or self-fusion controls contribute to the negative control activity. Because complementation might result in a stimulation of enzyme activity of only up to 50% of normal levels on a theoretical basis (1), the result is that a stimulation of only 2- to 3-fold over mutant levels might actually be observed in the fused cell population. This has been the level of complementation observed in some studies (5, 7). Such results are reliable but it is more difficult to judge negative results, particularly if no positive results have been obtained between other pairs of mutants for comparison. The greater sensitivity of the microtechnique should facilitate the interpretation of negative results and will probably validate results that indicate a failure to complement. With this method the question of whether or not the fusion was adequate in a failed test is eliminated because only multinucleate cells of a minimum nuclear content are assayed.

We suggest that whole cell procedures can be used for the complementation analysis of a wide variety of metabolic disorders. The only requirements are that substrates with sufficiently high specific activity be available and that the substrate cannot be metabolized through other pathways before the enzyme step in question. The product to be determined can be the immediate product of the reaction being assayed or it can be an end point of metabolism such as protein or DNA. In such assays care must be taken to completely separate the substrate or other precursors from the product.

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