mechanism for the acute production of axonal dystrophy. Mitochondrial swelling has been noted together with axonal dystrophy following neuronal injury by x-irradiation (Andres, see *2*; Forssmann *et al.*, *12*) and by plasmocid (D'Agostino, see *2*) and cyanide (*13*, *14*) poisoning. However, these two lesions have not hitherto been causally linked. I have found that several metabolic inhibitors with different modes of action, including cyanide, arsenious oxide, ouabain, and methionine sulfoximine, when injected into the lumbar theca of cat, cause mitochondrial swelling in spinal neurons and dystrophic changes in their axons (*14*).

Although the proximodistal transport of axonal material is a well-documented physiological phenomenon, the mechanism responsible for this transport is still obscure (*15*). The present study may shed some light on this subject. It is known that the respiratory enzymes embedded within the mitochondrial membrane, in addition to catalyzing the coupled generation of adenosine triphosphate, perform osmotic and mechanical work involving conformational changes in the mitochondrion (*11*). Respiration-linked mechanochemical changes in mitochondria analogous to the "small amplitude" swelling-contraction cycles produced in vitro (*16*) and the configurational and volumetric changes observed in fibroblast mitochondria in tissue culture (*17*) probably occur in nervous tissue. Thus it seems reasonable to envisage neuronal (and axonal) mitochondria as pulsating organelles with a pump-like action that serves to propel the axonal stream in a proximodistal direction.

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5. A crude synthetic preparation of DL-fluorocitrate containing about 11 percent of the active isomer and kindly supplied by Sir R. A. Peters was used for some of these experiments. The barium salt of DL-FC supplied by Sigma Chemical Co. was also used after removal of the barium by precipitation as the sulfate, with identical results. The drug was injected intrathecally after an L-7 laminectomy performed under pentobarbital anesthesia. The animal was kept in the head-up position to reduce rostral flow of FC.
6. The 4 percent paraformaldehyde fixative contained 7 percent sucrose and .015*M* potassium phosphate buffer, *p*H 7.4. The 4 percent glutaraldehyde solution contained 0.1*M* cacodylate buffer, *p*H 7.4. Perfuse-fixed spinal cord was placed in paraformaldehyde for 24 hours, or glutaraldehyde for 4 hours, at 4°C, rinsed, and stored in cacodylate-buffered 7 percent sucrose [D. Sabatini *et al.*, *J. Cell Biol.* **17**, 19 (1963)] or in a gum-sucrose medium [S. Holt, *Exp. Cell Res. Suppl. No. 7*, 1 (1959)] at 4°C until used. Paraffin sections were stained by standard Nissl and myelin methods. Aldehyde-fixed frozen sections were stained for the following enzyme activities: acid phosphatase [G. Gomori, *Microscopic Histochemistry* (Univ. of Chicago Press, Chicago, 1952); β-glucuronidase [M. Hayashi *et al.*, *J. Histochem. Cytochem.* **12**, 293 (1964)]; acid esterase in the presence of .0001*M* diisopropyl-phosphofluoridate [A. Pearse, *Histochemistry* (Little, Brown, Boston, ed. 2 1960)]; NAD diaphorase, lactate and malate dehydrogenases with nitro B tetrazolium (A. Pearse, *ibid.*). Tissues incubated for these activities in the absence of the specific substrates showed no reaction. For electron microscopy, tissues were post-fixed in 1 percent buffered OsO_4 at 4°C and embedded in araldite, and ultrathin sections were stained with uranyl acetate and/or lead citrate, and examined in an RCA EMU3F electron microscope. In a few experiments nonfrozen sections [R. Smith and M. Farquhar, *Nature* **200**, 691 (1963)] of paraformaldehyde-fixed tissue were incubated in Gomori's medium for acid phosphatase activity, and then processed for electron microscopy.
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Complementation Analysis on Virus-Fused Chinese Hamster Cells with Nutritional Markers

Abstract. *Cell fusion experiments have been carried out with Chinese hamster cell mutants with different nutritional growth requirements. Conditions have been devised in which approximately 1 to 2 percent of the cell population remaining after fusion are fused, hybrid cells. The all-or-none nature of the genetic markers employed and the extremely low reversion rates insure that no contamination of the hybrid population with parental forms occurs. Hybrids between glycine- and hypoxanthine-requiring mutants are prototrophic, which indicates that both mutations are recessive. Hybrids between a glycine-deficient mutant and a single-step mutant which requires glycine, hypoxanthine, and thymidine are relieved of the glycine dependency, an indication that the two loci associated with glycine dependence are different. This mutation to the triple-supplement requirement as well as a proline deficiency were also shown to be recessive mutations. The system appears applicable to a variety of genetic problems.*

Experiments have been described (*1*) in which single-step gene mutations could be induced in Chinese hamster cells grown in tissue culture for long periods and possessed of reasonably stable karyotypes. These mutations are produced by standard mutagens such as ethyl methanesulfonate and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; they have spontaneous reversion frequencies varying between 10^{-6} and 2×10^{-8}; their corresponding reverse mutations can also be elicited by standard mutagens; and the nutritional requirements introduced appear to be absolute, since no growth whatever is exhibited by deficient mutants in the absence of the specific supplement. In the case of a spontaneous proline-deficient mutation, it was demonstrated that the mutant makes no proline, that the block in the biosynthetic chain lies in the step converting glutamic acid to its gamma-semialdehyde, and that the revertant to proline independence obtained from the mutant cell behaves like a hetero- or a hemizygote with respect to this gene (*2*). Availability of these markers makes possible application of the cell fusion technique (*3*) to obtain rapid and convenient quantitation and isolation of hybrid cells carrying combined genetic markers, without contamination

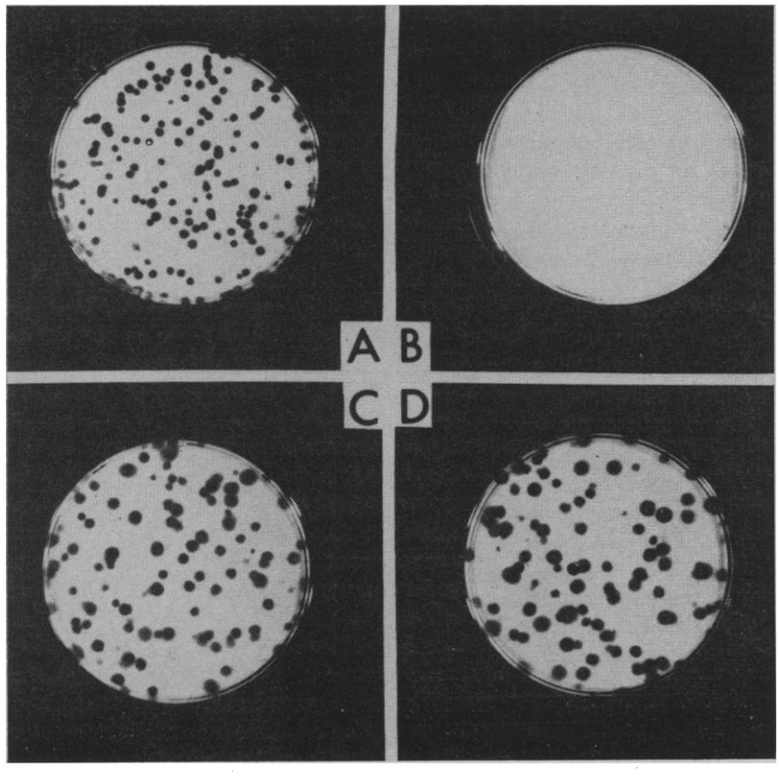
by the parental forms. It also permits conclusions about dominance and recessiveness, gene dosage effects, and linkage in relatively well-characterized genetic markers.

In all cases, the growth medium consisted of F12 (*4*) plus the macromolecular component of fetal calf serum, with specific omissions of metabolites as indicated. Mutants of the Chinese hamster ovary cell (CHO) were prepared by chemical or x-ray treatment, and clonal stocks were isolated by means of the 5-bromodeoxyuridine (BUdR)-"visible" light technique previously described (*5*). The parental form, CHO-K1, has a requirement for proline, a modal chromosome number of 20, and a reasonably constant karyotype which is shared by all the biochemical mutants derived from it through mutagenesis. These include glycine-requiring (gly^-) and hypoxanthine-requiring (hyp^-) mutants. In addition, a mutant derived from a Chinese hamster lung cell (CHL), in which a triple requirement for glycine, hypoxanthine, and thymidine was obtained (gly^--hyp^--thy^-) in a single treatment with ethyl methanesulfonate, was utilized. This cell and mutants derived from it have no proline requirement, and their growth rate is maximal even in the absence of added proline, an indication that they contain at least two functional proline genes (*2*). All of the mutant cells employed have plating efficiencies of zero in the absence of their specific nutritional supplements, while the wild types have a plating efficiency of about 80 percent either in the presence or absence of supplements.

The following standard cell fusion procedure was adopted: 5×10^5 cells of each desired type are added to a final volume of 1.0 ml of F12 minus the critical nutrilites. Ultraviolet-inactivated Sendai virus is added to produce a final titer of 200 hemagglutinating units. The mixture is kept at 4.0°C for 15 minutes, and then transferred to a shaker bath (37°C) for 15 minutes.

Experiments were designed to provide good yields of fused cells in which the binucleate forms predominate, thus avoiding the additional complications of the more highly polyploid forms. In a typical experiment, utilizing gly^- and hyp^- mutants, aliquots were plated out after the fusion period and the percentage of mono- and multinucleate cells present was counted microscopically. The resulting cell population contained 86.5 percent mono-, 8.5 percent bi-, 3.8 percent tri- and 0.9 percent tetranucleate cells.

When the entire cell population remaining after such fusions was plated in F12 lacking both glycine and hypoxanthine, approximately 1 to 2 percent of the plated cells grew into colonies. This represents a relatively high efficiency of hybrid recovery, since fusion is presumably random, so that homologous or heterologous cell fusion is equally likely. Control experiments carried out with cells of a single genotype yielded the same pattern of multinucleate cells as when two different auxotrophs were employed, but no growth whatever occurred when such



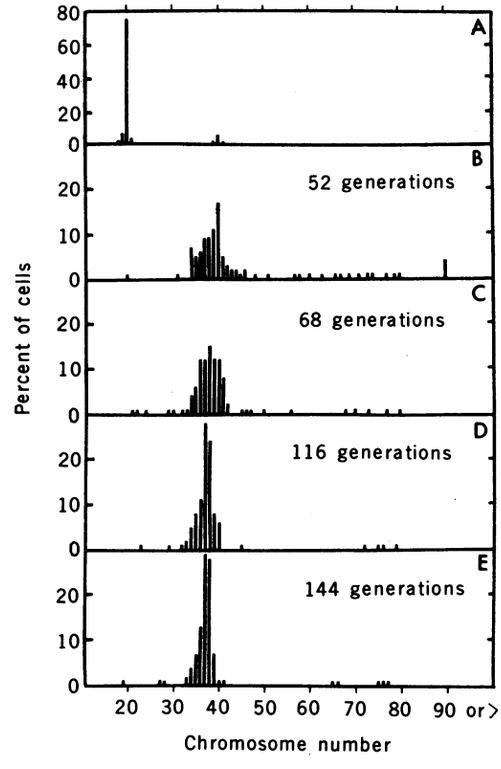


Fig. 1 (left). The appearance of plates seeded with identical numbers of single cells in the various selective media and incubated for 7 days. (A) The gly^- mutant in complete medium. (B) The gly^- mutant in the glycine-deficient medium. The hyp^- mutant exhibits a similar contrast in complete and hypoxanthine-deficient media, respectively. (C) The gly^-hyp^- hybrid in complete medium. (D) The gly^-hyp^- hybrid in medium lacking both glycine and hypoxanthine. Fig. 2 (right). Comparison of the chromosomal number distribution in the parental and hybrid cells. (A) chromosomal distribution of either parental cell. (B–E) Chromosomal distribution of a typical hybrid cell clone, as determined at various periods of growth after the fusion. The initial counts approximate the tetraploid number, but also contain appreciable numbers of more polyploid forms. With the passage of time, the modal number decreases by 5 to 10 percent, and most of the forms with chromosome numbers greater than 40 disappear. By the 116th generation, the distribution of chromosome counts appears to have stabilized, and has remained essentially unchanged after 200 generations. A concomitant increase in plating efficiency from about 50 percent to 70 to 80 percent occurs during this period, presumably because of the population drift to a distribution of forms with more stable chromosomal constitutions.

Table 1. Demonstration that while each of the mutant cells grows only in the supplemented medium, their fused hybrids grow without supplementation.

Clone	Plating efficiency		Generation time (hours)	
	Complete F12	F12 minus (gly + hyp)	Complete F12	F12 minus (gly + hyp)
Wild-type CHO-K1	86.5	84.8	12.2 ± 0.4	12.0 ± 0.3
Gly⁻ mutant	84.3	0	12.1 ± 0.4	∞
Hyp⁻ mutant	85.2	0	12.3 ± 0.5	∞
Hybrid clones:				
807–3	47.0	49.5	12.3 ± 0.4	12.3 ± 0.6
807–4	56.8	59.0	11.8 ± 0.3	12.0 ± 0.5
807–6	55.6	53.5	12.0 ± 0.5	12.1 ± 0.5

fused cells were plated in medium lacking both glycine and hypoxanthine.

Four colonies were picked and clonal cultures were established. All of these exhibited excellent single cell growth in the doubly deficient medium. Since the spontaneous reversion rate of each of the two parental auxotrophs is less than 5×10^{-8}, there is no doubt that all of these clones arose from the fusion process (Fig. 1 and Table 1). These data permit the conclusion that unless strange processes are operating, each of the mutations to glycine and hypoxanthine requirement, respectively, is recessive, since the fused cells show neither deficiency. Table 1 also demonstrates that the growth rate (as revealed by the generation time) of the colonies formed by the hybrids is maximally rapid and identical to the wild type, both in the deficient and the complete media.

In Fig. 2 a comparison of the parental and hybrid chromosomal counts is presented. As expected, the modal chromosome number of the hybrid clearly approximates twice that of the parental forms. With the passage of time, the hybridized cells at first lose chromosomes, eventually reaching a stable modal chromosome number of about 37 to 38. This behavior accords with that noted by previous investigators (*6*) [except for one case involving fusion of human and mouse cells which has been described, in which the loss of human chromosomes was extensive (*6*)]. Concomitant with these karyotypic changes, a definite increase in the plating efficiency of the clones occurs, raising their plating efficiency close to that of the parental forms in the supplemented medium.

It has been demonstrated (*1*) that a class of mutants can be produced which acquire simultaneously the need for supplementation with glycine, hypoxanthine, and thymidine for cell growth. It was postulated that only a single gene change had occurred, perhaps the one involved in the synthesis of tetrahydrofolic acid. If these three deficiencies result from a single gene change, it would necessarily be a different gene from that responsible for the glycine deficiency alone. In that case fusion between a mutant deficient for glycine only, and a triply deficient mutant, should produce a cell with no glycine requirement for growth.

A standard fusion experiment was performed between a glycine-deficient CHO-K1 cell and the triply deficient gly⁻-hyp⁻-thy⁻ CHL cell. Abundant colony formation from the fused cells was obtained in the absence of any added glycine. Four clones were picked and all exhibited excellent growth (plating efficiencies varying between 21 and 68 percent; generation times of about 12 hours) which was identical in the presence or absence of glycine. Hence the gene responsible for the single glycine deficiency must be different from that involved in the gly-hyp-thy requirement.

The CHO-K1 cell employed in this experiment also requires proline, while the CHL cell does not. The fused cells grew with maximum plating efficiency and growth rate in the presence or absence of any of the following combinations of supplements: proline; glycine; glycine plus thymidine plus hypoxanthine; or all four metabolites combined. Hence the proline gene defect as well as that responsible for the triple deficiency are recessive mutations.

It is of interest that the proline-independent hybrid cell obtained has a maximally high growth rate even in the absence of added proline, which is in contrast to the behavior of the pro⁺ revertant obtained from the pro⁻ cell (*2*). This result follows expectation, however, because as was shown previously, the pro⁺ revertant behaves like a hetero- or hemizygote, whereas the hybrid cell has presumably received at least two proline genes from the CHL cell so that it should have sufficient proline-synthetic capacity to be independent of any exogenous supplementation (*2*).

The change in chromosome number with time in these clones, together with the gradual increase in plating efficiency during the first several weeks following clone isolation, is consistent with the idea that the initial, chromosomally unstable hybrid cell eventually achieves a stable form.

Since the mutations to deficiency for glycine, hypoxanthine, proline, and the triple glycine-hypoxanthine-thymidine deficiency are all recessive by the complementation test utilized here, it is likely that each of the original mutations involves change such as inactivation of a structural gene, rather than activation of regulatory substances capable of shutting off intact structural genes.

The methodologies applied here can be used for further complementation studies. For example, experiments have shown that fusion of two glycine mutants of independent origin, each requiring glycine alone, has produced a fully glycine-independent hybrid. Therefore, these forms, with exactly the same nutritional requirement, are probably mutated in two different genes of the glycine synthetic chain (or may be exhibiting intragenic complementation, although the latter possibility is unlikely since a high frequency of prototrophs was obtained and these exhibited maximum growth rate in glycine-free medium). The highly selective nature of the genetic markers described here makes this system ideal for other investigations such as gene linkage, and the search for genetic recombination.

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