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Evidence of Normal Mitosis with Complete Cytokinesis in Central Nervous System Neurons during Sustained Depolarization with Ouabain

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Ouabain-induced depolarization of culture-matured spinal cord neurons from chick embryos causes the activation of DNA synthesis with subsequent mitotic nuclear division in a large fraction of the cells. Many of the activated neurons do not complete cytokinesis, and the binucleate cells thus formed have served as a convenient index for assaying the effectiveness of mitogenesis induction. However, the fact that the frequency of neurons induced to initiate DNA synthesis is consistently much larger than the frequency of binucleate neurons ultimately observed suggested the possibility that the difference may represent activated neurons that do complete cytokinesis, but go undetected in the binucleate assay cultures. This possibility was explored in the present study by determining the respective frequencies of induced neurons entering mitotic metaphase and completing mitotic telophase, and comparing them with the frequency of binucleate neurons ultimately formed. The results provide further evidence that normal mitogenic and mitotic processes are induced in central nervous system neurons by the depolarization treatment, and indicate that between 40 and 60% of the neurons which complete telophase subsequently undergo complete cytokinesis.

INTRODUCTION

Several recent reports from this laboratory provided evidence that normal mitogenesis and nuclear division are induced in culture-matured avian central nervous system (CNS) neurons by sustained depolarization

Abbreviations: CB—cytochalasin B; CNS—central nervous system; FUdR—5-fluoro-2′-deoxyuridine.

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with ouabain (3–5, 19). The primary study in this series (3) found that many mitogenically induced neurons did not complete cytokinesis, and thus formed binucleate cells. Such neurons have subsequently proven particularly useful for determining the effectiveness of mitogenesis induction, because the sister nuclei of a given nuclear division remain together in the original perikaryon and the resulting binucleate cells can be readily identified for quantitative assay. However, because complete divisions of neurons were also occasionally observed in induced cultures, the question arose as to whether or not a significant number of the initially activated neurons might actually be completing cytokinesis. The fact that the fraction of neurons initiating DNA synthesis in the above-cited studies was consistently much larger than the fraction of binucleate neurons ultimately found also suggested that many neurons may be proceeding through division. Such divisions may remain undetected, however, because the individual daughter cells subsequently either are assayed as part of the "nonresponding" (4) neuron population (in those cases where sufficient characteristic morphology is retained to permit positive identification) or are not recognizable as neurons per se for some time after division. Additional information was sought on this question in the present study, because the ability of mitogenically induced neurons to complete nuclear and somal division in substantial numbers (rather than being restricted to simple binucleate neuron formation) appears to be a matter of considerable theoretical and practical importance regarding the possibility of effecting true replication of mature CNS neurons. The primary approach involved determining the numbers of induced neurons that enter mitotic metaphase and complete mitotic telophase, respectively, and comparing them with the number of binucleate neurons ultimately formed. The results indicate that ~83% of the induced metaphase neurons proceed through telophase and that some 40 to 60% of this telophase neuron population is achieving complete cytokinesis in ouabain-treated cultures.

MATERIALS AND METHODS

Neuron Cultures. Neuron cultures were prepared as previously detailed (3, 10, 19) by plating pooled cell suspensions from several ganglion-free, collagenese-dissociated spinal cords of 9-day chick embryos. Two parallel experiments were run, each using a different culture substrate: collagen-coated 18 × 18-mm glass coverslips (6×10^5 cells/coverslip) in 60-mm plastic dishes with 3 ml medium (four cultures per dish), and uncoated 35-mm plastic culture dishes (8×10^5 cells/dish) with 2 ml medium. All cultures were maintained at *p*H 7.4 and 37°C in medium consisting of 80% Eagle's minimum essential medium with 2×10^{-3} M L-glutamine and added $NaHCO_3$ buffer, 5% fetal calf serum, 15% horse serum, 600 mg

glucose/100 ml, and 100 U/ml penicillin + 100 μg/ml streptomycin. Cultures were maintained 21 days before use to ensure full morphologic and electrophysiologic maturation of the neurons; detailed studies showed neurons in such cultures to be functionally mature (8–10) and entirely devoid of mitotic activity (3). In both culture forms at 21 days the neurons resided on top of a confluent monolayer of glial and fibroblastic "background cells," and were readily identified using criteria described previously (3, 4, 19). A few experiments were also conducted using (non-coated) dish cultures of fetal rat brain prepared according to the procedure of Godfrey *et al.* (11), but these cultures proved unsatisfactory for the purposes of the present mitogenesis study (see Results).

Assay of Ouabain-Induced Metaphase, Telophase, and Binucleate Neuron Frequencies. The percentage of neurons entering mitosis in response to ouabain depolarization was determined by adding 10^{-6} M colchicine (Sigma) to the ouabain induction medium and collecting the metaphase-arrested neurons. This colchicine concentration was determined in separate tests to be the maximum level permissible without causing significant morphologic alterations in the neuronal processes during a 6-h treatment period, but was adequate to arrest mitosis in nearly all of the dividing cells. The respective percentages of neuronal metaphases formed during hours 13 through 18 and 19 through 24 (inclusively) of a 24-h ouabain treatment period were determined separately, to preclude loss of arrested cells by too long an exposure to colchicine. A set of quadruplicate 21-day coverslip cultures was incubated 12 h in medium containing 10^{-7} M ouabain (Strophanthin-G, Sigma); this medium was then replaced with one containing 10^{-7} M ouabain + 10^{-6} M colchicine, and incubation was continued for 6 h before fixation. [In all cases where 10^{-7} M ouabain was used, additional NaCl was added to the medium to increase the total osmolarity by 4% (to 305 mosmol/liter). With this concentration of NaCl, 10^{-7} M ouabain was found to produce less rapid electroosmotic swelling (and neuron detachment losses) than 10^{-6} M ouabain, while still maintaining good induction effectiveness (3, 5).] A replicate set of four cultures was treated similarly, but exposed to colchicine during hours 19 through 24 of a 24-h ouabain treatment. Both culture sets were ethanol-fixed, Feulgen-stained by the "room-temperature" method (6), and assayed for the respective frequencies of metaphase (and binucleate) neurons. For dtermination of the frequency of binucleate neuron formation in response to treatment with ouabain alone during these two same time intervals, replicate culture sets were exposed to 10^{-7} M ouabain (0 through 18 and 0 through 24 h) and Feulgen-stained; the ouabain medium was replaced with fresh ouabain medium after 12 and 18 h, respectively. [Previous studies (3) showed that induced binucleate neurons do not appear before

12 to 14 h in 10^{-7} M ouabain medium; hence, the percentage of binucleate neurons formed in 0 through 18 h is equivalent to that formed during the 13- through 18-h period.] Controls consisted of two replicate sets of four cultures each, incubated in normal medium 18 and 24 h, respectively, with two cultures of each set receiving colchicine during the last 6 h of incubation; the other two received fresh medium replacements at the end of 12 and 18 h, respectively. An independent repetition of the above experiment was conducted using 21-day *dish cultures* of neurons (two cultures per experimental condition) and dilute (0.1%) cresyl violet for staining.

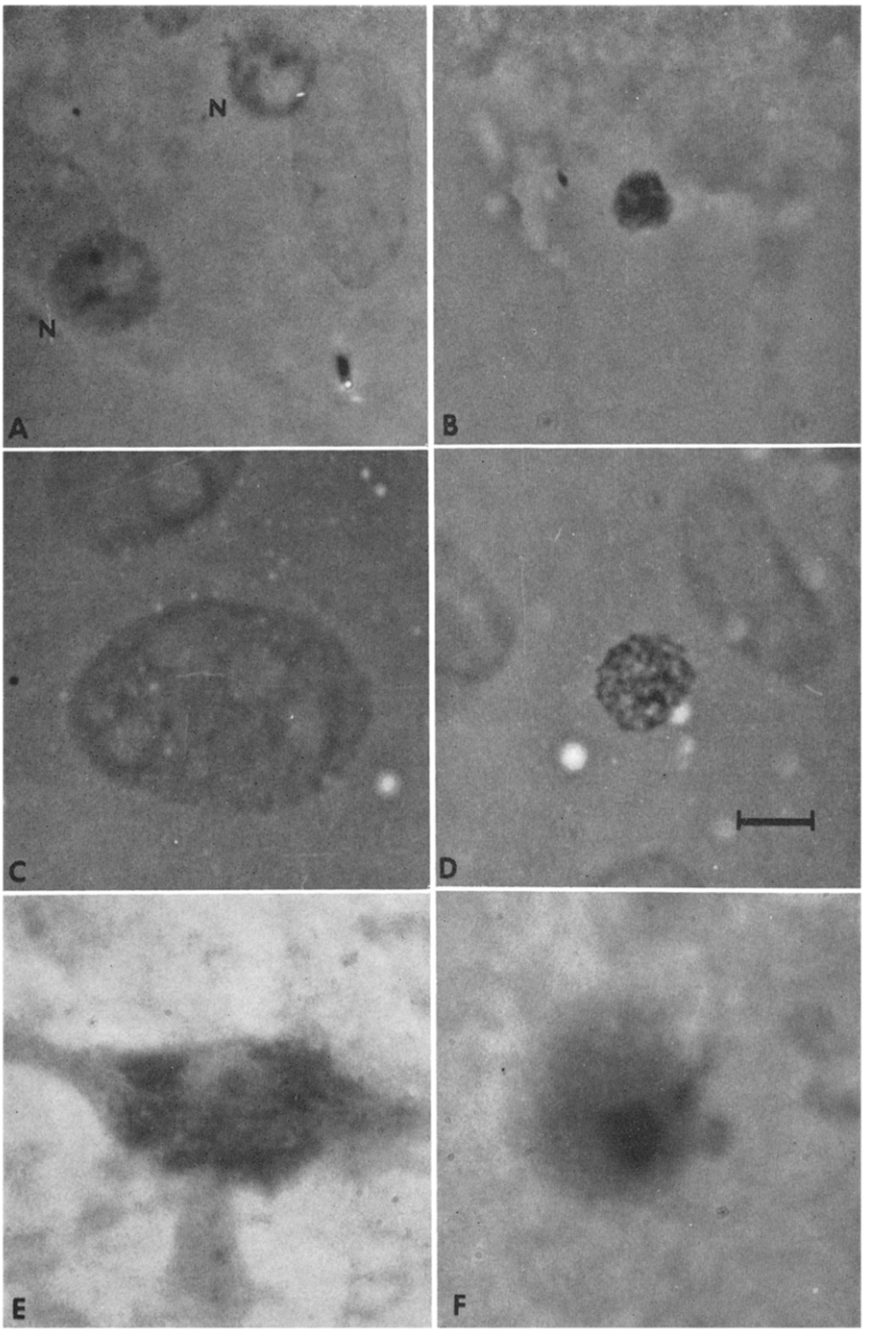
To determine the frequency of induced neurons completing telophase during the two successive time intervals, colchicine was replaced (in parallel experiments using dish cultures and cresyl violet staining) with 1.0 μg/ml cytochalasin B (CB; Aldrich Chemical Co.). Cultures were exposed to CB during 13 through 18 h of an 18-h ouabain induction period and 13 through 24 h of a 24-h induction period. In accord with previous observations of other cultured cell types (2), CB at a concentration of 1.0 μg/ml did not produce any discernible morphologic changes in the processes or attachment disposition of either the neurons or the background cells, but effectively blocked cytokinesis in all dividing cells in the neuron cultures. Hence, the frequency of binucleate neurons in CB-treated cultures permitted quantitative determination of the fraction of the induced metaphase neurons which actually completed mitosis up to the point of cytokinesis (i.e., through telophase). (A concentration of 5 μg/ml CB, however, caused a substantial distortion of the background cell monolayer, the layer deforming into numerous discrete groups of agglomerated cells. At 10.0 μg/ml CB, a gross distortion of the monolayer was produced, with the background cell bodies pulling into localized masses and leaving long cytoplasmic strands to points of attachment on the culture substrate. These pronounced distortions at the higher CB concentrations made accurate identification of individual neurons practically impossible.)

Identification of Metaphase Neurons. Because metaphase neurons apparently have not been the subject of assay in any prior investigation, a separate study was made to establish criteria for firm identification of true metaphase neurons in colchicine-treated cultures, and for distinguishing neuronal metaphase figures from those of colchicine-arrested background cells. To preclude any possible mistaking of neuronal RNA (Nissl body) aggregates for metaphase chromosomes, DNA-specific Feulgen staining (1, 5–7) was used in all initial experiments. Numerous rounded up, metaphase-arrested background cells in 21-day cultures treated 6 h with 10^{-6} M colchicine (with and without 10^{-7} M ouabain) were "location-coordinated" (3) with the microscope stage verniers, and the same cells were relocated after culture fixation and Feulgen staining. The character-

istic morphology and staining patterns of these naturally dividing, colchicine-arrested cells were used subsequently as the basis for identifying true "metaphase figures" under the test conditions used. [The presence of ouabain has no effect on the morphology of the background cells' metaphase figure, nor does it induce mitotic activity in them (4), but ouabain was included in the medium because it was a key agent in the neuron induction medium.] The same procedure was followed with populations of neurons, except that coordination was done prior to exposure to 10^{-7} M ouabain for 21 h; 10^{-6} M colchicine was added during the last 6 h of the ouabain treatment. Neuronal metaphases were carefully compared with background cell metaphase figures and basic criteria were established for differentiating the two (see Results). A similar comparative study was made using cresyl violet as the stain, and the Feulgen-stained results were used as a guide to ensure proper interpretation of the staining pattern for identification of true neuronal metaphase figures. Criteria for the identification of binucleate neurons (as distinguished from two closely apposed neurons) were detailed previously (5).

RESULTS

Identifying Characteristics of Neuronal Metaphase Figures. Photomicrographs representative of the Feulgen-stained interphase (G_0) and metaphase neurons are shown in Fig. 1 (A, B), along with those of background cells in the corresponding stages (C, D). The neurons (both G_0 and metaphase) were identified with phase-contrast optics in Feuglen-stained cultures by the morphology of their processes, small somal size, elevation above the background layer, and more intensely staining interphase nuclei and metaphase chromosome agglomerates (relative to the background). Neuronal G_0 nuclei were generally circular in outline with a relatively smooth periphery (Fig. 1A). The (magenta) staining intensity varied considerably throughout the nuclear area, with a clearly defined pale region where the nonstaining nucleolus was located (5). The mean nuclear diameter (±SD) for 101 cells was 7.4 ± 0.4 μm (area, 43.0 μm^2). In contrast, the neuronal metaphase figure consisted of a much smaller semicircular "knot" (diameter, 3.4 ± 0.3 μm; area 9.1 μm^2) of very darkly staining (deep purple to black) material, which at high magnification (× 100 objective) could be resolved as an assembly of closely apposed but discrete clumps of intensely stained chromosomes, with no enclosed nucleolar area (Fig. 1B). [The existence of discrete chromosomes in the metaphase neurons has been independently demonstrated by Feulgen staining of individual ouabain-induced, colchicine-blocked cells processed using procedures for conventional karyotype preparation (12) adapted for individual cells, with final dispersal of the chromosome complement by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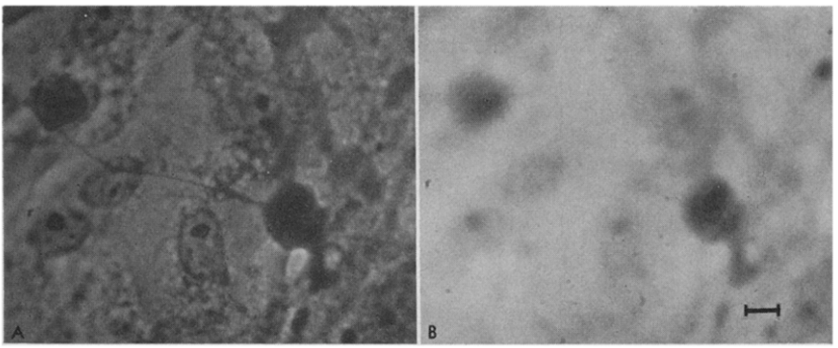

FIG. 2. Micrographs representative of metaphase neurons (colchicine arrested) in ouabain-induced cultures. Cells are stained with cresyl violet. A—Focus adjusted to show retained processes connecting center neuron with adjacent neurons (phase contrast). B—Same field as in A, but with focus on the metaphase chromosome figure of the center neuron (bright field). Calibration bar = 10 μm.

osmotic swelling and flaming; such karyotypes (*Gallus domesticus*) are particularly complex, containing some 77♀/78♂ micro- and macrochromosomes (5). Free metaphase chromosomes were also demonstrated in the living colchicine-blocked neurons by supravital staining with purified methyl green (studies to be reported).] Photomicrographs of representative G_0 and metaphase neurons stained with cresyl violet are shown in Figs. 1E and F, respectively.

The characteristic sequence of intracellular morphological changes occurring in neurons responding to ouabain was described previously (4). In general, with 10^{-7} M ouabain (+NaCl) the metaphase neurons retained their processes and network associations with other neurons, although the somata became characteristically enlarged (4) and quite refractive in phase contrast. Photomicrographs of a typical metaphase neuron are shown in Fig. 2. In Fig. 2A, a major process is seen connecting the center neuron with another neuron at the upper left, and the process extending to the right similarly connects with another neuron (not shown). The same (center) neuron is pictured in Fig. 2B, with the focus adjusted to reveal the chromosomal metaphase figure more clearly.

FIG. 1. Micrographs representative of Feulgen- and cresyl violet-stained interphase and metaphase neurons, and background cells. Metaphases were obtained by colchicine arrest of mitotic cells in 21-day cultures treated with 10^{-7} M ouabain (+NaCl). A—Feulgen-stained G_0 neuronal nuclei (N) with characteristic clear nucleolar areas. B—Ouabain-induced, colchicine-arrested neuronal metaphase figure (Feulgen stain). C—G_1 background cell nucleus with characteristic clear nucleolar areas (Feulgen stain). D—Metaphase figure of spontaneously dividing, colchicine-arrested background cell (Feulgen stain). E—G_0 neuronal nucleus (cresyl violet stain). F—Ouabain-induced neuronal metaphase figure (cresyl violet stain). All micrographs are at the same magnification; calibration bar = 5 μm.

The metaphase figure of the background cells (Fig. 1D) was morphologically similar to that of the neurons, but much larger in size (area, ~35 μm^2); the darkly staining chromosomes were generally less tightly clumped, and in many cells discrete chromosomes could be discerned. The body of the metaphase background cell was circular and considerably larger than the soma of the metaphase neuron. Occasionally, short cytoplasmic strands connecting the rounded-up metaphase background cells to the substrate were observed, but these strands were usually readily distinguished from neuronal processes with phase-contrast optics. Neuronal metaphase cells were always situated atop a monolayer sheet of background cells, often partially overlying a large G_1 nucleus, whereas metaphase background cells usually had an appreciable clear area around them constituting the "hole" left in the monolayer by the mitotic rounding up from the flattened interphase form. Moreover, the neuronal metaphase figures were characteristically situated in a focal plane some 3 to 4 μm above the background layer, whereas the background cells' metaphase figures were located in the focal plane of the monolayer.

Many of the identifying characteristics of Feulgen-stained metaphase neurons were also present in neurons stained with cresyl violet. With this stain, metaphase neurons were rapidly distinguished from G_0 neurons because the lightly staining nucleus and intensely staining nucleolus of the latter were replaced in the metaphase cell by an intensely staining knot of

TABLE 1

Frequencies of Metaphase and Binucleate Neurons in Ouabain-Induced Cultures[a]

Medium	Ouabain exposure[b] (h)	Colchicine or cytochalasin B exposure[c] (h)	Metaphase neurons (%)	Binucleate neurons (%)
Normal	—	—	0	2.1
Normal/colchicine	—	19–24	0	2.3
Ouabain (only)	0–18	—	0	15.3
Ouabain (only)	0–24	—	0	20.7
Ouabain/colchicine	0–18	13–18	27.5	2.7
Ouabain/colchicine	0–24	19–24	8.7	16.9
Cytochalasin B (CB)	—	19–24	0	2.4
Ouabain/CB	0–18	13–18	0	24.8
Ouabain/CB	0–24	13–24	0	31.9

[a] Pooled data for ~450 Feulgen-stained and ~1000 cresyl violet-stained neurons at each condition, except for CB-treated cultures (~700 neurons per condition).

[b] Inclusive period (hours) of exposure to 10^{-7} M ouabain before fixation.

[c] Inclusive period (hours) of exposure to 10^{-6} M colchicine or 1.0 μg/ml cytochalasin B (CB) before fixation.

chromosomes (with a diameter ~5 times that of the G_0 nucleolus), surrounded by a uniformly staining cytoplasm. The characteristic G_0 perinuclear Nissl substance became finely dispersed and uniformly distributed around the chromosome knot in the metaphase neuron. The cytoplasm of G_0 neurons stained much darker with cresyl violet than that of G_1 background cells. The cytoplasm of metaphase neurons stained less intensely than that of the G_0 neuron and, although the cytoplasm of metaphase background cells stained more deeply than that of cells in the G_1 phase, the cytoplasm of the metaphase neurons was still nearly always stained significantly darker than that of the metaphase background cells. This staining difference, along with the substantial differences in metaphase figure size and somal area–chromosome knot area ratio, the presence of characteristic processes in the neurons, and the clear area around metaphase background cells, generally permitted unambiguous identification of metaphase neurons in the cultures stained with cresyl violet.

Frequencies of Ouabain-Induced Metaphase, Telophase, and Binucleate Neurons. The frequencies of colchicine-arrested metaphase neurons and binucleate neurons in the ouabain-induced cultures are given in Table 1, and because there was no essential difference between the results obtained with the Feulgen-stained coverslip cultures and the cresyl violet-stained dish cultures, the pooled results are presented. The data indicate that there is no discernible neuronal mitotic activity in pretreatment (control) cultures, but that a significant fraction of the neuronal population (36.2%) enters metaphase during a 24-h exposure to ouabain (27.5% during 13 through 18 h + 8.7% during 19 through 24 h). This finding is consistent with previous results (3) on the frequency of neurons induced to initiate DNA synthesis under similar conditions, and indicates that those neurons activating DNA synthesis ultimately enter mitosis. Some 76% of the total metaphases were formed during the 13- through 18-h period, thus verifying the early activation of mitogenesis previously reported (3) using the DNA initiation of synthesis as the criterion. The fact that the frequency of binucleate neurons in induced cultures treated with colchicine during this period is essentially the same as that in the pretreatment cultures shows that the colchicine concentration used was adequate to effect complete metaphase blockage of the induced neuron population. From Table 1, it appears that nominally 18.6% of the neuron population formed binucleate cells in 24 h [20.7 −2.1% of the preexisting (control) binucleate neurons; see Appendix for method of calculation] and, under the prerequisite that each induced binucleate neuron came from a metaphase neuron, it is clear that only a fraction of the total 36.2% metaphase neurons subsequently formed binucleate cells. The results obtained with CB (13- through 24-h treatment period, Table 1) indicate that 82.3% of the ouabain-induced metaphases formed binucleate cells [(31.9 −2.1)/36.2] during this period,

whereas with ouabain alone only 51.4% of the metaphases formed binucleates [(20.7 −2.1)/36.2]. Thus, nominally 30.9% of the induced metaphases [82.3 −51.4] are completing mitosis up to the point of cytokinesis, but are not forming binucleate neurons. Unless these neurons are dying during cytokinesis per se, the most reasonable conclusion that can be drawn from the data is that they have completed division.

Although the foregoing percentages indicate that a significant fraction of the induced neurons may be completing cytokinesis, consideration must be given to the fact that the identifiability of the daughter neurons of such divisions will affect the *numerical value* determined for the percentage of the initial neuron population which forms binucleate (or dividing) cells. For example, if all the induced metaphase neurons which do not form binucleate cells actually divided to produce daughter cells subsequently assayed as nonresponding (initial) neurons in the induced cultures, the calculated percentage of induced binucleate neurons would be numerically less than if the daughter cells were not identifiable as neurons and hence not tabulated as neurons in the final assay. However, relatively precise calculations of the fraction of binucleate and dividing neurons under each of these possible limiting conditions, based on the data in Table 1, still indicate that a significant portion of the metaphase neurons is dividing (40 to 60%; see Appendix).

Another observation germane to neuronal division was made during determination of the colchicine concentration required for complete blocking of binucleate neuron formation in ouabain-treated cultures. In cultures exposed 12 through 18 h to (weak) 10^{-7} M colchicine, the binucleate frequency was nearly "normal" (14.6%) and only a few neuronal metaphase figures were seen (1.2%). However, many widely distributed pairs of discrete, but closely situated, cells having nuclear and cytoplasmic staining properties (cresyl violet) identical to those of immature neurons in recently plated neuron cultures were observed. As no such obvious *neuron pairs* were seen in control cultures (containing ouabain only), we concluded that these cells most probably represented daughters of neurons that had completed cytokinesis, which remained in close proximity, possibly as a result of some inhibitory effect of the colchicine on neuronal locomotion after division. Daughter neurons are particularly motile just after division [(14); personal time-lapse observations], and pronounced effects of colchicine on neuronal cytoskeletal microtubules associated with process extension and shape maintenance have been described (21).

Experiments with mammalian CNS neurons, using dish cultures of fetal (18-day) rat brain prepared according to the procedure of Godfrey *et al.* (11), proved unfeasible because of spontaneously continuing neuronal mitotic activity even in 21-day-old (control) cultures. Such cultures not treated previously with fluorodeoxyuridine (FUdR) (11), although con-

taining many morphologically mature neurons, also showed numerous metaphase figures characteristic of neuronal cells when exposed 6 h to 10^{-6} M colchicine (alone). Moreover, such metaphase figures were still observed, although in lesser number, in 21-day cultures which had previously received the 4-day FUdR treatment (11) at 7 days. Unlike chick embryo cord cultures at 21 days, the rat brain cultures contained many cells which appeared to be immature neurons, possibly mitotically active neuroblasts which had failed to fully mature *in vitro* and continued to proliferate. The possibility of continuing neuronal proliferation in fetal rat brain cultures was recently suggested, on the basis of decreased development of total choline acetyltransferase activity (15) in cultures treated with FUdR. Moreover, relatively extensive spontaneous divisions in neurons cultured from fetal (18- to 20-day) mouse brain and spinal cord were reported even earlier (13).

DISCUSSION

The present results, demonstrating the formation of typical colchicine-arrested metaphase chromosomal figures in ouabain-depolarized CNS neurons, constitute further evidence of the induction of normal mitogenesis and initiation of mitosis in such cells. The similar induced frequencies of DNA-synthesizing (3, 4) and metaphase neurons implies that those neurons initiating DNA synthesis in response to ionic depolarization proceed into mitotic metaphase, and the subsequent development of metaphase neurons into binucleate cells with sister nuclei each containing the G_1 DNA content (5) indicates the completion of a normal mitotic nuclear division, but without final cytokinesis. Of perhaps greater significance, however, is the implication of the present results that a large fraction of the induced neurons (up to 60% of the telophase cells) is completing cytokinesis to form individual daughter cells. The CB results show that a large majority of the ouabain-induced metaphase neurons is reaching telophase, but only about half of these cells form binucleate neurons. Ostensibly, the remaining half completes cytokinesis to form individual daughter neurons. The occasional observations of complete division in induced neurons clearly show that such cells are in fact capable of undergoing normal cytokinesis, so there appears to be no reason to expect a high level of cell death during the cytokinesis process per se. Complete mitoses arising spontaneously in cultured sympathetic neurons from *human adults* under conditions allowing firm identification of the dividing cells were reported previously (14). The observation, in the present study, of numerous isolated pairs of cells having the characteristic appearance of immature neurons is also considered indicative of appreciable neuronal cytokinesis activity in the induced cultures. Under the usual induction

conditions, it is probable that the pronounced increase in motility (14) seen in daughter neurons following division, coupled with the very small size and changed appearance of these cells (3), precludes their being identified and assayed as neurons (much less their being discernible as products of a recent neuronal division) for some time after their formation. Under these conditions, the present results may be interpreted as indicating that the higher percentages of dividing neurons apply (i.e., condition (iiib) as cited in the Appendix, obtains).

The requirement for complete cytokinesis is obviously of central importance if actual replicative regeneration of mitogenically induced CNS neurons is to be effected. The present results, suggesting that cytokinesis is being achieved by an appreciable fraction of the mitotically activated neurons, thus appear significant in their implication that ouabain-induced neuronal mitogenesis is not inherently limited to the formation of just binucleate cells, with the consequent possibility that full division might be achieved by most mitogenically activated neurons under appropriate conditions. Thus, studies for evaluating the effectiveness of various conditions which might enhance the frequency of neuronal cytokinesis are encouraged, e.g., delineation of the effects of relative and absolute intracellular concentrations of cations on the initiation and functional efficiency of the cleavage–furrow microfilament system which effects cytokinesis (16, 17, 20). The CB procedure developed in the present study for determining the relative percentages of telophase neurons completing, and failing to complete, cytokinesis would appear particularly valuable for use in such cytokinesis enhancement investigations. An alternate approach of possible merit lies in the assay of total neurons (13) in previously induced cultures, after the presumptive neuronal daughter cells have had an adequate period of growth to become distinguished again as neurons (4). In a recently reported study (18) using this total-neuron assay procedure, the conclusion was reached that a significant fraction of mouse sensory ganglion neurons in dispersed-cell cultures may be dividing in response to a medium which induces depolarization of those cells. Although the present study utilized neurons of embryonic origin, the demonstrated functional maturity of these cells at 21 days *in vitro* (5, 8–10) lends support to the expectation that similar responses may be elicited in CNS neurons from fully adult sources, when adequately depolarized (4).

APPENDIX

In regard to the fate of the metaphase neurons which do not form binucleates, three primary possibilities may be considered: (i) All metaphase neurons not forming binucleate cells ("residual metaphase neurons") actually completed division, and each of the daughter cells was then either

(a) assayed as if it were an individual nonresponding neuron of the initial population, or (b) unrecognizable as a neuron and not counted in the assay; (ii) the residual metaphase neurons all perished or were lost during mitosis per se and hence were not counted; (iii) a combination of the above wherein some of the initial metaphase cells were lost during mitosis, while the rest reached telophase, with those telophase cells not forming binucleate neurons dividing to produce daughter cells which were then either (a) assayed as individual (nonresponding) neurons or (b) unrecognizable as neurons and not counted at all. From the data in Table 1 for the 13 through 18-h interval, it is seen that, for each 100 neurons in the pretreatment cultures, 27.5 metaphase neurons were induced by ouabain and, from these, 22.7 binucleate neurons (i.e., 24.8 −2.1) were formed in the presence of cytochalasin B. Consequently, 82.6% of the metaphase neurons (22.7/27.5) formed during this period completed mitosis through telophase, with 17.4% presumably being lost (through death or detachment). Thus, condition (iii) above apparently obtains. With this information, the true numerical values for the percentages of binucleate and dividing neurons forming from the initial neuron population in the 13- through 18-h time interval can be calculated for each of the conditions (iiia) and (iiib) above using the data in Table 1.

Let x represent the number of neurons (per 100 pretreatment neurons) which forms binucleate cells during the 13- through 18-h period of ouabain treatment. Then in the final binucleate assay cultures, there will be $(x + 2.1)$ binucleate neurons per 100 pretreatment neurons (taking 2.1% as the level of binucleate neurons existing in pretreatment cultures; Table 1). Because 82.6% of the 27.5 induced metaphase neurons complete telophase (per 100 pretreatment neurons) during this period (see above), there will be $2(22.7 - x)$ identifiable daughter neurons formed from these metaphases if condition (iiia) applies. Thus, in the binucleate assay cultures there will be $(x + 2.1)$ binucleate neurons and $[2(22.7 - x) + x\,(100 - 27.5)]$ total neurons for each 100 pretreatment neurons. The experimentally assayed frequency of binucleate neurons is 15.3% for this time period; consequently,

$$\frac{(x + 2.1)}{[2(22.7 - x) + x + (100 - 27.5)]} = 0.153,$$

and $x = 13.9$, or 13.9% of the initial neuron population formed binucleate neurons. Then 13.9/22.7, or 61.2%, of the telophase neurons became binucleates and (22.7 −13.9)/22.7, or 38.8% divided. A similar procedure can be used to determine the corresponding results for condition (iiib) during this same period, and for conditions (iiia) and (iiib) during the 19- through 24-h period.

The essential results of such calculations for the two time periods covered

TABLE 2

Summary of Binucleate and Cytokinetic Neuron Frequencies in Ouabain-Induced Cultures

Time interval (h)	Condition of daughter neurons[a]	Frequency of binucleate neurons[b] (%)	Frequency of cytokinetic neurons[b] (%)	Frequency of dividing telophase neurons[c] (%)
13–18	(iiia)	13.9	8.8	38.8
	(iiib)	10.6	12.1	53.3
19–24	(iiia)	4.1	2.9	41.4
	(iiib)	2.5	4.5	64.3

[a] Condition (iiia): Residual telophase neurons give daughter cells which are assayed as nonresponding neurons of initial culture. Condition (iiib): Residual telophase neurons give daughter cells which are not recognizable as neurons, and are not counted in assay.

[b] Frequency is given in terms of binucleate or cytokinetic neurons formed per 100 *initial* (preinduction) neurons during the respective time intervals.

[c] Percentage of induced neurons reaching telophase which go on to divide completely.

by the data in Table 1 are summarized in Table 2. It is thus evident that, even with an accounting for the fraction of neurons lost during mitosis and for the effect of the assayability conditions on the daughter neurons produced [i.e., conditions (iiia) and (iiib)], a significant fraction of the telophase neuron population is completing cytokinesis (between 40 and 60%), excluding the seemingly unlikely possibility that all of the "residual" neurons are dying during cytokinesis per se.

REFERENCES

1. Barka, T., and P. J. Anderson. 1965. *Histochemistry: Theory, Practice, and Bibliography.* Harper and Row, New York.
2. Carter, S. B. 1967. Effects of cytochalasins on mammalian cells. *Nature (London)* **213**: 261–264.
3. Cone, C. D., Jr., and C. M. Cone. 1976. Induction of mitosis in mature neurons in central nervous system by sustained depolarization. *Science* **192**: 155–157.
4. Cone, C. D., Jr., and C. M. Cone. 1978. Blockage of depolarization-induced mitogenesis in CNS neurons by 5-fluoro-2′-deoxyuridine. *Brain Res.* (in press).
5. Cone, C. D., Jr., M. Tongier, Jr., and C. M. Cone. 1977. DNA content of daughter nuclei from ouabain-induced nuclear divisions in central nervous system neurons. *Exp. Neurol.* **57**: 396–408.
6. Decosse, J. J., and N. Aiello. Feulgen hydrolysis: effect of acid and temperature. *J. Histochem. Cytochem.* **14**: 601–604.
7. DeRobertis, E. D. P., W. W. Nowinski, and F. A. Saez. 1965. *Cell Biology.* W. B. Saunders, Philadelphia and London.

8. FISCHBACH, G. D. 1970. Synaptic potentials recorded in cell cultures of nerve and muscle. *Science* **169**: 1331–1333.
9. FISCHBACH, G. D. 1972. Synapse formation between associated nerve and muscle cells in low density cell cultures. *Develop. Biol.* **28**: 407–429.
10. FISCHBACH, G. D., AND M. A. DICHTER. 1974. Electrophysiologic and morphologic properties of neurons in dissociated chick spinal cord cell cultures. *Develop. Biol.* **37**: 100–116.
11. GODFREY, E. W., P. G. NELSON, B. K. SCHRIER, A. C. BREUER, AND B. R. RANSOM. 1975. Neurons from fetal rat brain in a new cell culture system: A multidisciplinary analysis. *Brain Res.* **90**: 1–21.
12. HSU, T. C. 1973. Karyology of cells in culture. Pages 764–767 in P. F. KRUSE, JR., AND M. K. PATTERSON, JR., Eds., *Tissue Culture: Methods and Applications.* Academic Press, New York.
13. MURPHY, W. H., AND C. BULLIS. 1973. A simple method for the growth of mouse neuron monolayer cultures. *Proc. Soc. Exp. Biol. Med.* **142**: 1–6.
14. MURRAY, M. R., AND A. P. STOUT. 1947. Adult human sympathetic ganglion cells cultivated in vitro. *Am. J. Anat.* **80**: 225–273.
15. SCHRIER, B. K., AND D. L. SHAPIRO. 1974. Effects of fluorodeoxyuridine on growth and choline acetyltransferase activity in fetal rat brain cells in surface culture. *J. Neurobiol.* **5**: 151–159.
16. SCHROEDER, T. E. 1968. Cytokinesis: Filaments in the cleavage furrow. *Exp. Cell Res.* **53**: 272–318.
17. SCHROEDER, T. E. 1969. The role of "contractile ring" filaments in dividing Arbacia egg. *Biol. Bull.* **137**: 413–414.
18. SCOTT, B. S. 1977. The effect of elevated potassium on the time course of neuron survival in cultures of dissociated dorsal root ganglia. *J. Cell Physiol.* **91**: 305–316.
19. STILLWELL, E. F., C. M. CONE, AND C. D. CONE, JR. 1973. Stimulation of DNA synthesis in CNS neurones by sustained depolarisation. *Nature [New Biol.]* **246**: 110–111.
20. SZOLLOSI, D. 1970. Cortical cytoplasmic filaments of cleaving eggs: A structural element corresponding to the contractile ring. *J. Cell. Biol.* **44**: 192–209.
21. WESSELLS, N. K., B. S. SPOONER, J. F. ASH, M. O. BRADLEY, M. A. LUDUENA, E. L. TAYLOR, J. T. WRENN, AND K. M. YAMADA. 1971. Microfilaments in cellular and developmental processes. *Science* **171**: 135–143.