THE DISSOCIABILITY OF DEOXYRIBONUCLEIC ACID SYNTHESIS FROM THE DEVELOPMENT OF MULTINUCLEARITY OF MUSCLE CELLS IN CULTURE

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ABSTRACT

The effects of a nitrogen mustard on both the morphology and several synthetic capacities of embryonic chick skeletal muscle cells in monolayer culture have been examined. Concentrations of nitrogen mustard which profoundly inhibit deoxyribonucleic acid synthesis specifically do not inhibit the development of multinuclearity in the contractile "ribbons" which form rapidly in culture. Nitrogen mustard affects the nuclear morphology of "fibroblast-like" and multinuclear muscle cells differentially. The mononucleated cells in treated cultures exhibit extreme nuclear enlargement which distinguishes them from the multinuclear cells as well as from both types of cells in control cultures. The nuclei of the multinuclear cells which form after nitrogen mustard treatment, however, do give evidence of having been affected by the treatment. They exhibit somewhat less uniformity of size than similar cells in control cultures. Analogous differences were described by Bodenstein (3) between potentially proliferating cells and postmitotic differentiating cells, marked nuclear enlargement being characteristic of cells in the proliferative zone. The results are more compatible with the hypothesis that multinuclearity arises through successive cell fusion than through amitotic nuclear multiplication, since it is unlikely that any form of nuclear replication could occur in the absence of DNA synthesis.

Two alternate mechanisms have, at various times, been suggested to explain the origin of the multinuclear character of skeletal muscle. One suggested mechanism invokes amitotic or cryptomitotic nuclear division in the absence of cytokinesis (mitotic division being a rarely reported event). Antithetically it has been suggested that skeletal muscle is a true syncytium which arises from successive fusion of initially mononucleated cells. (See Murray (22), Godman (12), Lash *et al.* (18), and Moscona (21), for reviews of the development of these hypotheses).

In the present study the capacity of muscle cells in monolayer culture to form multinuclear elements has been tested in the presence of an agent (methyl-bis[beta chlorethyl]-amine) reported

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by Bodenstein and Kondritzer (4) to inhibit DNA synthesis. The efficacy of the inhibitor in specifically inhibiting DNA synthesis in the experimental situation employed here has been tested. Despite pretreatment with concentrations of nitrogen mustard which proved effective in blocking DNA synthesis, the process of multinuclear cell formation is not inhibited. Since it is difficult to imagine any normal form of nuclear replication in the absence of DNA synthesis the data support the fusion hypothesis.

MATERIALS AND METHODS

Chick embryos of a Delaware × White Leghorn strain (Hyline) of 12 days incubation age (at 37.5°C.) served as the source of explant tissue. After removal of the embryo from the egg and extra-embryonic membranes, the skin was removed from both legs and the legs severed from the body at the level of the ischial crests. The muscle tissue was removed from the bone and minced with a sharp pair of scissors. Muscle from two to three embryos was trypsinized for 10 minutes in 15 cc. of 0.05 per cent trypsin (N.B.C. Co. 1-300) made up in a calcium, magnesium-free saline (19). During trypsinization the temperature was maintained at 37°C. and the suspension was agitated with a magnetic stirrer. Trypsinization was stopped by the addition of 15 cc. of chilled, complete growth medium and the suspension filtered through six layers of cheese cloth to remove macroscopic clumps. The supernatant was removed after 5 minutes' centrifugation at 1,000 R.P.M. and the cell pellet resuspended in complete growth medium. A second filtration through 200-mesh bolting silk results in a suspension consisting largely of single, dispersed cells. Further clarification of the suspension could be achieved by allowing the cells to attach to the bottom of large, 14-cm. petri plates for 24 hours after which time the plates were washed with a phosphate-buffered saline (23) and the cells trypsinized and resuspended. Replicate cultures were set up in 50 mm. diameter petri plates by procedures similar to those developed in Earle's laboratory (9), except that aliquots of the cell suspension were delivered with a volumetric pipette (see also Harris (13)).

For histological examination small chambers were prepared by attaching small (19 mm. inside diameter) siliconed glass rings to 1 × 1½ inch microscope slides with Dow-Corning silicone grease. One-milliliter aliquots of cell suspensions were delivered to the chambers which were placed in a 50 mm. diameter petri plate arranged as a moist chamber.

Complete growth medium consisted of 40 per cent of a modified Waymouth medium (19), 10 per cent horse serum, 5 per cent embryo extract (8) made up to 100 per cent with Hank's solution and contained 100 units of penicillin and 0.1 mg. streptomycin per cc. of final medium. Solutions of methyl-bis-[beta chlorethyl]-amine HCl (nitrogen mustard)[1] were made up in phosphate-buffered saline immediately before use. Cultures were exposed to solutions of nitrogen mustard for 1 hour at 37.5°C. in normal atmosphere after which time the solution was withdrawn and the plates washed once with saline before replacement with complete growth medium. Control cultures were treated similarly except that the phosphate-buffered saline was substituted for the nitrogen mustard solutions during the 1-hour treatment period.

At 24-hour intervals after nitrogen mustard treatment two cultures from each group of treated cells and from the control group were washed twice with saline and fixed with 0.5 ml. of 5 per cent TCA. The cells were scraped off the petri plates with a rubber policeman and transferred to 2 ml. centrifuge tubes. Each plate was washed and scraped twice more with 0.5-ml. portions of 5 per cent TCA. After centrifugation the supernatant was decanted and the precipitate washed successively with 1 ml. of 5 per cent TCA and with 1 ml. of 80 per cent ethanol. Lipids were removed by heating the samples at 42–45°C. for 15 minutes in a 1:1 mixture of 95 per cent ethanol and absolute ether. Traces of ether were removed with an additional wash of 95 per cent ethanol. Nucleic acids were removed by two extractions at 70°C. for 15 minutes each with 1 N perchloric acid. The two extracts (0.1 ml. followed by 0.05 ml.) were pooled and made up to 0.4 ml. in a calibrated micro-deproteinization tube (misco). Two tenths of a cc. aliquots of the perchloric acid extracts were analyzed for deoxyribonucleic acid by the method of Burton (7), modified for the sample size used. Standards prepared from beef thymus DNA (Schwarz) were run at five different levels from 1 to 20 μg. with each assay.

Glycine-1-C^{14} incorporation was studied by replacing the medium over the cultures with complete growth medium made up to contain 0.306 μC of labeled amino acid per ml., (unlabeled glycine being omitted from the modified Waymouth's medium). After the addition of the labeled medium the cultures were returned to the CO_2-gassed incubator. At hourly intervals after the addition of label, cultures were removed from the incubator, the medium withdrawn by aspiration, and the cells treated as described above for DNA determinations. The residue remaining after extraction of nucleic acids was washed three

[1] The authors wish to express their indebtedness to the Sharp and Dohme division of Merck, Inc., for a generous supply of methyl-bis-[beta chlorethyl]-amine HCl.

times with 1-ml. portions of 5 per cent TCA followed by a brief wash with 1 ml. of double distilled water. The precipitates were then transferred in 1 ml. o 6 N HCl to small pyrex test tubes which were sealed and the contents hydrolyzed at 160°C. for 3 hours. After opening the tubes the hydrolysates were evaporated to dryness and redissolved in 1 ml. o double distilled water. Four-tenth ml. aliquots were plated at infinite thinness on aluminum planchets which were then counted in a gas-flow "micromil" window counter (D47, Nuclear-Chicago).

Incorporation into DNA thymine was studied by replacing the culture medium with complete growth medium containing 2 μC of C^{14}-formate per ml. At regular intervals (2, 4, 6, and occasionally 8 hours) incubation was interrupted, the labeled medium withdrawn, and the cells treated as for DNA determinations (see above) through the lipid extraction step. Four to six cultures were pooled for each determination.

After lipid extraction, separation of DNA and RNA nucleotides was achieved by essentially the method of Siminovitch and Graham (25), with the following modifications:

1. The DNA fraction was hydrolyzed in 0.5 ml. of 98 per cent formic acid at 175°C. for 30 minutes (27).

2. Adsorption to and subsequent elution from activated charcoal were omitted. Instead, desalting and removal of charred material were simultaneously achieved by passage through the Dowex-1-Cl^- column whose height was increased to 3.5 cm. The hydrolysate after evaporation to dryness *in vacuo* was taken up in 1.0 cc. of distilled water and adjusted to pH 8 with 1 N NH_4OH before being transferred to the Dowex column.

The effluent solution which contained adenine, guanine, and thymine (cytosine was eluted from the column by the initial water wash) was evaporated to a convenient volume and applied *in toto* to a strip of Whatman No. 1 filter paper. Good chromatographic separation was achieved by a single descending run in butanol-ammonia (20). The bases were visualized under a mineralight lamp and their positions marked. Identification was based on both relative Rf values and on position relative to simultaneously run standards. After elution of the thymine spot by shaking in 0.1 N HCl, the sample was evaporated, made up to volume, and aliquots taken for determinations of thymine concentration and radioactivity. Thymine concentration was calculated from absorption at 260 mμ employing a suitable standard curve and blank chromatographed simultaneously with the sample. Aliquots were plated and counted as for glycine-C^{14} (see above).

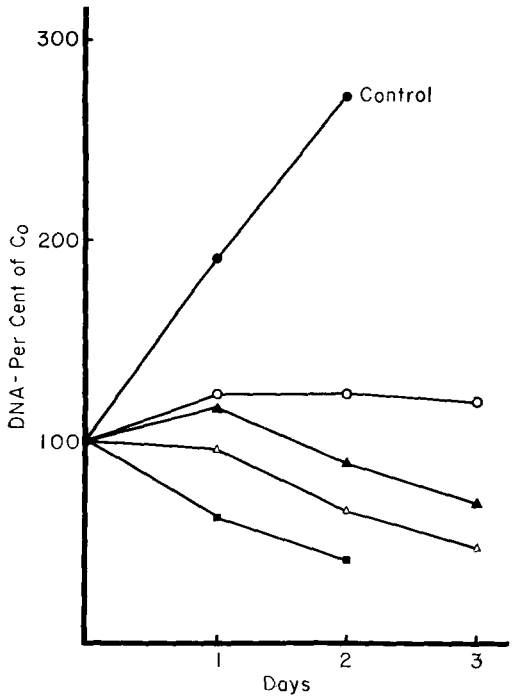

FIGURE 1

Deoxyribonucleic acid per culture, expressed as percentages of the control level at the time of nitrogen mustard treatment (C_o). Control, closed circle (●); 0.52 × 10^{-7} M mustard, open circle (○); 1.56 × 10^{-7} M mustard, closed triangle (▲); 2.6 × 10^{-7} M, open triangle (△); 5.2 × 10^{-7} M mustard, closed square (■).

RESULTS

The appearance of the cultures 24 hours after plating suggests a typical culture of fibroblast-like cells. As growth proceeds, the cultured cells become a confluent monolayer. With the attainment of confluency there occurs a rapid formation (within a 24-hour period) of extremely long, ribbon-like multinuclear cells. Such cells have been observed in monolayer cultures of embryonic cardiac and breast muscle as well (24).

In testing the effects of nitrogen mustard on cell cultures the inhibitor was applied just prior to massive formation of multinuclear cells. With the inoculum sizes employed this usually occurred between the 3rd and 4th day in culture. A concentration range of from 0.52 to 5.2 × 10^{-7} M was surveyed.

Figure 1 represents a typical experiment on the accumulation of DNA in nitrogen mustard–treated cultures. Deoxyribonucleic acid doubles during the first 24 hours in control cultures. An increase

of approximately 90 per cent occurs during the second 24-hour period in the controls. After mustard treatment an increase in DNA occurs during the first 24-hour period only in those cultures treated with the two lowest concentrations used (0.52×10^{-7} M) and (1.56×10^{-7} M). These increases, however, are small and at most represent only one-third of the DNA increase of the controls. After the first 24 hours post-treatment, DNA per culture decreases steadily and approximately at the same rate except for cultures treated with the lowest concentration of nitrogen mustard. In these cultures, the DNA content appears to remain constant. The losses in DNA which are observed after treatment with the three higher concentrations of mustard could mask some degree of simultaneously occurring DNA synthesis.

To evaluate the magnitude of DNA synthesis under these conditions the incorporation of C^{14}-formate into DNA thymine was measured in control and experimental cultures from the 20th through the 26th hour after mustard treatment. The data are expressed as counts per minute per micrograms of DNA thymine. It can be seen from Fig. 2 that DNA synthesis is reduced by the application of 1.56×10^{-7} M solutions to less than 15 per cent of the control level.

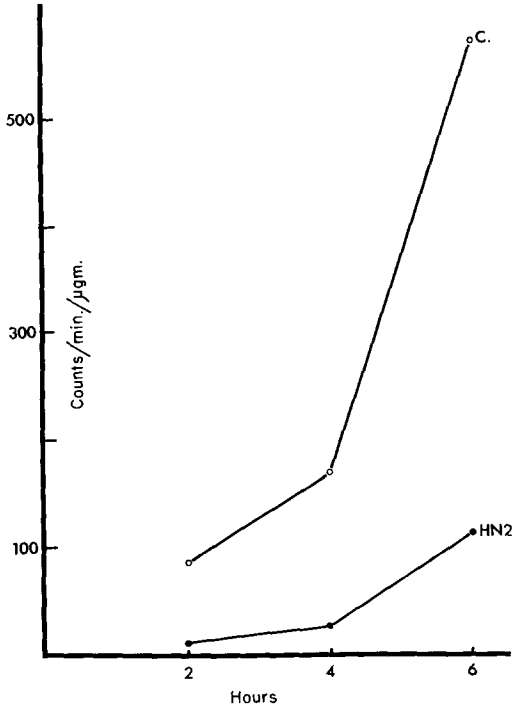

FIGURE 2

Specific activity of DNA-thymine in control and nitrogen mustard-treated cultures after exposure for short periods to C^{14}-formate. Control curve marked *C*, nitrogen mustard-treated values marked *HN2*.

The nitrogen mustards are a group of highly reactive compounds which would be expected to react, at some level of concentration, with many constituents of living cells. In the present investigation the incorporation of glycine-C^{14} into the total cell protein fraction has been used as a measure of the over-all metabolic state of the cell. Impairment of energy generation, RNA synthesis, amino acid activation, transport of activated amino acids to sites of protein synthesis as well as synthesis itself might reasonably be expected to be reflected in the rate of incorporation.

Preliminary experiments using twice the level of glycine-1-C^{14} indicated that incorporation was linear only for the first 3 hours. Thereafter the rate of incorporation fell off progressively. Reduction of the concentration of isotope resulted in linear rates of incorporation for at least 5 hours.

Comparison was also made of the rates of incorporation obtained by separating the glycine from the final hydrolysate chromatographically (the method of Krol (17) as modified by Herrmann and Schultz (15)) and counting this fraction separately. In two experiments of this kind the rates obtained with the protein-glycine fraction did not differ from the rates observed with the whole protein hydrolysate in parallel experiments. Conversion of glycine (*i.e.*, to serine) must occur at too slow a rate to affect the results of these short-term incorporation experiments. The comparison of incorporation into the protein fraction of control and nitrogen mustard-treated cultures suggests that nitrogen mustard, at levels which profoundly affect DNA accumulation (0.52×10^{-7} M and 1.56×10^{-7} M), has no significant effect on glycine incorporation. Only at the next higher concentration of mustard (2.6×10^{-7} M) were inhibitions of the rate of glycine incorporation evident and even at this concentration inhibition was not observed in every experiment.

Table I summarizes the results of eight experiments in which glycine incorporation was determined. The time interval represented is from the 20th to the 24th hour after treatment. It will be noticed that the control rates vary among the

TABLE I

Rates of Incorporation of Glycine-1-C^{14} into the Protein Fraction of Control and Nitrogen Mustard–Treated Cultures

	Control	0.52×10^{-7} M	1.56×10^{-7} M	2.6×10^{-7} M
$\mu C \times 10^{-4}$/hr./μg. DNA	0.78	0.68	0.72	0.16
per cent of control	—	87	92	21
$\mu C \times 10^{-4}$/hr./μg. DNA	0.67	0.80	0.76	0.28
per cent of control	—	119	113	42
$\mu C \times 10^{-4}$/hr./μg. DNA	0.66	0.84	0.59	0.31
per cent of control	—	127	89	47
$\mu C \times 10^{-4}$/hr./μg. DNA	1.03	1.32	1.20	0.57
per cent of control	—	128	117	55
$\mu C \times 10^{-4}$/hr./μg. DNA	1.02	0.98	0.86	0.70
per cent of control	—	96	84	69
$\mu C \times 10^{-4}$/hr./μg. DNA	0.95	1.24	0.89	0.80
per cent of control	—	131	94	84
$\mu C \times 10^{-4}$/hr./μg. DNA	0.47	0.56	0.58	0.55
per cent of control	—	119	123	117
$\mu C \times 10^{-4}$/hr./μg. DNA	1.19	1.54	1.23	1.41
per cent of control	—	129	103	118
Average per cent of control		117	102	69

individual experiments. This variation is due, perhaps, to the fact that the labeled molecule was presented to the cells in a medium containing such heterologous materials as horse serum and embryo extract. Variable amounts of amino acids in different batches of these components could dilute the labeled glycine to varying degrees which would, in turn, produce differences in rates of incorporation.

Within each experiment the three categories of experimentals have been compared with the respective controls. An analysis of variance of regression has been applied to determine if the differences between experimental and respective control are significant. With the two lower concentrations of nitrogen mustard none of the small differences are significant. Only with the highest concentration used in this series (2.6×10^{-7} M) do significant deviations from control rates occur. The first four rates listed in the 2.6×10^{-7} M column are significant below the 1 per cent level, the next value is significant only below the 5 per cent level, and the last three rates do not differ significantly from their controls.

This lack of absolute consistency of cultures treated with 2.6×10^{-7} M mustard may indicate a threshold above which mustard exerts an effect on glycine incorporation as well as DNA accumulation. On the other hand, it may reflect a higher percentage of necrotic cells in the cultures treated with the higher concentration of nitrogen mustard. The data in Fig. 1 suggest that with higher concentration of mustard the onset of cell loss occurs earlier.

When applied just prior to massive multinuclear cell formation even the highest concentrations of nitrogen mustard studied do not inhibit the development of multinuclearity (see Figs. 3 to 6). Although it appeared unlikely, the possibility was explored that a 24-hour lag period occurred prior to inhibition of DNA synthesis. This lag might permit multinuclear cell formation by a process of nuclear replication during the first 24 hours after treatment. The capacity for the development of multinuclearity in nitrogen mustard–treated cells after a lapse of 24 hours post-treatment was tested. For this purpose cell suspensions were plated in high dilution in 14 cm. diameter petri plates. The cells were treated with 1.56×10^{-7} M nitrogen mustard 18 to 20 hours later, after cell attachment had occurred. After treatment the cultures were washed with phosphate-buffered saline, fed with complete growth medium, and returned to the incubator for an additional 24 hours. During this period, due undoubtedly, to the high dispersion of the cells, no noticeable degree of multinuclearity had occurred. The cells were then resuspended by trypsinization, concentrated by centrifugation, counted, diluted to an appropriate level, and

plated in siliconed ring cultures (see Methods). The cell number was varied from 2.5×10^5 to 2×10^6 cells per culture in doubling steps. With these high cell numbers, relative to the culture surface area, confluency was observed immediately after attachment was complete (24 hours) in all but the lowest cell concentration. Multinuclear cell formation had occurred at all concentration levels within the first 24 hours (see Figs. 3 to 6). Grossly the density of multinuclear cells paralleled cell concentration except for the highest concentration in which excessive acid production had occurred.

An examination of the ring cultures under low magnification (120) reveals an obvious and extreme enlargement in the mononucleated "fibroblast-like" cells. At this magnification, however, the nuclei in the multinuclear cells do not appear strikingly altered. Owing to the decrease in density of the giant fibroblast nuclei the elongated multinuclear cells are very prominent and appear in these preparations as strings of small basophilic nuclei. A comparison of Figs. 7 and 8 demonstrates the similarity in the size of nuclei in control "fibroblast-like cells" and both control and experimental multinuclear cells as compared to the extreme enlargement of the "fibroblast-like" cell nuclei after mustard treatment.

At higher magnifications (570) it becomes apparent, however, that a more moderate degree of nuclear enlargement has, in fact, occurred in the nuclei of the multinuclear cells which form after nitrogen mustard treatment. The nuclei of control multinuclear cells are all fairly regular in size (see Fig. 9). In the multinuclear cells which form between the 24th and 48th hours after nitrogen mustard treatment there exists a wider variation of nuclear size (Fig. 10). This size variation, however, is of a lower order of magnitude than the observable difference between mono- and multinucleated cells in the treated cultures.

In addition to these size differences between intact nuclei the cytoplasm of both cell types after nitrogen mustard treatment contains groups of dense basophilic bodies, smaller in size than normal nuclei and lacking any pronounced internal detail (see Fig. 10). These bodies fit the description of nuclear fragments or micronuclei described previously (2, 11, and 16).

One further difference between mono- and multinucleated nitrogen mustard–treated cells is illustrated in Figs. 3 to 6. At 48 and 72 hours after treatment the cultures present the appearance of

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Photomicrographs of control cultures and cultures treated with 5.2×10^{-7} M nitrogen mustard. Living cultures growing in 50 mm. petri plates were photographed through an inverted optics microscope using ordinary light optics.

FIGURE 3

Control cultures at the time of application of nitrogen mustard to the experimental plates. Multinuclear cell formation has been initiated. Relatively small multinuclear cells are apparent (see arrow).

FIGURE 4

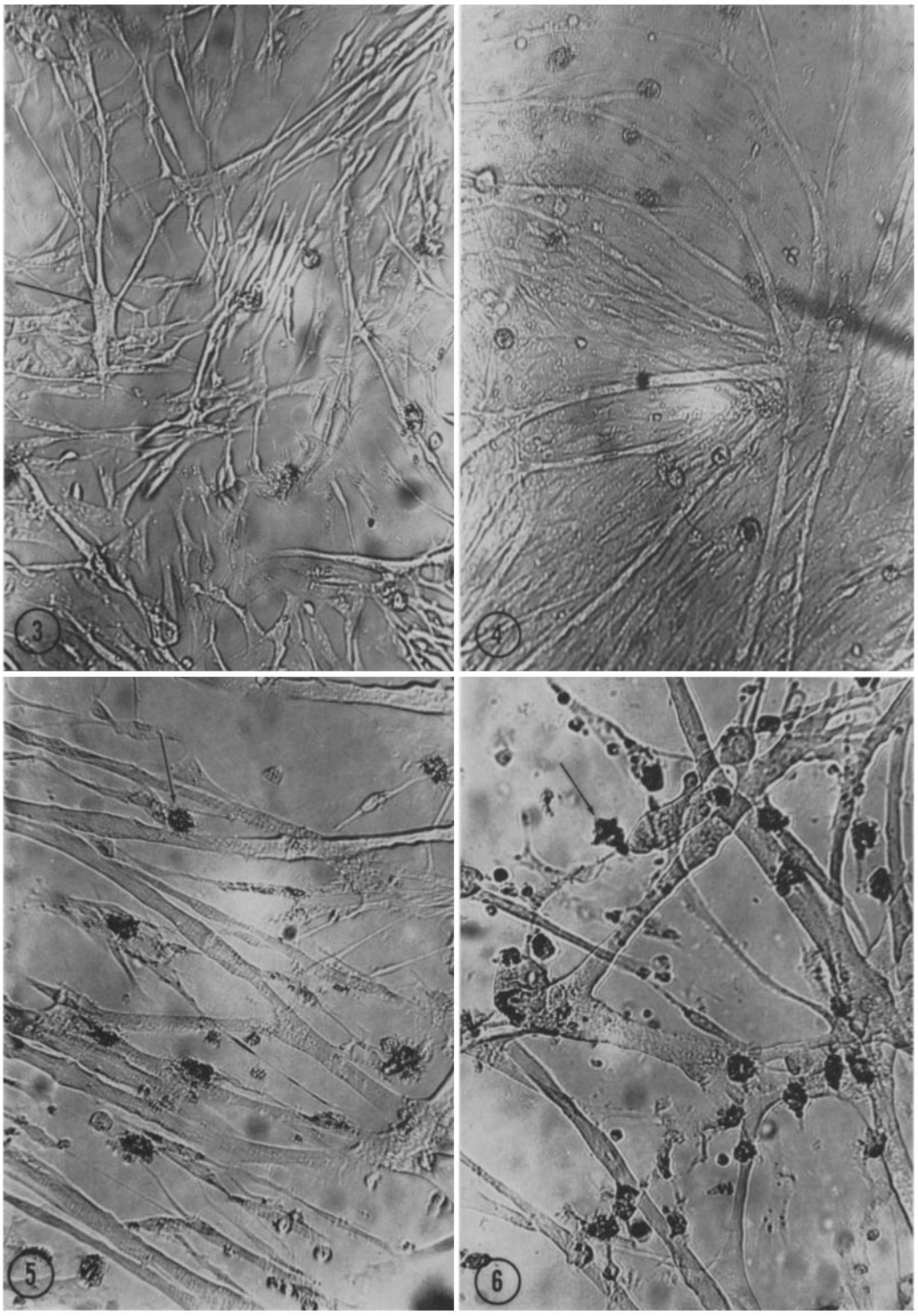
Nitrogen mustard–treated culture 24 hours after treatment. Multinuclear cell formation has continued producing typical elongated broad ribbon-like cells.

FIGURE 5

Mustard-treated culture 48 hours after treatment. The mononucleated cells observed between the multinuclear ribbon in Fig. 4, have by this time greatly decreased in number. The loss of mononucleated cells does not occur in control cultures. Arrow indicates one of several dark, crenated cells presumed to be necrotic.

FIGURE 6

Mustard-treated culture 72 hours after treatment. The loss of mononucleated cells has continued; few normal mononucleated cells remain.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an interlacing network of multinuclear "ribbons" with bare interstices. In control cultures such interstices are occupied by contiguous sheets of "fibroblast-like" cells. These results are clear cut only at the highest concentration level used (5.2×10^{-7} M). Although the fate of the cells disappearing from the interstices is unknown, from the obvious loss of DNA which occurs in nitrogen mustard–treated cultures (Fig. 1), it seems most likely that the mononucleated cells are more sensitive to the lethal effects of nitrogen mustard than are the multinucleated cells.

The greater resistance of the multinucleated cell may be related to the less profound effects of nitrogen mustard on nuclear morphology in these cells or may merely reflect the obvious advantage of multinuclearity in the presence of an agent whose toxicity is exerted primarily via nuclear damage.

DISCUSSION

Despite the profound effects on DNA accumulation observed with even the lowest concentration of mustard used, the rates of glycine incorporation are remarkably unaffected except for the variable depression observed at 2.6×10^{-7} M. It would appear that in the system under study, the alkylating agent preferentially affects DNA accumulation. This finding is in accord with the results of Bodenstein and Kondritzer (4) on amphibian larvae.

The more direct measurement of the incorporation of a labeled precursor (C^{14}-formate) into DNA thymine indicates that the effect on DNA accumulation is not due to a high rate of cell death masking an otherwise normal rate of DNA synthesis. Whether the observed specific inhibition of DNA synthesis is due to direct or indirect effects of nitrogen mustard is beyond the scope of the present study (see Biesele, Mitotic Poisons and the Cancer Problem (1)). It is clear, however, that none of these effects interferes with the process of multinuclear cell formation. Such cells which form after nitrogen mustard treatment resemble the multinuclear cells formed under control conditions with respect to their ribbon-like appearance, the large numbers of nuclei involved,

Photomicrographs of control and nitrogen mustard–treated cells grown in siliconed-ring cultures. Cultures were fixed briefly (5 minutes) in 10 per cent neutral formalin and stained with Harris hematoxylin. No counterstain was used.

FIGURE 7

Control culture in which multinuclear cell formation has occurred. × 120.

FIGURE 8

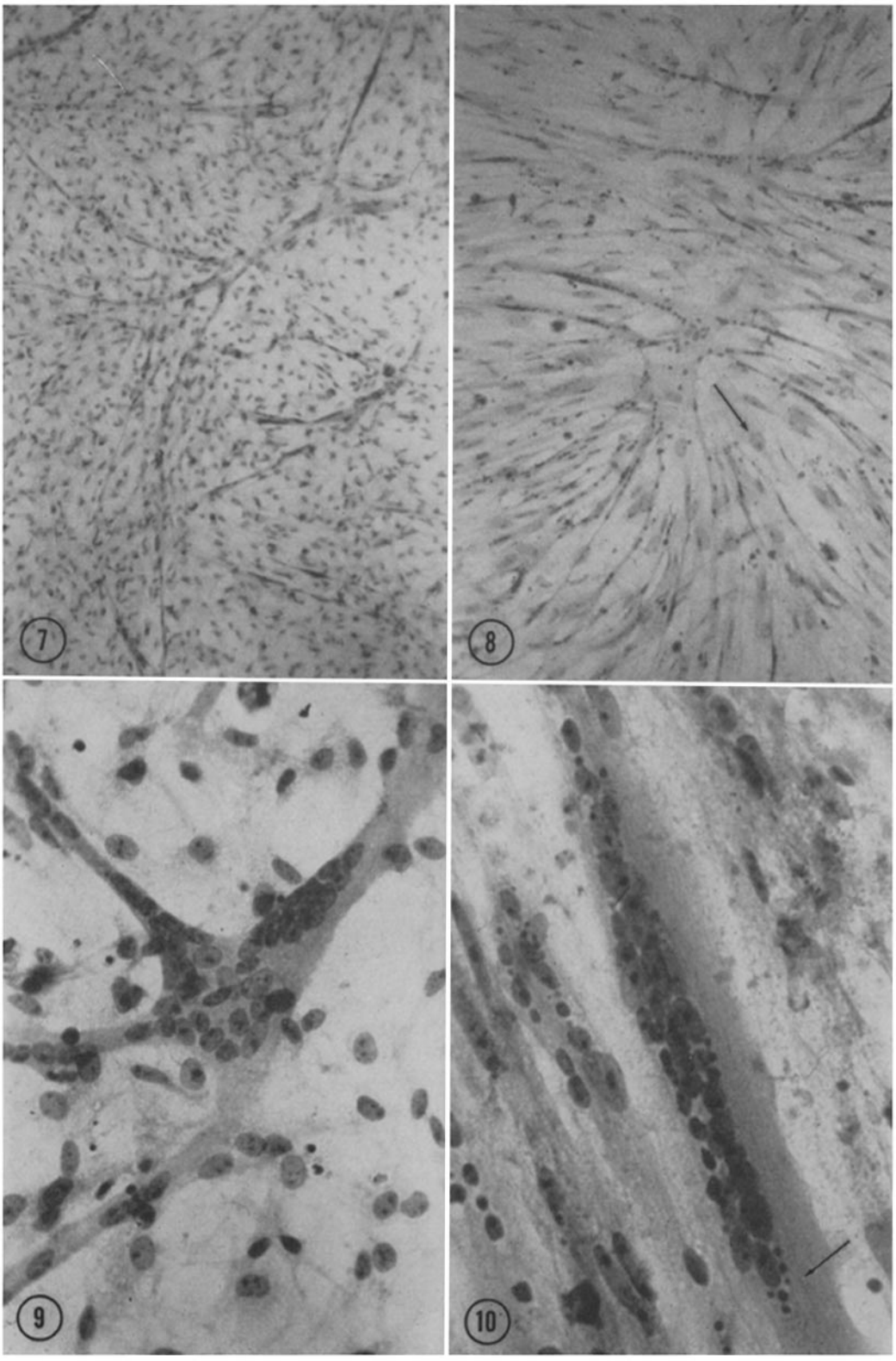
Culture of cells treated *in vitro* with nitrogen mustard 24 hours prior to resuspension and replating in ring cultures. Cultures were fixed 24 hours after replating. Despite treatment multinuclear cell formation has occurred. The multinuclear cells can be recognized by the linear arrangement of their nuclei and their basophilic ribbon-like cytoplasm. At this magnification the size of the nuclei of the multinuclear cells are not strikingly different from the nuclei of either the multinuclear or mononuclear cells of the control culture (see Fig. 7). However, between the multinuclear "ribbons" in Fig. 8 the giant nuclei of mononuclear cells can be clearly observed (note arrow). × 120.

FIGURE 9

Control culture at higher magnification. The photomicrograph more clearly demonstrates the more basophilic cytoplasm and sharper cell boundary of the multinuclear cells. Note the relatively uniform size of the nuclei in the multinuclear cells. × 570.

FIGURE 10

Multinuclear cells which have formed in culture during the second 24-hour period after nitrogen mustard treatment. Note the wide variability of size among the nuclei of the multinuclear cell. The presence of small basophilic bodies ("nuclear fragments") is indicated by the arrow. × 570.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the linear arrangement of nuclei, and the marked basophilia of their cytoplasm. Multinuclear cells have been observed in fibroblast cultures to occur both spontaneously (6) as well as after mustard treatment (10); however, such cells appear to involve far fewer nuclei and fail to meet the cytological criteria cited above.

The formation of such cells in the absence of DNA synthesis rules out amitotic division as an exclusive or significant mechanism. These results are in agreement with the microspectrophotometric study of Lash, Holtzer, and Swift (18) on the distribution of ploidy classes in developing muscle cells during muscle regeneration. Only an occasional nucleus was found to contain the tetraploid quantity of Feulgen-DNA. Wilde's (26) reported experimental production of muscle cell chimera containing both mouse and chick nuclei would also necessitate a process of cell fusion. It was previously reported by Herrmann *et al.* (14) that the deoxyribonucleic acid content of embryonic muscle increases at a rate faster than can be accounted for by the mitotic index. Although the nature of this phenomenon remains unclarified, in view of the present results, "amitosis" appears to be a less likely explanation.

For fusion to occur after nitrogen mustard treatment would require that the agent not alter the cell surface to an appreciable degree. One would infer from the work of Bodenstein (2) that such is the case. The development of the lateral line organ in amphibia involves extensive cell migration along specific pathways. Treatment with nitrogen mustard, although inhibiting mitosis, does not interfere with the normal pattern of cell migration involved in the development of lateral line organs.

In addition to the observation that the block in DNA synthesis does not interfere with the development of multinuclearity, several cytological aspects of the mustard-treated muscle cells are more compatible with the fusion hypothesis than with an "amitotic" mechanism. The differences in the degree of nuclear enlargement in mononucleated and multinucleated cells resemble the situation described by Bodenstein (5) which attains in the developing amphibian larva treated with nitrogen mustard.

Bodenstein (3, 5) demonstrated differential responses between cells of the same embryonic origin which appear clearly related to the developmental stage reached by a particular cell at the time of treatment. This is most striking in the larval eye of amphibia in which the zones of proliferation are spatially separated from zones of postmitotic differentiating cells. Nitrogen mustard both inhibits mitosis and induces cell and nuclear enlargement in the proliferative zone. The postmitotic cells, however, either show little effect or with higher concentrations or younger larvae break down without any preliminary enlargement. In the nitrogen mustard–treated cultures the nuclei of the multinucleated cells are, by comparison to the "fibroblast-like" cells, relatively unaffected. By analogy to the *in vivo* situation described by Bodenstein, these would be the nonproliferating nuclei. However, even these nuclei have undergone, to variable extents, some nuclear enlargement. This is quite apparent from a comparison of nuclear size variation in control and nitrogen mustard–treated muscle cells.

The greater size variation observed among the nuclei of the multinuclear cells which form after nitrogen mustard treatment may simply be an expression of individual differences in response to the agent. However, it is tempting to speculate that these size differences may reflect temporal differences in the incorporation of the cells bearing individual nuclei into the syncytium. Since mononucleated cells show marked nuclear enlargement and multinucleated cells very little, it seems reasonable that some enlargement might occur prior to fusion. The assumption that proliferative capacity (and consequently the nuclear enlargement induced by nitrogen mustard) is lost at the time of fusion must be tested since it is equally possible that such loss occurs earlier. Fusion may, in fact, be a consequence of the loss of the capacity to proliferate.

The mechanism of the nuclear enlargement produced by nitrogen mustard treatment is at present unknown. Although a similar response occurs after x-irradiation, in view of the vastly different properties of these two agents it would seem premature to assume a common mechanism. The examination of DNA synthesis reported here as well as the work of Bodenstein and Krondritzer (4) would indicate that the enlargement is most probably due to a nuclear constituent or constituents other than DNA. Whatever the nature of this constituent it would seem to be unaltered by mustard treatment in postmitotic cells.

The presence of "nuclear fragments" in mul-

tinuclear cells formed after nitrogen mustard treatment again seems more compatible with fusion than with "amitosis." These fragments which appear at about 1 week after treatment of cells *in vivo* appear to be produced by the rupture of enlarging nuclei (2, 11). Hughes and Fell (16) have described a condition which arises soon after the application of sulfur mustard to cells *in vitro* which, although it differs from it temporally, produces the same end result as "nuclear fragmentation." Abnormal mitoses were observed shortly after treatment, (abnormal mitotic figures having also been described by Bodenstein (2) in his nitrogen mustard-treated larvae). Cinematographic records of these abnormal mitoses in the treated cultures revealed that they frequently result in the reconstitution of several micronuclei. These micronuclei can arise from either a disorganized metaphase plate or from lagging during anaphase movement.

In the absence of time-lapse records, the origin of the basophilic inclusions in the multinuclear cell is open to question. However, irrespective of whether they are micronuclei or nuclear fragments their presence in the multinuclear cells seems more compatible with fusion than with an *in situ* origin of plural nuclei.

If they are indeed micronuclei it seems reasonable that they were formed in a mononucleated cell and secondarily incorporated into a syncytium since it is extremely doubtful that mitosis occurs in multinucleated muscle cells. Although somewhat less unequivocal, it is doubtful that nuclear fragmentation of enlarged nuclei arises *in situ* in the multinuclear cell since relatively little nuclear enlargement occurs in these cells.

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