In vitro Effects of 2,4-Dichlorophenoxy Acetic Acid (2,4-D) on Bovine Cells

S. V. Basrur, R. A. Fletcher and P. K. Basrur*

ABSTRACT

Bovine fetal muscle cells were exposed to culture media containing 2 mg and 20 mg per liter of 2,4-dichlorophenoxy acetic acid (2,4-D) for varying intervals to determine the in vitro response of mammalian cells to this compound. The concentrations of 2,4-D used were comparable to those used in spray programmes although the residues normally found in pasture are much lower since 2,4-D is rapidly degraded under field conditions. Untreated and treated cultures were analyzed for total cell count, mitotic index and the percentages of differentiating and degenerating cells. The response of cultures to treatment was similar irrespective of the concentrations of 2,4-D used although in higher concentrations there was an initial drop in mitotic index. Other changes noted in treated cultures included an increase in differentiating and degenerating cells compared to those in control. The mitotic cells in treated cultures exhibited unipolar and tripolar spindles and a variety of other abnormalities including malorientation of the mitotic apparatus in relation to the axis of the cell. Myoblasts in initial stages of myogenesis were noted to be in mitosis in treated cultures suggesting that 2,4-D may have a stimulatory effect on myoblasts which in normal myogenesis are in post mitotic stage.

RÉSUMÉ

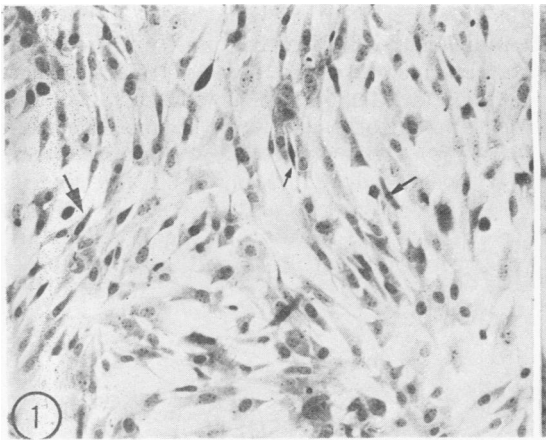
Cette expérience visait à ensemencer des cellules musculaires de foetus bovins dans des milieux de culture contenant respectivement 2 et 20 mg/l de 2,4-dichlorophenoxy acide acétique (2,4-D) et à les y laisser pour différents intervalles, afin de déterminer la réponse in vitro de cellules de mammifères à cet herbicide. Les concentrations de 2,4-D utilisées se comparaient à celles des programmes de vaporisation, bien que les résidus retrouvés sur les pelouses soient beaucoup moins considérables, puisque le 2,4-D se dégrade rapidement dans la nature. On rechercha dans les cultures cellulaires expérimentales et témoins le nombre total de cellules, l'index mitotique et le pourcentage de cellules en voie de différentiation ou de dégénérescence. La réaction des cultures cellulaires au 2,4-D s'avéra similaire, indépendamment des concentrations utilisées; toutefois, les plus élevées produisirent une baisse initiale du taux de mitoses. Comme autres changements des cultures cellulaires soumises à l'action de cet herbicide, on nota une augmentation du nombre des cellules en voie de différentiation ou de dégénérescence, comparativement aux cultures témoins. Les cellules en mitose des cultures expérimentales manifestèrent des fuseaux uni et tripolaires, ainsi qu'une variété d'autres anomalies incluant une mauvaise orientation de l'appareil mitotique par rapport à l'axe de la cellule. On remarqua que les myoblastes des phases initiales de la myogénèse étaient en mitose, dans les cultures expérimentales; cette observation laissa supposer que le 2,4-D pourrait exercer un effet stimulant sur les myoblastes qui, dans la myogénèse normale, ont déjà atteint la phase post-mitotique.

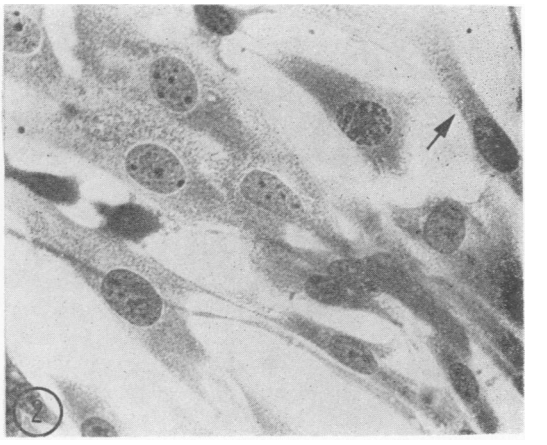
INTRODUCTION

Phenoxy herbicides which include 2,4-dichlorophenoxy acetic acid (2,4-D) and 2,4,5-trichlorophenoxy acetic acid (2,4,5-T) are used for eradicating broadleaf weeds. At low concentrations their action is similar to indole acetic acid, a natural plant growth hormone (10). Both 2,4-D and 2,4,5-T increase the RNA levels by more than 200% and lower the protein/RNA

*Department of Environmental Biology, Ontario Agricultural College (S. V. Basrur and R. A. Fletcher) and Department of Biomedical Sciences, Ontario Veterinary College (P. K. Basrur), University of Guelph, Guelph, Ontario N1G 2W1.

Submitted September 3, 1975.





Figs. 1 and 2. A two day old culture. Note that a majority of cells are fibroblast-like. A few myoblasts characterized by uniform shape and intense staining reaction may be noted (arrows). Fig. 1 — X100, Fig. 2 — X450.*

ratio in cucumber which is extremely sensitive to these herbicides (3). It has been proposed that the phytotoxicity of these herbicides which also change the nucleohistone patterns may be based on the alteration of RNA species and the interference with protein synthesis (4). In addition to their effects on plants, the phenoxy herbicides including 2,4-D and 2,4,5-T have been reported to be toxic to man and animals at high concentrations (8, 14) and teratogenic to animals (5, 8, 11, 12, 15, 17). Some of the fetal malformations have been attributed to the presence of 2,3,7,8-tetrachlorodibenzo-p-dioxin (dioxin) as a contaminant in these compounds (5, 6, 16). In the present study pure 2,4-D containing no dioxin was used for determining its effects at relatively low concentrations on bovine cells grown *in vitro*.

MATERIALS AND METHODS

Limb muscles of bovine fetuses ranging in age from 2.5 to 4.5 months were cultured in Hanks' Minimum Essential Medium (HMEM) supplemented with 15% calf serum, 100 μg/ml streptomycin and 100 I.U. of potassium penicillin G according to the method of Basrur and Gilman (1). Twenty-seven Leighton tubes were inoculated with 2.0 ml each of cell suspension containing approximately 200,000 cells per ml. Cultures were incubated at 38°C for 24 hours and were then divided into three groups, one of which remained as controls. The media in the other cultures were replaced with fresh media containing 2.0 mg or 20 mg per liter of 2,4-D which on mass spectrometric analysis revealed no dioxin and was 99.6 ± 0.08% pure. The 2,4-D sample and the statement of purity were obtained from the Division of Chemical Standards, National Physical Laboratory, Teddington, Middlesex, England. Cultures were terminated at 24, 48 and 96 hours after treatment and were stained with May-Grünwald-Giemsa stain (13). These experiments were repeated on two more sets of cultures after an interval of three weeks.

The percentage of dividing, degenerating and differentiating cells (with two or more nuclei) were estimated from the total cell counts obtained in 20 randomly selected microscopic fields (under X40 objective) from each treated and control culture of each experiment. In order to ensure randomization of the fields the counting was done in a standardized pattern starting with a field on the upper left-hand side of the coverslip culture and counting cells in three fields at 500μ apart in each of six rows and two fields from a seventh row. The percentage of mitotic cells was estimated from counts of all cells at various stages of mitosis including those in metaphase which were also grouped separately. For the purpose of classification, interphase cells with nuclei two or more times larger than the majority of cells in the field were grouped together as polyploid

*Figs. 1 to 6 — Bovine fetal muscle cell cultures. May-Grunswald-Giemsa stain.

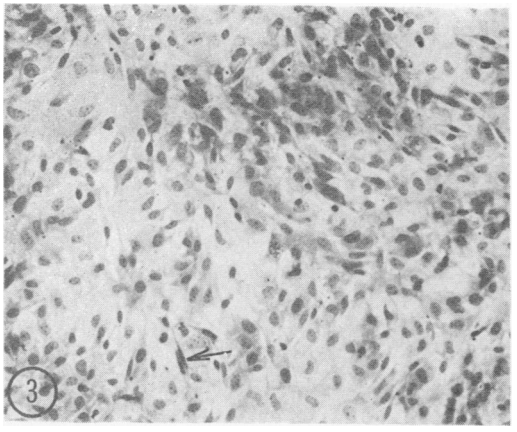


Fig. 3. A three day old culture, showing a few mitotic cells and cells in the process of myogenesis (arrow). X100.

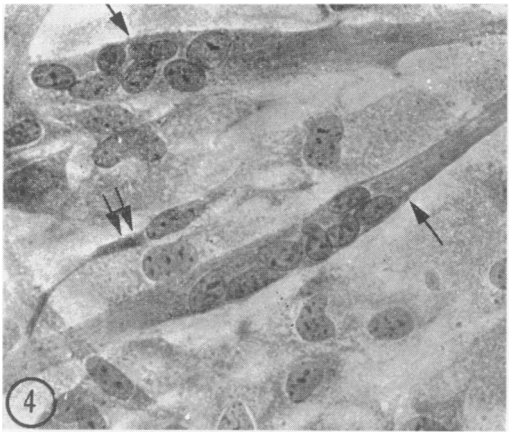


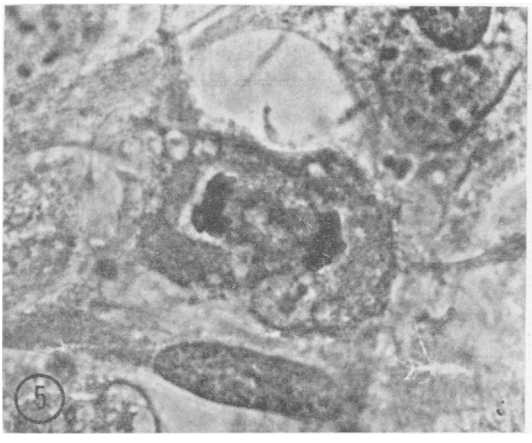
Fig. 4. A higher magnification of myotubes and myoblasts in the process of differentiation. Note the multinucleated myotubes (single arrow) and myoblasts in the process of fusion (double arrow). X450.

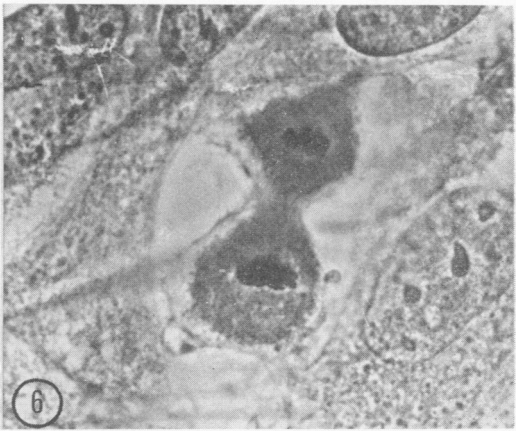
cells, cells with two or more nuclei and indications of longitudinal and/or cross striations were grouped as differentiating cells and interphase cells with pyknotic nuclei and mitotic cells with abnormal spindle and/or chromosome groupings were grouped as degenerating cells. The growth rates for treated and untreated cultures were estimated from the mean cell counts per field obtained for cultures terminated at various times after the initiation of the experiment.

RESULTS

Cultures of bovine fetal muscle cells consisted mainly of fibroblast-like cells during the first 24 to 48 hours (2). These cells formed a confluent sheet between 48 to 72 hours after initiation of the cultures. Mitotic cells were abundant in these cultures although by 72 hours myoblasts in the process of fusion were also frequently detected (Figs. 1-6).

The total cell counts in cultures treated with 2,4-D dropped below the controls initially but at 96 hours after treatment there was very little difference between controls and treated cultures (Table I). In cultures exposed to 20 mg/l of 2,4-D for 24 hours the percentage of mitotic index cells was strikingly low compared to the other cultures of this series but by 48 hours the mitotic index rose to well above that of controls (Table I). The percentage of differentiating cells in cultures exposed to 2.0 mg/l of 2,4-D for 96 hours was greater





Figs. 5 and 6. Cells in anaphase and telophase, respectively. X1000.

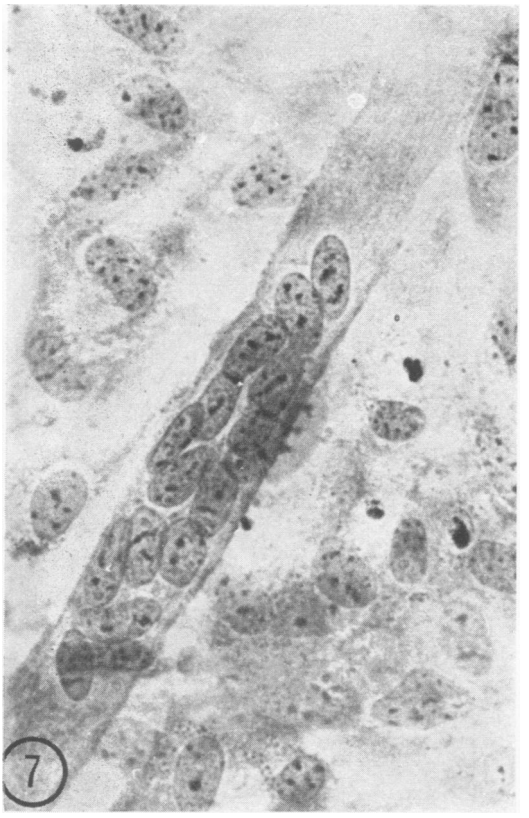


Fig. 7. A multinucleate myotube in a five day old culture 96 hours after exposure to 20 mg/l of 2,4-D. Note the pyknotic uninucleate (degenerating) cells and the relatively well differentiated myotube exhibiting distinct longitudinal fibres and indications of cross striations at the periphery. X450.*

than that of controls (Table I) and the myotubes containing larger numbers of nuclei were also more abundant. Cultures treated with 20 mg/l of 2,4-D exhibited a more striking increase in the percentage of differentiating cells (Table I) and in the extent of differentiation characterized by the number of nuclei per myotube (Fig. 7). The size of the nuclei in these myotubes was remarkably similar and the cytoplasmic differentiation was reminiscent of the initial stages of normal *in vitro* myogenesis (1, 9).

In cultures exposed to the lower concentration of 2,4-D the contractile elements of some of the large myotubes appeared less distinct and strap-like cells with irregularly distributed nuclei and poorly differentiated cytoplasm increased in number (Fig. 8). This is in contrast to cultures exposed to similar concentration of 2,4-D for shorter periods (Fig. 9) where the myotubes generally appeared intensely basophilic although the distribution of nuclei was irregular. Another noteworthy feature of the cultures exposed to the lower concentration of 2,4-D was that cells at early stages of myogenesis often exhibited nuclei at different stages of the cell cycle. In some instances (Fig. 10) one of the nuclei was in interphase while the other was distinctly in mitosis.

Cultures treated with high and low concentrations of 2,4-D exhibited a high frequency of degenerating cells (Table I). Other changes in 2,4-D treated cultures included an increase in the percentages of metaphase cells, polyploids (Table I), unipolar, tripolar, tetrapolar and pentapolar spindles, C-mitoses and cells exhibiting malorientation of the mitotic apparatus relative to the long axis of the cell (Figs. 11 to 16). Some of these mitotic irregulari-

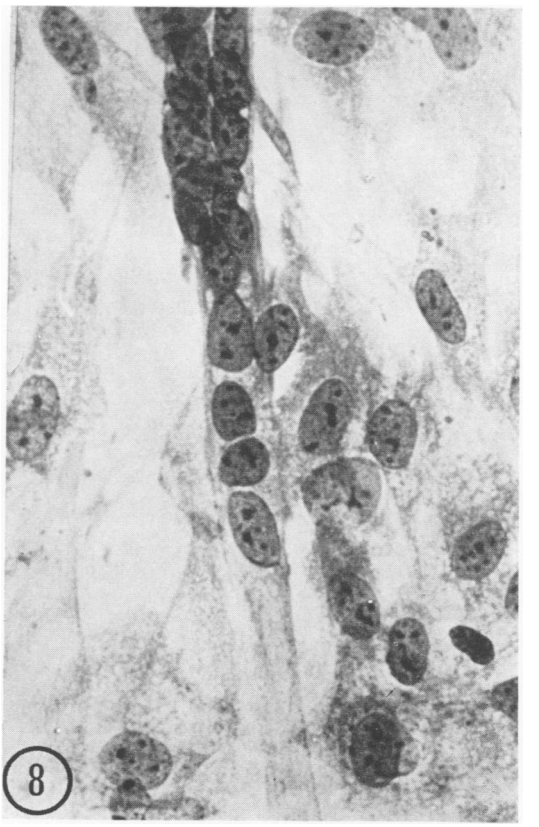


Fig. 8. A five day old culture 96 hours after exposure to 2 mg/l 2,4-D. Note the myotube on the left which is practically devoid of nuclei and has fewer contractile elements as compared to the myotube on the right with two rows of nuclei. X450.

*Figs. 7 to 10 — Bovine fetal muscle cultures treated with 2,4-D. May-Grunwald-Giemsa stain.

ties appeared to have led to unequal nuclear divisions and, in some instances, to unequal cytokinesis in which one daughter cell was often without any chromosomes (Fig. 11). The frequency of these anomalies was similar in cultures exposed to high and low concentrations of 2,4-D whereas in control cultures of corresponding age they were extremely rare.

DISCUSSION

The most interesting observation in this study is that of myoblasts undergoing mitosis (Fig. 10). As illustrated in Fig. 10, one of the nuclei in these myoblasts was unmistakably in metaphase while the others were in interphase. The mitotic nuclei in some of these myoblasts were not of normal orientation in that the metaphase plate was

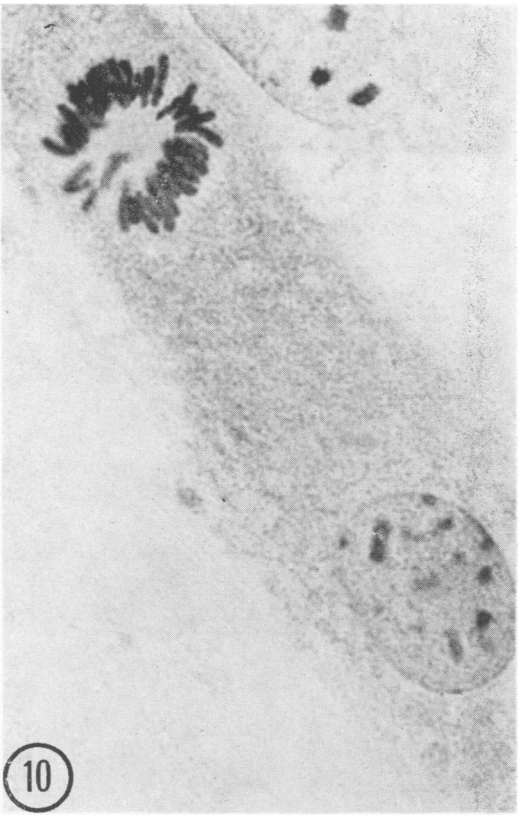


Fig. 10. A binucleated myoblast with one of the nuclei in metaphase in a culture 48 hours after exposure to 2 mg/l of 2,4-D. Note the cytoplasm between the two nuclei without interruption and the bidirectional growth pattern characteristic of myoblasts. X1000.

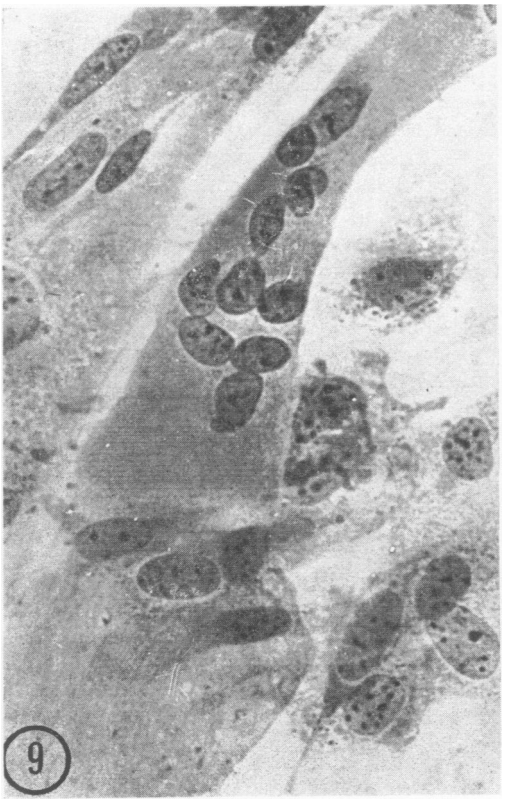


Fig. 9. A multinucleated myoblast in a three day old culture exposed to 2 mg/l of 2,4-D for 48 hours. Note the contractile elements in the bulged lower portion of the myoblasts with very few nuclei. X450.

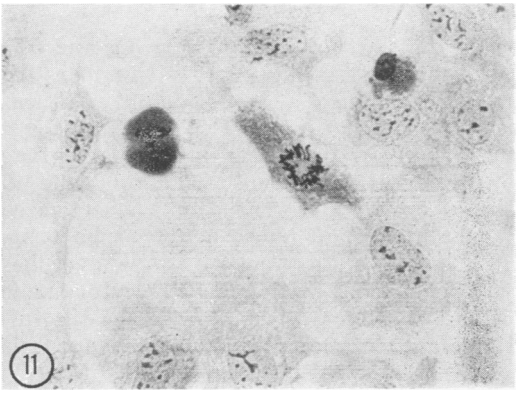


Fig. 11. A three day old culture treated with 20 mg/l 2,4-D for 48 hours. Note the abnormal mitotic figures. The cell on the left is in mitosis with the chromosomes segregating to one pole and the cell to the right exhibits metaphase chromosomes in polar view with the mitotic apparatus located perpendicular to the long axis of the cell. X450.*

*Figs. 11 to 16 — Bovine fetal muscle cultures treated with 2,4-D, showing mitotic aberrations including multipolar spindles and improper chromosome segregations. May-Grunwald-Giemsa stain.

TABLE I. Total Cell Counts and the Distribution of Dividing, Differentiating and Degenerating Cells in 2,4-D Treated and Untreated Cultures of Fetal Bovine Muscle

Treatment	Age of cultures (hr)	Duration of treatment (hr)	Total cell count[a]	Mitotic cells[a] (%)	Metaphase[a] (%)	Polyploid cells[a] (%)	Differentiating cells[a] (%)	Degenerating cells[a] (%)
Untreated	48	0	2306	3.99	1.50	0.48	4.47	0.74
	96	0	3964	3.94	1.54	0.95	10.82	0.96
	120	0	5945	4.21	1.41	1.20	16.87	0.84
2 mg/l 2,4-D	48	24	1485	4.24	2.57	3.60	4.71	2.36
	96	48	2267	3.84	2.83	4.98	8.73	3.66
	120	96	5443	4.91	3.90	5.76	19.90	7.94
20 mg/l 2,4-D	48	24	1623	2.59	1.90	4.67	8.87	3.27
	96	48	2504	5.39	4.34	5.82	12.98	6.83
	120	96	6023	5.10	4.01	7.32	24.74	9.00

[a]Mean of three experiments

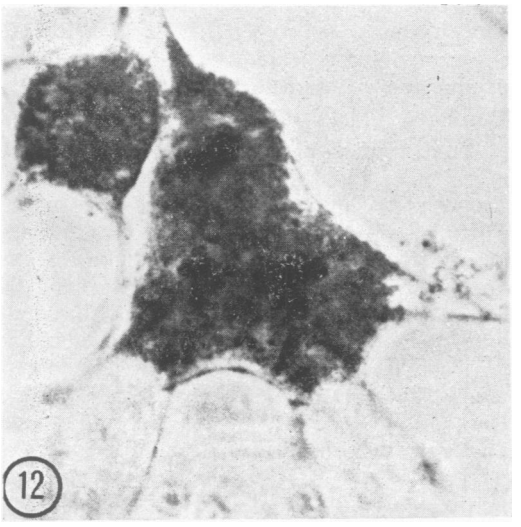
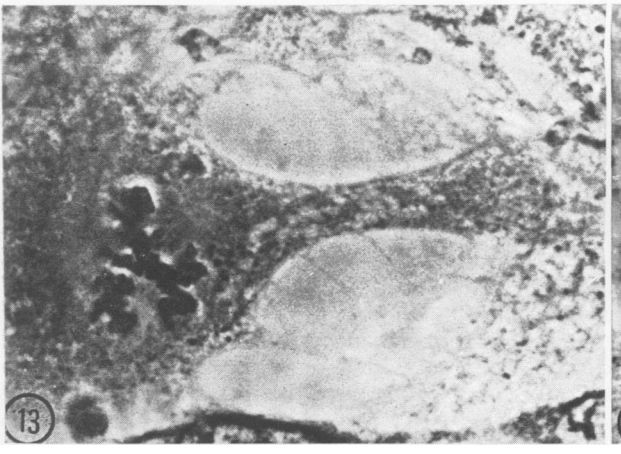
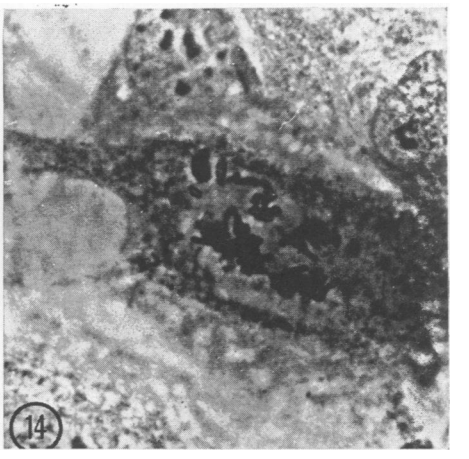


Fig. 12. A cell exhibiting a tripolar spindle in a culture treated with 2 mg/l of 2,4-D for 24 hours. Note the long cytoplasmic processes of the tripolar cell. X1000.

often discernible in a polar view. These cells were unequivocally myoblasts since the integrity of the cell membrane between the two nuclei was lost and the contour of the myoblast was uniform. The cytoplasm surrounding the mitotic nucleus was often darker than the remaining cytoplasm, suggesting either that the myoblast fusion had occurred recently or that the cytoplasm in that region was in the process of RNA synthesis in preparation for nuclear division. It would appear that the "mitogenic" effect of 2,4-D prompted nuclear division in some myoblasts after they had become "committed" myoblasts. Although the number of cells exhibiting this phenomenon was low, the fact that it happened at all is very interesting since division of cells after differentiation is usually associated only with pathological conditions including neoplastic





Figs. 13 and 14. Mitotic cells with tetrapolar and pentapolar spindles respectively in cultures treated with 20 mg/l of 2,4-D for 48 hours. X1000.

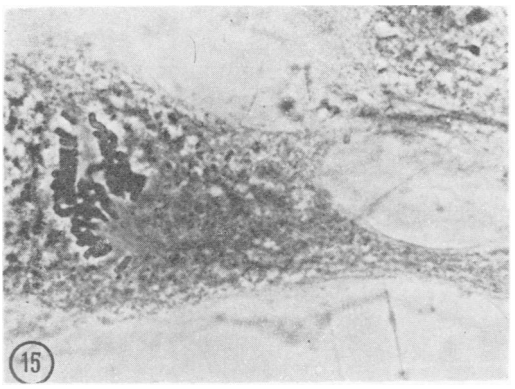


Fig. 15. A cell with abnormal mitotic apparatus in a culture treated with 2 mg/l of 2,4-D for 24 hours. Note the long cytoplasmic extension containing contractile elements. X1000.

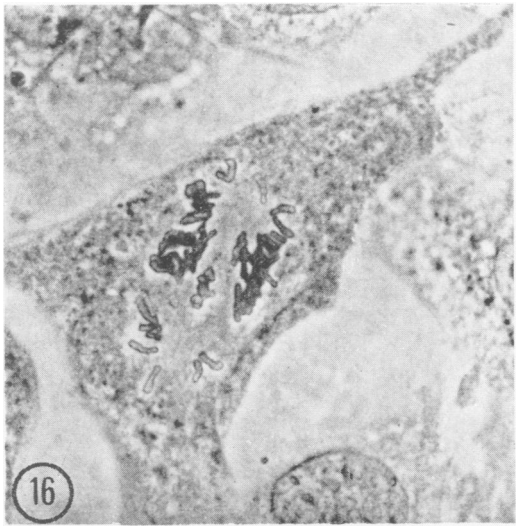


Fig. 16. A cell with improper orientation of the chromosomes in a culture treated with 20 mg/l of 2,4-D for 24 hours. X1000.

growth. The mitogenic effect of 2,4-D may be the result of a stimulus to synthesize proteins involved in mitotic processes: structural proteins which make up the microtubules of the mitotic apparatus or the enzymes required for the onset of division and the movement of chromosomes. The relatively high incidence of polyploid cells noted in 2,4-D treated cultures suggests that the compound may be interfering with the mitotic spindle. The increase in cells at metaphase (Table I) and those exhibiting abnormal cytoplasmic divisions may all be related to the effects of 2,4-D on the protein synthesizing machinery of the cells. This possibility has also been proposed for plants where the phytotoxic action of 2,4-D and 2,4,5-T has been attributed to their effects on nucleic acid and protein metabolism (3, 4).

The percentage of differentiating myoblasts was greater in treated cultures as compared to controls (Table I). It is conceivable that while 2,4-D promotes the synthesis of mitotic proteins which may be abnormal and thus cause aberrations in mitotic cells, the formation of myoblasts which involves the synthesis of specialized proteins is probably normal at least in the initial stages. It is possible that the two main processes in myogenesis (myoblast fusion and differentiation of contractile proteins) are not adversely affected since there is an overall increase in myoblasts with two or more nuclei in treated cultures. The higher degree of differentiation in these myoblasts probably indicates that the production of muscle proteins is uninterrupted in the presence of 2,4-D.

Whatever the mode of action of 2,4-D the effect is of short duration since the mitotic drop and the drop in total cell counts noted initially disappeared by 96 hours of exposure to 2,4-D. This may be due to the depletion of 2,4-D from the medium by 96 hours. Alternatively, it is conceivable that only a small population of cells which are at a sensitive phase in cell cycle at the initial stage of treatment are susceptible to the action of 2,4-D while the older and post mitotic cells are able to withstand the adverse effects of this herbicide. Thus, the myoblasts in 2,4-D treated cultures may be able to fuse unimpeded while the mitotic cells are detained at metaphase in greater percentage than those found in controls (Table I). The increase in degenerating cells in treated cultures may be partly attributable to the mitotic interference at metaphase leading to the destruction of dividing cells by 2,4-D. It would appear, therefore, that 2,4-D exerts a mitostatic effect on dividing cells and a mitogenic effect on post mitotic myogenic cells. The effect on differentiation *per se* may be incidental and not a direct result of 2,4-D since it was noted that the cells in which the nuclei were obviously affected by 2,4-D were also able to proceed with fusion. Cell fusion, which concerns mainly cell membrane and the phenomenon of self recognition by myoblasts destined to form myotubes, is probably not adversely affected by this herbicide.

ACKNOWLEDGMENTS

This investigation was supported by a grant from the National Research Council of Canada. The authors thank Dr. R. R. Collerson, Division of Chemical Standards, England for supplying the purified sample of 2,4-D for this investigation. Our thanks are also extended to Dr. T. A. Bongso, Mr. Ed Reyes and Mrs. Helen Randall for their technical help.

REFERENCES

1. **BASRUR, P. K. and J. P. W. GILMAN.** Behaviour of two cell strains derived from rat rhabdomyosarcomas. J. natn. Cancer Inst. 30: 163-207. 1963.
2. **BONGSO, T. A. and P. K. BASRUR.** Alterations in bovine cells exposed to 2,4-dichlorophenoxy acetic acid. Proc. Am. Ass. Tissue Culture, Boston. 1973.
3. **CHEN, L. G., C. M. SWITZER and R. A. FLETCHER.** Nucleic acid and protein changes induced by auxin-like herbicides. Weed Science 20: 53-55. 1972.
4. **CHEN, L. G., A. ALI, R. A. FLETCHER, C. M. SWITZER and G. R. STEPHENSON.** Effects of auxin-like herbicides on nucleohistones in cucumber and wheat roots. Weed Science 21: 181-184. 1973.
5. **COLLINS, T. F. X. and C. H. WILLIAMS.** Teratogenic studies with 2,4,5-T and 2,4-D in the hamster. Teratol. 4: 229. 1971.
6. **COURTNEY, K. D., D. W. GAYLOR, M. D. HOGAN, H. L. FALK, R. R. BATES and I. MITCHELL.** Teratogenic evaluation of 2,4,5-T. Science 168: 864-866. 1970.
7. **EMERSON, J. L., D. J. THOMPSON, C. G. GERBIG and B. V. ROBINSON.** Teratogenic study of 2,4,5-trichlorophenoxy acetic acid in the rat. Toxic. appl. Pharmac. 17: 317. 1970.
8. **ERNE, K.** Studies on the Analytical Chemistry and Toxicology of Phenoxy Herbicides. A. B. Uppsala, Sweden: Appelbers Boktryckeri. 1966.
9. **HOLTZER, H., J. M. MARSHALL and H. FINCH.** An analysis of myogenesis by the use of fluorescent antimyosin. J. Biophys. Biochem. Cytol. 3: 705-709. 1957.
10. **KEY, J. L.** Hormones and nucleic acid metabolism. A. Rev. Plant Physiol. 20: 449-474. 1969.
11. **KHERA, K. S. and W. P. McKINLEY.** Pre- and postnatal study on 2,4,5-trichlorophenoxy acetic acid, 2,4-dichlorophenoxy acetic acid and their derivatives in rats. Toxic. appl. Pharmac. 22: 14-28. 1972.
12. **KING, C. T. G., E. A. HORIGAN and A. L. WILK.** Screening of the herbicides 2,4,5-T and 2,4-D for cleft palate production. Teratol. 4: 233. 1971.
13. **MERCHANT, D. J., R. H. KAHN and W. H. MURPHY.** Handbook of Cell and Organ Culture. 2nd Edition. Minneapolis, Minnesota: Burgess Publishing Company. 1964.
14. **PALMER, J. S.** Chronic toxicity of 2,4-D alkanolamine salts to cattle. J. Am. vet. med. Ass. 143: 398-399. 1963.
15. **SCHWETZ, B. A., G. L. SPARSCHU and V. K. ROWE.** The effects of 2,4-dichlorophenoxy acetic acid (2,4-D) and esters of 2,4-D on rat fetal and neonatal growth and development. Toxic. appl. Pharmac. 19: 399 (abstract). 1971.
16. **SPARSCHU, G. L., F. L. DUNN and V. K. ROWE.** Teratogenic study of 2,3,7,8-teratochlorodibenzo-p-dioxin in the rat. Toxic. appl. Pharmac. 17: 317-318. 1970.
17. **THOMPSON, D. J., J. L. EMERSON and G. L. SPARSCHU.** Study of the effects of 2,4,5-trichlorophenoxy acetic acid (2,4,5-T) on rat and fetal rabbit development. Teratol. 4: 243. 1971.

BOOK REVIEW

ALLGEMEINE CHIRURGIE FÜR TIERÄRZTE UND STUDIERENDE. *(General Surgery For Veterinarians And Students). Edited by H. Schebitz und W. Brass. Published by Verlag Paul Parey, Berlin, Germany. 1975. 620 pages. Price DM240,—*

The outstanding feature of this multi-authored textbook is its wealth of up-to-date information on basic subjects required for the evaluation of a surgical patient. Special emphasis has been placed on the histopathology and the healing of lesions of the locomotor system, the skin and the circulatory system including shock, and shock therapy.

The history of veterinary surgery and the chapter on Infectious Diseases and Antibiotics have received considerable and detailed attention, occupying approximately 8 and 15% respectively of this volume. One wonders however if this is justified when at the same time, methods of diagnoses are not covered in detail. The description of surgical procedures comprising approxomately six percent of the book volume, is limited to some suture patterns and materials, osteosynthesis and some special procedures. Analgesia and anesthesia are not considered. Under these circumstances, perhaps a more suitable title would have been "General Surgical Pathology".

Not surprisingly, and considering that this textbook is written in German, by far the majority of the references are from German literature, the one exception being the chapter written by Dr. Peter Suter.

This book at an approximate cost of $95-98 at the present rate of exchange will probably not sell too well, except to those who have a working knowledge of the German language. — *E. Tolksdorff and F. J. Milne.*