EFFECTS OF FLUORIDE ON CELLS AND TISSUES IN CULTURE
A REVIEW

by

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The fluoride ion is capable of inhibiting a wide range of enzymes. Metabolically inactive complexes are formed by fluoride, particularly with enzymes dependent upon calcium, magnesium and iron (1). However, the fact that the highly-reactive fluoride ion is an enzyme inhibitor in vitro and in vivo, when administered at dose levels producing acute toxicity, is irrelevant to the study of any potential toxicity of minute amounts of fluoride administered on a long term basis to the intact animal or to humans.

In assessing the risk of toxicity arising from the chronic administration of any pharmacologically-active agent, two factors must be examined separately and independently:

(a) The sites of concentration of the drug within the body and the maximum concentrations and persistence of the drug in those sites after various patterns of administration, and

(b) The sensitivity to the drug of cells of the organs in which the concentration of the drug is maximal.

The former question is beyond the scope of this review; the latter question can best be approached by the use of tissue culture model systems. However, it remains an unanswered question whether the particular tissue culture systems which have been studied so far represent the best models from which to extrapolate potential risks to the intact animal.

Tissue Cultures in Toxicology

Early work in tissue culture used the relatively crude parameter of the survival or failure of histological integrity and gross "growth" of large pieces or organs explanted into culture media to which the drug in question had been added. Organ function was usually difficult to assess in vitro. The "survival" of a tissue explant was often the result of the successful growth of fibroblasts derived from the normally reproductively dormant connective tissues of the organ, rather than from the survival of the parenchymal cells which represented the real interest of the toxicity study. The development of the method of clonal cell culture for mammalian cells by Puck et al. (2) provided a highly sensitive method for deter-

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Presented at Frankfurt Conference of I.S.F.R., October 23-25, 1967.

mining the survival of the reproductive capacity i. e. the ability to divide an unlimited number of times not only for the ubiquitous fibroblasts, but also for epithelial cells derived from various organs, and for cells originating in malignant tumors.

Significance of Cell Growth in Toxicology

A by-product of studies of the effects of ionizing radiation upon mammalian cells was the finding that most irradiated cells which died did so because of failure to complete mitosis. The few exceptions were cell types particularly sensitive to radiation e. g. lymphocytes, occytes which died a rapid, pyknotic "interphase" death unrelated to their position in the cell division cycle. Other cells, if not called upon to divide after being irradiated, could continue to carry on their many normal biochemical functions unhindered; only their failure to divide revealed the accumulated latent radiation damage. This loss of cell reproductive capacity was far more sensitive as a biological end-point than any other which had been studied. Cells could be rendered reproductively inert by tens of rads*, while depression of the complex synthetic process which replicated deoxyribonucleic acid (DNA) took hundreds of rads (3, 4).Depression of protein synthesis by irradiation required thousands of rads; the inactivation of isolated enzyme systems required even higher doses up to hundreds of thousands of rads. Other recent studies have shown that not only the ability to divide but also the rate at which surviving mammalian cells divide (even several divisions after irradiation) is affected by relatively small radiation doses(5). Thus, the complex phenomenon of cell growth has emerged as the most sensitive biological end-point for the study of radiation damage, a rather non-specific type of damage to mammalian cells. Cell growth has hence been used as criterion for assessing the effects of many other, more specific, kinds of damage, such as the effects of pharmacological agents used in cancer therapy (6, 7).

Sodium Fluoride in Cell and Tissue Cultures

1. Early Studies with Avian Embryo Tissue Cultures: Short-term exposure (one hour) to sodium fluoride added to the balanced salt solution used as temporary incubation medium affected the pulsatile function of the developing hearts of chick embryos only at concentrations above 1125 ppm F^- (2500 ppm NaF); concentrations below 450 ppm of F^- (1000 ppm NaF) did not affect the functional end-point of phenol red concentration by the embryonic chick mesonephros (kidney). Failure of growth of embryonic heart and kidney explants after this short-term exposure to sodium fluoride occurred only above 675-720 ppm F^- (1500-1600 ppm NaF) (8). By contrast, in another study the growth of chick embryo bone was inhibited by a concentration of 94. 5 ppm F^- (210 ppm NaF). The highest concentration at which no interference with bone formation was seen was 37. 8 ppm F^- (84 ppm NaF) (9).

*A rad is the unit of absorbed radiation dose.

2. Mammalian Organ Cultures: An extremely comprehensive study of effects of added sodium fluoride upon two biochemical parameters in explants of metacarpal bones from young growing rats was reported in a deceptively brief communication by Proffit and Ackerman (10). Their data are shown in Figures 1 and 2. Various concentrations of sodium fluoride were continuously maintained in the culture medium throughout the experiment. Total protein synthesis during the experiment was measured by the incorporation of ^{14}C-labelled proline into collagen, while synthesis of DNA was measured concommitantly by the incorporation of ^{3}H labelled thymidine. No significant depression of protein synthesis was seen when the concentration of fluoride in the medium was below 10-20 ppm F^-; no depression in DNA synthesis took place below 20 ppm F^- (44.5 ppm NaF). Concurrent autoradiographic studies showed no structural disturbance in bone cultures grown in medium containing 15 ppm F^- (33.3 ppm NaF). Thus the lack of effect on protein and DNA synthesis could not have been due to a small surviving proportion of bone cells working harder to maintain the total tissue production of DNA and protein.

3. Isolated Mammalian Cells: Carlson and Suttie (11) have shown that the addition of sodium fluoride (30 ppm F^-) to proliferating monolayer cultures of HeLa cells caused a rapid drop in the cellular adenosine triphosphate (ATP) level. This compound contains a high-energy phosphate bond which acts as the immediate energy reserve for many synthetic processes in the cell. There was no specific direct inhibition of glycolysis by this growth-inhibiting concentration of fluoride. Similar results of equivalent inhibition of the growth of HeLa cells on glucose utilization, and lactate and CO_2 production were obtained by appropriate concentrations of fluoride and by the inhibitors ethionine and iodoacetate. However, this does not necessarily imply a common site of action for all three. The specific effect of fluoride on ATP concentration was not shared by the other inhibotors.

Three types of studies have been reported on effects of sodium fluoride upon the growth of isolated mammalian cells in vitro:

(a) Suspension Culture: Albright (12) used murine leukaemic lymphoblasts which grow in suspension in culture and do not attach to the surface of the culture vessel. He failed to note a significant decrease in the number of cells harvested after 4 days' growth (circa 7 population doublings) unless the growth medium contained fluoride in excess of 5-6 ppm (11.1 - 13.3 ppm NaF).

(b) Monolayer growth of surface-attached cells: Berry and Trillwood (13) studied two strains of mammalian cells which grow when attached to the glass or plastic surface of the culture vessel to produce a confluent monolayer of cells. A small but significant decrease in the number of cells harvested after seven days' growth (circa 4-5 population doublings) was seen in glass-attached cultures of human carcinoma-derived HeLa $S-^{3}$ cxf. cells

Fig. 1

Uptake of thymidine H^3 by Metacarpal Bones in Organ Culture as a Function of Fluoride Concentration from Proffit and Ackerman (10)

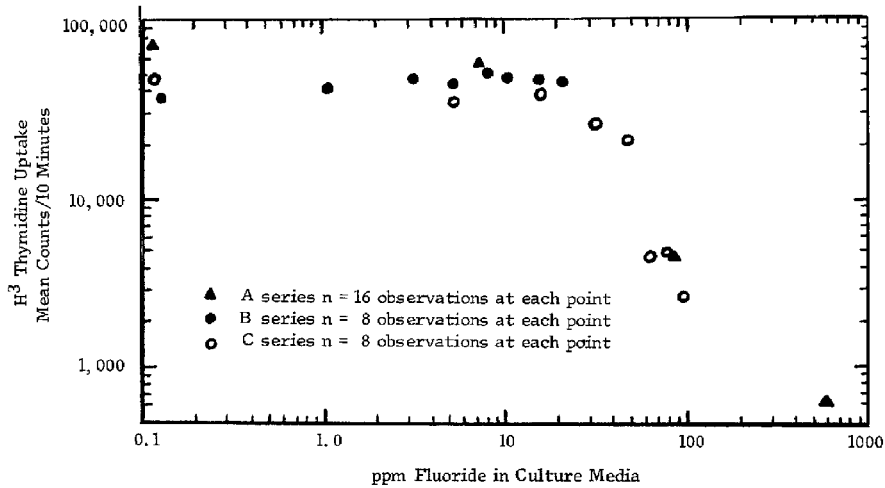
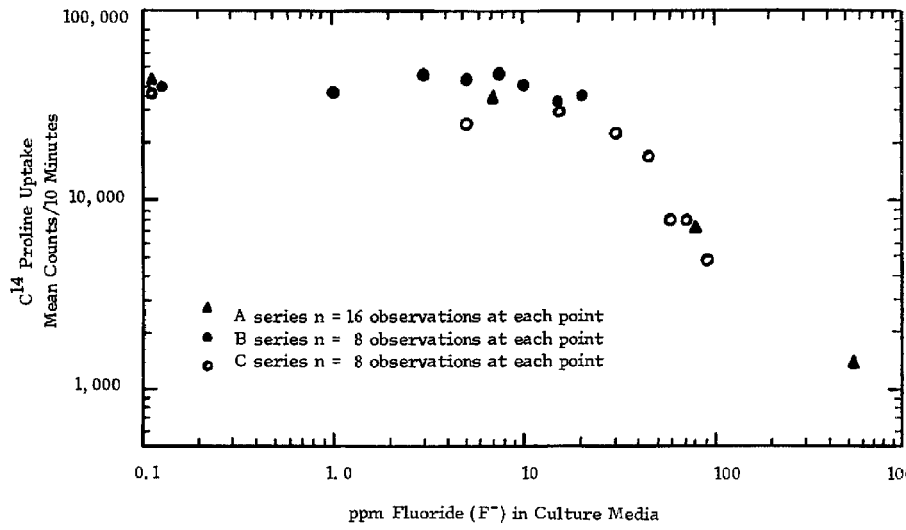


Fig. 2

Uptake of Proline -C^{14} by Metacarpal Bones in Organ Culture as a Function of Fluoride Concentration from Proffit and Ackerman (10)



and mouse Strain $L_{oxf.}$ dermal fibroblasts to which as little as 0.045 ppm F^- (0.1 ppm NaF) had been added. Their data are shown in Table 1.

TABLE 1

Action of Dilute Solutions of NaF on Growth of Mammalian Cells In Vitro

Cell Type	Percent of Control Growth in 7 Days Concentration of Sodium Fluoride in Medium		
	0.1 mg/l (1/10 ppm)	1.0 mg/l (1 ppm)	10.0 mg/l (10 ppm)
Human Carcinoma (HeLa S-3_{oxf})			
Experiment 1	-	72.5	69.8
2	82.3	89.8	73.0
3	92.5	90.2	70.9
4	85.5	85.9	75.5
Average:	86.8	84.6	72.3
Mouse Fibroblast (L, clone 12/oxf.)			
Experiment 1	-	78.4	72.6
2	68.5	77.0	53.0
3	95.0	81.1	65.7
4	92.1	94.3	65.6
Average:	85.2	82.7	64.2

From Berry and Trillwood (13)

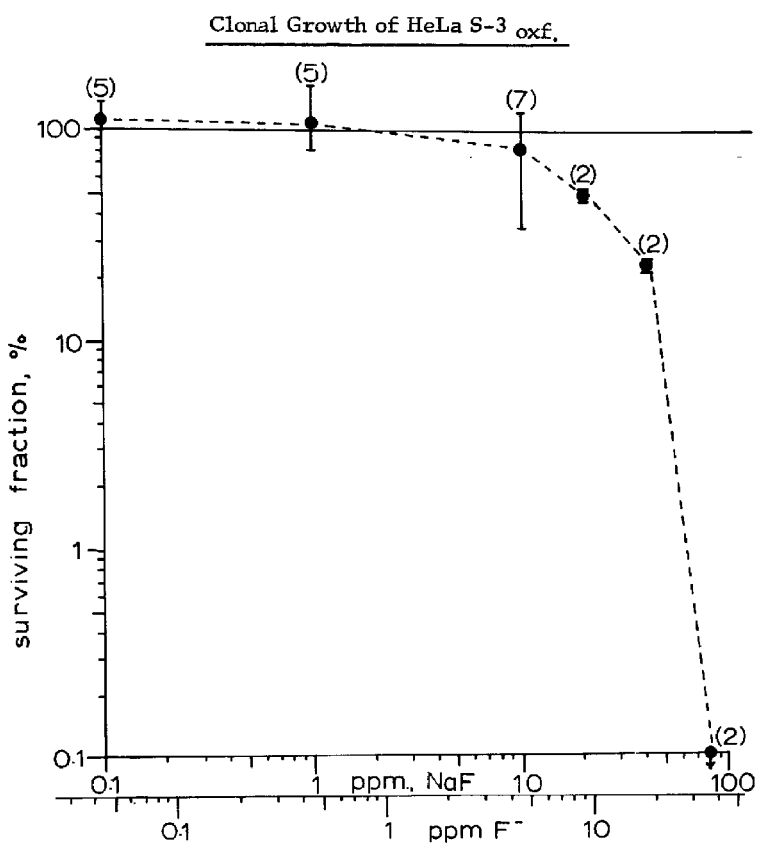
The reduction in cell growth was not due to altered ionic strength of the growth medium: Addition of as much as 10 ppm NaCl to this medium did not affect the number of cells harvested. This fluoride-induced depression of growth rate was also reflected in microcinematographic observations of murine Strain L fibroblasts in a study by the Time-Lapse Research Foundation which was terminated prematurely. For the mouse fibroblasts a decrease in the frequency of cell divisions, and a possible increase in the proportion of abnormal divisions were seen when either 0.018 or 0.038 ppm F^- (0.038 or 0.078 ppm NaF) were added to the culture medium. When similar amounts of sodium fluoride were added to cultures of human tooth pulp cells, no such effect was noted. However, these latter cultures were sufficiently crowded so that it was unlikely that the cells would attempt many

further divisions. A similar criticism has been made by Berry and Trillwood (14) of the failure of Armstrong et al. (15) to detect any decrease in growth of glass-attached HeLa or Minnesota EE (human esophageal epithelium-derived) cells in media to which sodium fluoride had been added in concentrations up to 10 ppm F^- (22.2 ppm NaF). However, Nias (16) used glass-attached HeLa cells which had completed over six population doublings in the one-week experimental period. When 0.45 ppm F^- (1.0 ppm NaF) was added to the growth medium, the mean number of cells harvested in five replicate experiments was 4% lower than that in the control cultures. The probability, that this difference was due to chance alone, was 0.20, rather greater than the accepted level of statistical significance, $p \leq 0.05$. Carlson and Suttie (17) also used glass or plastic-attached HeLa cells. Although they did not study the effects of concentrations lower than 10 ppm F^-, they found no difference from control growth at this concentration and only a slight depression of growth rate when 15 ppm F^- was maintained in the growth medium for at least 4 days (circa 4 population doublings). They showed that the decrease in growth was unaffected by alteration in the calcium and magnesium ion concentration in the medium over the range 0.1 to 2 times the normal level. They noted that fluoride-induced growth inhibition was not due to a failure of cells to attach to the surface of the culture vessel but rather to a true decrease in the rate of cell proliferation. Cells grown continously in high concentrations of sodium fluoride (15-25 ppm F^-) showed adaptation which reduced their sensitivity to an acute challenge with higher concentrations of fluoride; while 75 ppm F^- decimated "naive" HeLa cells, the fluoride-adapted cell line, suffered only a 53% reduction in growth at this concentration. The adapted cell line grew more slowly than the "naive" parent cell line, however, in the absence of added sodium fluoride in the growth medium.

(c) Clonal growth: Armstrong, et al. (18) studied the effect of addition of sodium fluoride (1-10 ppm F^-) to the culture medium, on the numbers of cells in individual clones of HeLa cells growing attached to the polystyrene surface of tissue culture flasks (Falcon TC). Their results indicated that there was no reduction in the rate of growth of the faster-growing clones at a concentration of 5 ppm F^-, although there was a detectable decrease in the growth rate when the concentration of added fluoride was raised to 10 ppm F^-. They rejected slowly-growing clones by abandoning further examination of all clones which failed to reach 4-cell size after 2 days' growth. They also measured survival of cell reproductive capacity by scoring the number of macroscopically-visible clones after 11 days' growth. "The mean number of clones formed in the flasks which contained media of 1 ppm and 5 ppm fluoride equalled or exceeded (my italics) the mean clone count of the control flasks"; at 10 ppm F^-, the mean number of clones was respectively 85% and 79% of the control.

The present author's data for clonal growth of HeLa $S\text{-}3_{oxf.}$ cells attached to Falcon TC polystyrene Petri dishes are shown in Figure 3. As in the experiments reported earlier (13), the growth medium was 199 (Glaxo), but it was supplemented with 20% type AB human serum to support clonal

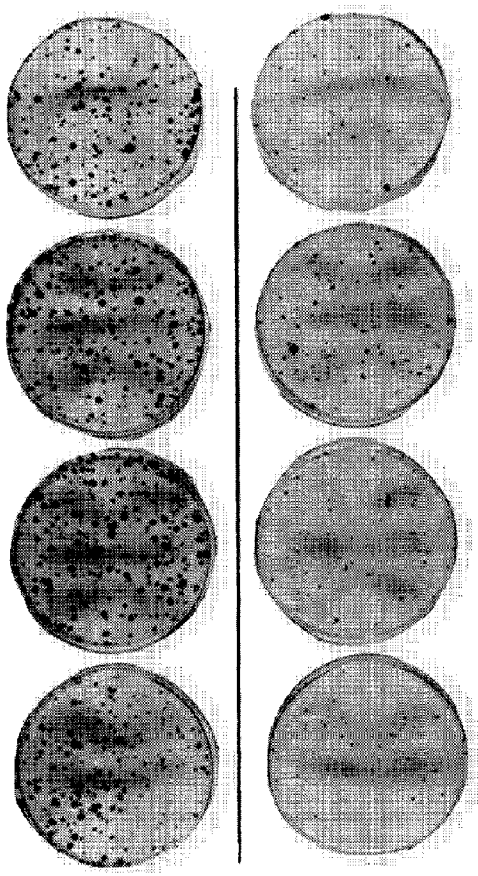
Fig. 3



growth from inocula containing only 125 cells per ml of medium. Both the cells and the appropriate amounts of sodium fluoride were added to the medium in bulk before it was divided into individual aliquots in the Petri dishes. In each experiment, the number of clones was scored on 4-5 dishes to establish one experimental point. Incubation was at 37° C in a humidified 5% CO_2 - 95% Air atmosphere, and the dishes were fixed and stained with Leishman's stain 14 days after plating. The plating efficiency (clones grown/cells initially plated) averaged 43%. When sodium fluoride at a concentration of 0.045 ppm F^- or 0.45 ppm F^- (0.1 or 1.0 ppm NaF) was added to the growth medium, the plating efficiency regularly exceeded that of three controls; only above 4.5 ppm F^- (10 ppm NaF) was there a significant decrease in the number of macroscopic colonies grown. The complete set of culture dishes from one such experiment is shown in Figure 4. Note that at 4.5 ppm F^- (10 ppm NaF) not only the number of clones, but also the size of the individual clones is reduced.

Fig. 4

Typical Growth of HeLa S3 Cells



CONTROL

4.5 ppm F^-
(10 ppm NaF)

Experiment 98

Discussion

The extreme sensitivity to sodium fluoride of the clonally-selected Oxford HeLa and Strain L cell sub-lines now appears to be somewhat idiosyncratic. Results obtained by several other investigators show similar retardation of cell growth only at concentrations of fluoride ranging upwards from 5 ppm F^-. However, the findings by Carlson and Suttie (17) that cells chronically exposed to fluoride in high concentrations adapt themselves to its presence so that their sensitivity to subsequent growth inhibition by fluoride is reduced, offers the possibility that some cell lines (conceivably even some of the widely-used HeLa lines) may have undergone similar adaptive changes by chance.

An increased plating efficiency of HeLa cells when low concentration of sodium fluoride (0.045-5.0 ppm F^-) were added to the growth medium has been noted both by Armstrong et al. (18) and by the present author. This may, paradoxically, represent a demonstration of a small amount of cell death due to these low concentrations of fluoride. When plating efficiency of a given cell line is less than 100%, the addition of a "feeder layer" of reproductively inert but metabolically active cells has been shown in many cases to increase the proportion of potentially viable cells which grow into macroscopically visible colonies (19). The "dead" cells supply the specific minimal nutritional requirements which the growth medium may lack (despite all the effort and skill which has gone into compounding the particular witch's brews in which mammalian cells thrive in vitro). Only when more marked degrees of cell-killing supervene, in the case of sodium fluoride at levels above 4.5 ppm F^-, would the apparent plating efficiency begin to decrease.

We have previously questioned whether the tissue culture systems which have been used to study effects of fluoride are the best ones from which to extrapolate any potential hazard to the intact animal. From the teleological viewpoint, the types of cells whose response to sodium fluoride is most likely to be relevant are those of bone (where fluoride is concentrated for excretion). Single-cell cultures of true bone cells, as distinct from bone marrow (haematopoietic) cells, are not generally available, but well-documented lines of cells originating from the kidneys of the Rhesus and African Green monkeys, the cow, the pig, the dog, the Syrian hamster and the marsupial Potorous are available from the American Type Culture Collection (20). This author is unaware of published studies in which the response of these cells to fluoride has been reported.

Finally, although the generally accepted levels of plasma fluoride around 0.1 - 0.2 ppm F^- (21) have recently been challenged as being too high (22), it is generally agreed that the concentration of fluoride ion in the urine is of the order of 3 ppm F^- (23). It would not be surprising if cells originating from renal epithelium proved less sensitive to the effects of added fluoride than other cell types which have been studied, due to adaptation to this environmental factor. This would be a reassuring find-

ing, comparable to the decreased sensitivity to high concentrations of fluoride of the HeLa cells grown by Carlson and Suttie (17) after a prolonged period of growth in fluoride-containing medium.

Summary

Various authors have studied the effect of fluoride ion upon mammalian cells using many different biological end-points, of which inhibition of the function of specific cellular enzymes, survival of cell reproductive capacity, production of abnormalities of cell division and alteration in the rate of cell proliferation are a representative sample. In this review the minimum fluoride ion concentration at which biological effects are detectable are compared for each of these end-points. The usefulness of such in vitro studies for evaluation of potential whole-animal toxicity is discussed.

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Acknowledgment

The work of the Radiobiology Laboratory is supported by a grant from the Medical Research Council. The studies of effects of sodium fluoride on mammalian cells were initiated during the time the author was Helen Hay Whitney Fellow in Radiobiology, and the laboratory was at that time aided by a grant from the Damon Runyon Fund for Cancer Research.