phosphatase "release" with and without CPZ was compared to control suspensions in air. Oxygenation produced no noticeable effect on phosphatase release. These results confirm the earlier reports of deDuve and Beaufay[7] and of Sledge and Dingle.[8] CPZ, 5×10^{-4} M, inhibited the "release" of enzyme equally well in oxygenated, nitrogenated and aerated lysosomal suspensions. Spirtes *et al.*[9] reported the absence of lipoperoxidation, as measured by the thiobarbiturate method, in mitochondria undergoing swelling. It should be noted that these mitochondria were in rather dilute suspension.

In order to test the possibility that CPZ stabilization of lysosomal membrane involves interactions with metals, disodium EDTA (1×10^{-3} M) was added to the incubation medium. In two experiments, EDTA did not alter either the "release" of lysosomal acid phosphatase or the inhibition of that release produced by CPZ (5×10^{-4} M).

Likewise, ouabain in concentrations of 1×10^{-4}M, 5×10^{-4}M, 1×10^{-6}M and 1×10^{-8}M had no effect on enzyme "release" or on CPZ (5×10^{-4}M) inhibition of enzyme "release". An ATPase not inhibited by ouabain has been suggested by Duncan[3] to be of importance in the labilization of the lysosomal membrane. Thus, it is not possible to dismiss the importance of an ATPase in lysosomal membrane function. In this regard it may be recalled that CPZ itself inhibits the activity of several ATPases such as the mitochondrial Mg^{2+}-activated ones, but not others.[10] Since divalent cations are required for the activation of certain ATPases, the lack of evidence of interaction of certain ATPases, the lack of evidence of interaction between CPZ and EDTA tends to cast further doubt on the involvement of an ATPase in lysosomal membrane function.

Of the three hypotheses, only the one concerning lipid peroxidation has been examined under sufficiently stringent conditions to warrant its rejection. The evidence concerning the other two hypotheses, although consistently negative, is not sufficient to permit their complete rejection. In our opinion, however, the mechanism of stabilizing action of CPZ on lysosomes must be sought elsewhere such as on biophysical grounds.

Department of Pharmacology,
Tulane University School of Medicine,
New Orleans, La., U.S.A.

P. S. GUTH
J. AMARO

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Repair of sub-lethal damage of L5178Y lymphoblasts *in vitro* treated with dimethyl myleran and nitrogen mustard

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IN STUDYING mechanism of cell kill by physical or chemical agents, one may examine either the damaging or the recovery process.[1] With chemicals the damaging process includes such considerations as dose-dependence, membrane permeability, inactivation of drug, and reduction in cytocidal effect

by protective agents (e.g. thiol groups in the presence of nitrogen mustard). The recovery process may be examined by modifying post-treatment conditions and by making the assumption that improvement in survival results from repair of the initial injury rather than the injury being bypassed.[1]

Previous studies have indicated that recovery from dimethyl myleran(DMM)-induced cell injury resembles that seen following ionizing radiation in that the dose-response curve has a prominent shoulder and post-treatment storage at sub-optimal temperatures facilitates cell recovery.[2] Evidence for similar recovery processes following nitrogen mustard (HN2) treatment was lacking since reduction of temperature following treatment did not alter survival and the dose-response curve for HN2 appeared to be "single-hit" in nature with a small or negligible shoulder.[2]

Elkind *et al.*[3–5] using the dose fractionation technique have shown that mammalian cells repair damage following sub-lethal injury. More recently Elkind *et al.*[6] have suggested that sub-lethal and lethal damage are qualitatively equivalent both with respect to damage registration and damage repair and that if a cell survives it will have repaired a significant amount of sub-lethal damage. In this investigation the dose fractionation technique of Elkind[1] has been used to study repair of sub-lethal damage following treatment with DMM and HN2. The object is to compare the cytocidal effect of a given dose of drug, divided into two or more fractions separated in time, with the effect achieved by the same dose of drug delivered at one time.

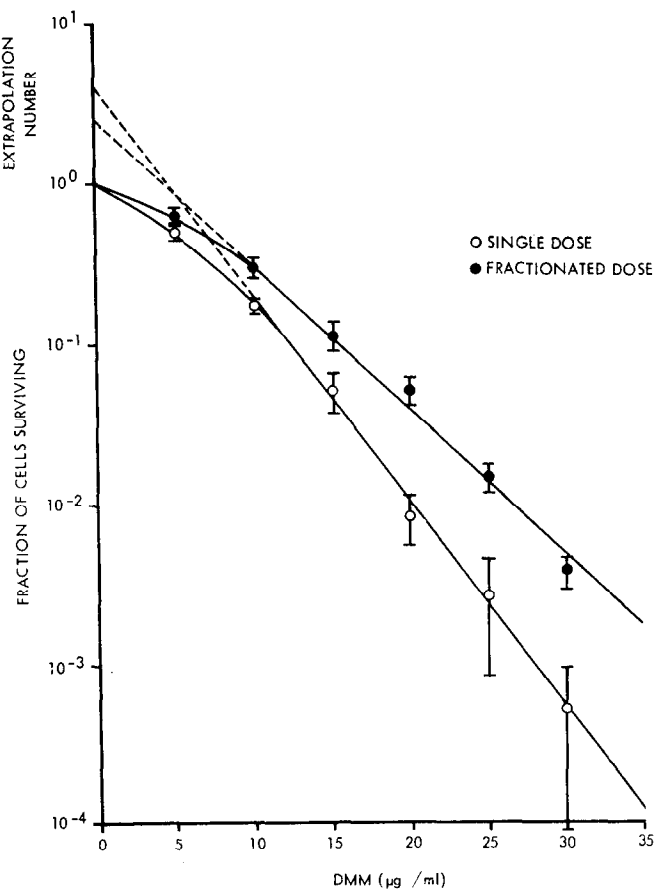

FIG. 1. Dose-survival curves of L5178Y lymphoblasts *in vitro* following DMM treatment, ○ single dose and ● fractionated dose schedules (¼ dose administered every 8 hr × 4 doses). Data are expressed as mean surviving cell fraction ±S.E.

Murine leukemia L5178Y lymphoblasts were grown in tissue culture and solutions of dimethyl myleran* and nitrogen mustard† were prepared as previously outlined.[2] Paired experiments in which cells were treated with either single or fractionated dose schedules of drug were employed. The fractionation program consisted of dividing the dose into 4 equal fractions, with ¼ dose being administered at 8-hr intervals. The first fraction of drug was added to the cell cultures at the same time that the single dose was given; thus the single and fractionated dose experiments shared the same zero time.

Dose-survival curves were determined using the extrapolation technique of Alexander.[2, 7] The limitations and possible errors of this method have been discussed in detail previously.[2] In brief, the extrapolation method tends to exaggerate cell kill if a significant fraction of surviving cells has a doubling time longer than that of the untreated controls (slow-growing variants), or if the surviving cells are arrested for some time in interphase before undergoing cell division (division delay). Both these phenomena have been observed at higher drug concentrations, than those used here, in which cell kill is 4-logs or greater. Slow-growing variants are recognized by prolongation of the

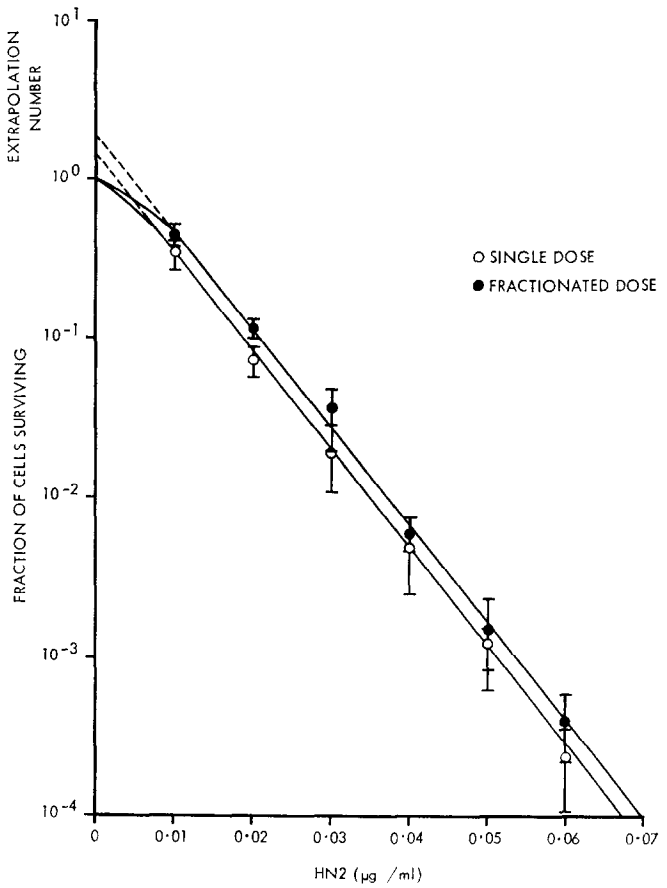

FIG. 2. Dose survival curves of L5178Y lymphoblasts *in vitro* following HN_2 treatment, ○ single dose and ● fractionated dose schedules (¼ dose administered every 8 hr × 4 doses). Data are expressed as mean surviving cell fraction ±S.E.

doubling time and division delay by curvilinear deviation of the dose-survival curve (i.e. surviving cell fraction is lower than that expected assuming an exponential dose-survival curve throughout). At the drug concentrations used in this experiment the dose-survival curves were exponential, viz. followed first-order kinetics down to approximately 3·5 log cell kill and the surviving cells grew up

* DMM was kindly supplied by Mr. J. L. Everett, Chester Beatty Research Institute.

† HN2 "Mustargen" was kindly supplied by the Merck Sharp & Dohme drug company.

promptly with a doubling time identical to that of the untreated controls. Dewey *et al.*[8] compared the cloning and extrapolation technique for estimating survivors following X-irradiation of fibroblasts. The dose-survival curves obtained by the extrapolation technique were lower but parallel to those obtained by colony counts so that although the extrapolation number was higher with colony counts little difference was observed in the Do (the dose required to reduce survival by 1/e along the exponential portion of the dose-survival curve).

The cytocidal effect of DMM administered as a single dose (Do = 3·37 μg/ml) was 1·43 times more marked than that achieved by dose-fractionation (Do = 4·81 μg/ml) as illustrated by the dose-survival curves in Fig. 1. With HN2 there was no difference in cytocidal effect using the single dose (Do = 7·08 mμg/ml) or fractionated dose (Do = 7·14 mμg/ml) programs (Fig. 2). The extrapolation number n, was lower with HN2 treatment than with DMM for both dose schedules.

Linear regression analysis of the exponential portion of the dose-survival curves was performed for each of 4 categories (single and fractionated doses for dimethyl myleran and nitrogen mustard) using an Olivetti-Underwood programma 101 table-top computer. The lowest dose for each of the 4 categories was excluded from the regression analysis since these values fell within the shoulder region of the curves (Figs. 1 and 2). The correlation coefficients ranged from −0·90 to −0·95 for each of the regression analyses indicating the data conformed to a first-order kinetic relationship. The *t*-test for comparing the difference between slopes of the single and fractionated dose-survival curves following DMM treatment was highly significant ($P < 0·001$) but a similar comparison for HN2 showed no significant difference.

The Do obtained for DMM in this study approximated the value reported previously for the radioresistant cell line.[2] However, the Do obtained for HN2 is approximately 4 times lower than the value previously found for the radioresistant line.[2] This observation that sensitivity to HN2 may increase several-fold with no apparent change in response to DMM suggests that the two drugs act by independent mechanisms. This alteration in drug sensitivity also suggests that mutations of L5178Y may arise *in vivo* and underlines the need for continual checks on the responsiveness of stock cell lines to various chemotherapeutic agents.

The cytocidal effect of DMM administered as a single dose was 1·43 times that observed by administration of the same total dose divided into 4 fractions administered at 8-hr intervals. By altering the dose-fractionation schedule with respect to number of fractions and time interval between doses an even more pronounced difference might be obtained between single and fractionated dose programs as Elkind has demonstrated for radiation.[1, 4, 5]

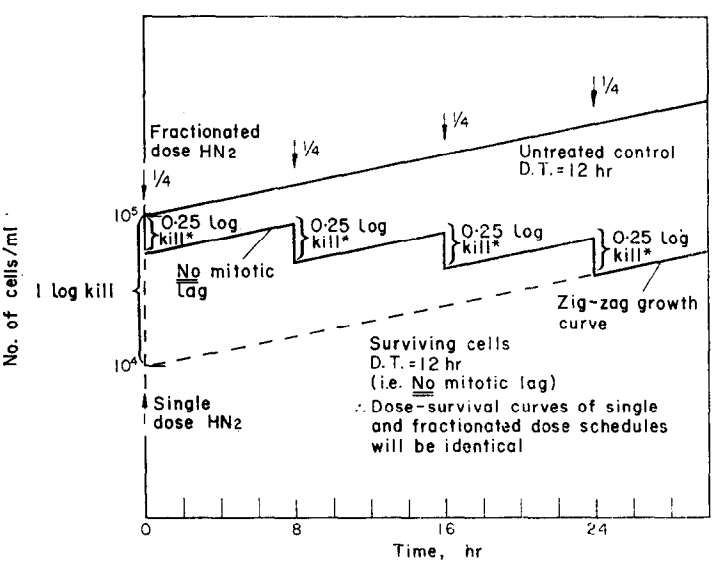

FIG. 3. Discussion of extrapolation method. Extrapolation of single and fractionated dose growth curves gives identical surviving cell fraction. * Note: surviving fraction is independent of initial cell concentration between 4×10^4 to 4×10^5 cells/ml (see ref. 2).

The diminution of the cytocidal effect of DMM by dose-fractionation is similar to that observed with dose-fractionation of radiation.[3-5, 9] The effect is predictable from the nature of the dose-survival curve which in the case of DMM and radiation is characterized by an initial shoulder followed by an exponential decline. With each dose fraction a portion of drug will be "wasted" traversing the shoulder portion of the dose-survival curve, the more prominent the shoulder the greater will be the loss of cytocidal effect by dose-fractionation. In the case of HN2, in which the dose-survival curve is exponential throughout with only a slight shoulder, the cytocidal effect is similar whether the drug is administered as a single dose or divided into several fractions.

The reduction in cytocidal effect of DMM on dose-fractionation can also be explained by repair of sub-lethal damage inflicted by each dose fraction and such cumulative repair exceeds that observed following single dose therapy. This study supplies further evidence that mechanisms exist to repair DMM-induced cell damage but fails to reveal evidence for analagous repair processes following HN2 therapy.

The possibility that division delay is more marked following single dose than fractionated dose DMM therapy has not been entirely excluded but this interpretation of the results is considered unlikely for the reasons stated above (see discussion on limitations of extrapolation method, and Fig. 3).

If this explanation was valid then it would also follow that division delay following single and fractionated dose HN2 therapy was identical.

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Department of Internal Medicine,
University of Manitoba and
The Manitoba Cancer Foundation,
Winnipeg, 3, Canada

GERALD J. GOLDENBERG

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Hyposensitivity to 5-hydroxytryptamine in the isolated stomach fundus of the newborn rat—I.

Organ preparation and neonatal quantitative behaviour of the hyposensitivity*

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THE PHARMACOLOGICAL experimentation on the newborn rat is lacking in precise quantitative data, if compared with that on the adult animal, and our current state of knowledge is based mostly upon toxicological and teratological studies of reviews by Done,[1] Yeary *et al.*,[2] Yaffe and Back.[3] The data concerning the sensitivity and the responsiveness of newborn animals towards different drugs are particularly scarce (Yaffe and Back[3]). Although it is generally assumed that the animal in the period

* Part of this data was presented at the XIV Congresso Nazionale della Società Italiana di Farmacologia, Trieste, June 5–7, 1967.